TRYPTOPHAN AVAILABILITY MODULATES SEROTONIN RELEASE

by

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ABSTRACT

Serotonin synthesis in the brain varies with brain tryptophan levels because tryptophan hydroxylase is unsaturated with its substrate. It was unresolved whether changes in brain tryptophan levels within the physiologic range are paralleled by changes in serotonin release. Therefore, an *in vitro* experimental system, utilizing superfused rat hypothalamic slices, was developed to examine the relationship between tryptophan availability and the release of endogenous serotonin. This experimental system was characterized with respect to time-dependent changes in medium tryptophan concentrations, tissue indole levels, ratio of tissue protein to DNA levels, tissue lactate dehydrogenase activity and 5-hydroxyindole release with slice superfusion. Slices superfused with physiologic medium, which contained a serotonin reuptake inhibitor, released low amounts of serotonin spontaneously. The rate of serotonin release increased, in a Ca^{2+}-dependent and tetrodotoxin-sensitive manner, with the delivery of low-frequency electrical field-stimulation. Increasing the availability of tryptophan to the slices, by superfusing them with medium supplemented with low concentrations of tryptophan (1 - 10 μM), increased dose-dependently spontaneous and electrically-evoked serotonin release. The magnitude of the tryptophan-induced potentiation of evoked serotonin release was independent of the frequency of electrical field-stimulation. Decreasing the availability of tryptophan to the slices, by superfusing them with medium supplemented with a high concentration of another large neutral amino acid (100 μM leucine), reduced the amounts of serotonin released spontaneously and with depolarization. The leucine-induced decreases in tissue tryptophan levels and serotonin release were suppressed by co-supplementing the medium with both leucine and tryptophan. These data demonstrate that tryptophan availability modulates serotonin release from hypothalamic slices, and suggest a physiologic coupling between brain serotonin synthesis and the amounts of serotonin released from nerve terminals.

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1. INTRODUCTION

1.1 Historical Background

Serotonin was originally characterized, as the word connotes, as a component of blood serum which affects vascular tone (0'Connor, 1912; Freund, 1920; Gaddum, 1936). The source of this biologically active substance was determined to be blood platelets, from which serotonin was purified and its chemical structure subsequently elucidated to be 5-hydroxytryptamine (5-HT) (Rapport et al., 1948; Rapport, 1949). Independently, stemming from an interest in characterizing the substance which imparts particular histochemical properties to enterochromaffin cells of the gastrointestinal tract, enteramine was isolated and later shown to be chemically identical to serotonin (Erspamer, 1952, 1954). The presence of 5-HT in the mammalian brain was discovered by Twarog and Page (1953) by application of a bioassay utilizing a molluscan heart (Welsh, 1954). Scientists were soon discussing the possibility that 5-HT was used by brain neurons to communicate messages across synaptic junctions (Brodie and Shore, 1957), and this idea gained enthusiasm with the development of the Falck-Hillarp histofluorescent method to visualize directly monoamines in neuronal tissue (Falck et al., 1962). Using this method, Dahlstrom and Fuxe (1964) demonstrated clearly the presence of serotonin-containing cell bodies in the mammalian brain, and found that they were congregated in the brainstem raphe nuclei. The functional significance of the existence of serotoninergic neurons was reinforced by certain pharmacological observations: The potent psychotomimetic lysergic acid diethylamine (LSD) blocked the constrictive action of 5-HT in a variety of smooth muscle preparations (Gaddum, 1953); the centrally-acting anti-

hypertensive drug reserpine depleted the rabbit brain of 5-HT (Shore et al., 1957); the tranquilizing drug imipramine depleted blood platelets of 5-HT (Marshall et al., 1960). These observations raised the possibility that 5-HT might have a role in cerebral function.

Recognition of the effects on brain 5-HT of certain mood-altering pharmacological agents led to the postulate that a 5-HT deficiency in the brain might underlie certain psychiatric disorders such as schizophrenia (Wooley and Shaw, 1954) and depression (Lapin and Oxenkrug, 1969). The role that this neurotransmitter may play in mood states has continued to be a central issue addressed by neurochemists; it has also become apparent that serotoninergic neurons are likely to be involved in a remarkable diversity of brain functions. These brain functions have been characterized as serving to initiate behaviors which protect the organism from effects of potentially damaging environmental stimuli (Vogt, 1982). For example, sleep (which permits optimal performance during the waking state) is profoundly disrupted by administration of an inhibitor of 5-HT synthesis, and is restored by treatment with the immediate precursor of 5-HT, 5-hydroxytryptophan (5-HTP) (Jouvet and Pujol, 1974). Also, maintenance of normal body temperature in response to changes in environmental temperature is apparently dependent on serotoninergic neuronal activity (Myers et al., 1969). Other self-preserving functions attributed to serotoninergic neurons include pain sensitivity and appetite.

Fundamental to understanding how serotoninergic neurons may mediate various behavioral states is determining that exogenous 5-HT applied to a sensitive brain region induces a behavior which mimics that produced when endogenous 5-HT is released from local nerve terminals. This principle was first addressed in the context of the involvement of serotoninergic neurons in temperature regulation. Feldberg and Myers (1965) demonstrated that injecting exogenous 5-HT into the anterior hypothalamus of anesthetized cat elevates its

body temperature. They then showed that endogenous 5-HT could be detected in the medium perfusing the cat third ventricle (which borders the hypothalamus and may thus contain some 5-HT which has diffused from hypothalamic synapses), but increasing its release by intraperitoneal injection of 5-HTP caused respiratory failure rather than the expected rise in body temperature (Feldberg and Myers, 1966). Shortly thereafter it was demonstrated successfully that locally perfusing the third ventricle with 5-HTP (El Hawary and Feldberg, 1966) or a monoamine oxidase (MAO) inhibitor (El Hawary et al., 1967) increased 5-HT release in association with shivering and a rise in body temperature.

A further series of experiments strengthened the hypothesis that the activity of serotoninergic neurons is associated with 5-HT release from their nerve terminals and thereby modifies behavioral outputs. Aghajanian et al. (1967) demonstrated that electrical stimulation of the rat midbrain raphe causes forebrain levels of 5-HT to decrease and those of its major metabolite 5-hydroxyindoleacetic acid (5-HIAA) to increase; these observations suggested that depolarization of serotoninergic raphe perikarya causes an increase in 5-HT release and turnover in their distal nerve terminals. That the activity of serotoninergic neurons has a behavioral consequence was supported by the observations that electrically stimulating the midbrain raphe inhibited the habituation process which normally occurs with repetitive sensory stimulation in awake, unrestrained rats (Sheard and Aghajanian, 1968), and increased the amounts of 5-HT actually released from forebrain nerve terminals (Eccleston et al., 1969).

1.2 Anatomy of Serotoninergic Neurons in Mammalian Brain

1.2.1 Serotoninergic Perikarya and Their Projections

The distribution of serotoninergic neurons in the mammalian brain, as originally localized by Dahlstrom and Fuxe (1964) using the formaldehyde-induced fluorescence technique of Falck-Hillarp, has been confirmed using a variety of more modern techniques (immunohistochemistry, autoradiography, retrograde transport tracing, microenzymatic, spectrofluorimetric). Dahlstrom and Fuxe characterized nine groups (B1 - 9) of serotoninergic cell bodies in the brainstem raphe nuclei, from the caudal medulla oblongata to the midbrain. Since then, serotoninergic perikarya have also been found to reside outside the B1 - 9 localizations, in: the area postrema (Fuxe and Owman, 1965); locus coeruleus and subcoeruleus (Sladek and Walker, 1977); habenular nucleus (Chan-Palay, 1977); interpeduncular nucleus (Chan-Palay, 1977); several hypothalamic nuclei (Chan-Palay, 1977; Kent and Sladek, 1978); and in the subpial surface of the ventral medulla oblongata (Gorcs et al., 1985).

The general organization of the originally designated 'B' serotoninergic cell groups in the raphe complex and their projections are outlined here (for details, see Dahlstrom and Fuxe, 1964; Dahlstrom and Fuxe, 1965; Fuxe, 1965; Chan-Palay, 1976; Steinbusch, 1981). Neurons from B1 - 9 cell groups project either rostrally, along a dorsal bundle or the ventrally sweeping medial forebrain bundle, or caudally along the bulbospinal pathway. Serotoninergic fibers of the bulbospinal tract originate largely from the caudal raphe nuclei B1 (raphe pallidus), B2 (raphe obscurus) and B3 (raphe magnus), but also from B5 (raphe pontis). These descending projections innervate various structures of the medulla oblongata, with particularly dense

terminations in the visceral efferent and afferent nuclei, and the grey matter of all spinal cord levels. The serotoninergic projections ascending along the dorsal bundle have cell bodies residing in B3, B5, B7 (raphe dorsalis) and B8 (raphe medianus); these fibers terminate in the central grey substance, cerebellum (via the brachium conjunctivum and pontis), locus coeruleus, preoculomotor complex, superior colliculus, ventricular supra- and subependymal plexuses, and in the leptomeninges. Serotoninergic fibers ascending along the medial forebrain bundle have cell bodies in B3, B5, B6 (raphe pontis), B7, B8 and B9 (mesencephalic reticular formation), and distribute themselves widely over many forebrain structures: to all cortical areas, amygdala, basal ganglia, thalamus, hypothalamus and septum.

1.2.2 Extrinsic and Intrinsic Serotoninergic Innervation of the Hypothalamus

The hypothalamus is innervated by ascending serotoninergic fibers arriving largely from raphe cell groups B7 (dorsalis) and B8 (medianus), but probably also from B9 (mescencephalic reticular formation) (Anden et al., 1966; Kuhar et al., 1972*a*; Halaris et al., 1976). Axonal terminations of these neurons are very dense in the suprachiasmatic nucleus, and scarce to dense in most other hypothalamic areas (Loizou, 1972; Steinbusch, 1981). Biochemical mapping of the rat hypothalamus has shown each nuclei to contain detectable levels of 5-HT, with a five-fold range between areas containing the highest concentration (37 ng 5-HT/mg protein, internal portion of the suprachiasmatic nucleus) to the lowest (7 ng 5-HT/mg protein, periventricular portion of the preoptic nucleus) (Saavedra et al., 1974); the average concentration of 5-HT in the rat hypothalamus is approximately four-times that in the cerebral cortex, and two-times that in the basal ganglia (Saavedra, 1977).

Histological studies, coupled with selective pharmacological manipulations, have also revealed the existence of serotoninergic neurons intrinsic to the rat hypothalamus. Chan-Palay (1977) localized, by autoradiography following MAO inhibition, hypothalamic neuronal perikarya that selectively accumulate intraventricularly-infused [^{3}H]5-HT. These labeled neurons were in the suprachiasmatic nucleus, median eminence, arcuate nucleus, ventromedial nucleus, paraventricular nucleus and periventricular nucleus. A later study by Beaudet and Descarries (1979), utilizing methodology similar to that of Chan-Palay, found labeled hypothalamic neurons restricted to the ventral portion of the dorsomedial nucleus. A clear explanation for this discrepancy is lacking, but may have resulted from the fact that while both studies applied a ten-fold excess of norepinephrine to compete with the nonspecific uptake of [^{3}H]5-HT into catecholaminergic nerve terminals, the latter study used the more selective l-isomer, whereas the former used an equimolar concentration of the racemic mixture (i.e., 50% of which is the l-isomer). The serotoninergic perikarya described by Beaudet and Descarries were estimated to be a population of 1000 neurons residing on each side of the third ventricle; electron microscopic examination of the autoradiographs revealed these neurons to be relatively small (i.e., a diameter of 10 μm as compared to 15 - 24 μm for those in the dorsal raphe nucleus). A more recent study utilizing immunocytochemistry, after pretreating rats with a MAO inhibitor and tryptophan, confirmed the existence of approximately 350 serotonin-immunoreactive cells of 9 μm in diameter in the dorsomedial nuclei on either side of the ventricle (Frankfort et al., 1981). Kent and Sladek (1978) applied microfluorescence to brain tissue from rats pretreated with a monoamine storage inhibitor (reserpine) and a MAO inhibitor to find dull-yellow fluorescent cells in the ventrolateral portion of the arcuate nucleus; they suggested that the failure of Beaudet and Descarries to demonstrate the presence

of 5-HT accumulating neurons in the arcuate nucleus may have been due to the tight junctions between ependymal cells which line the ventricle in the region of the arcuate nucleus but not the dorsomedial nucleus.

1.2.3 Terminal Arborizations

Serotoninergic axons arborize profusely within most brain regions, and terminate at multiple varicose swellings along their paths. High resolution autoradiography has revealed that the density of serotoninergic innervation in the rat cerebral cortex is about 1×10^6 varicosities/mm^3 of cortex; this figure represents a mean incidence of about 0.1% of all cortical nerve endings, which is ten-times higher than that estimated for noradrenergic innervation and one-thirtieth lower than that of GABA-ergic nerve terminals (Beaudet and Descarries, 1976). Varicosities containing radiolabeled 5-HT in the cerebral cortex seldom (c.a. 5%) possess synaptic specializations of their surface membrane (Descarries et al., 1975). Based on the substantial amounts of 5-HT released *in vivo* in the rat cerebral cortex (Eccleston et al., 1969), it is probable that endogenous 5-HT can be released from all axonal varicosities which concentrate the amine, not only from the small proportion that make classic synaptic contacts. The relative paucity of membranous differentiations associated with serotoninergic varicosities has also been described in several other mammalian brain regions, including the median eminence (Calas et al., 1974), caudate nucleus (Calas et al., 1976), locus coeruleus (Leger and Descarries, 1978), cerebellar cortex (Beaudet and Sotelo, 1981), and hypothalamus (Beaudet and Descarries, 1979). This feature of 5-HT varicosities (which is similar to that observed for catecholaminergic nerve terminals in some brain regions) has led to the suggestion that 5-HT released into the extracellular space has its effect at local postsynaptic

5-HT receptors and, notably, may diffuse to reach relatively distant 5-HT receptors (Beaudet and Descarries, 1978). In this manner, 5-HT may exert a widespread influence on neuronal activity of the brain.

1.3 Regulation of Serotonin Biosynthesis

1.3.1 The Biosynthetic Pathway

Serotonin is formed from the essential amino acid l-tryptophan in a two-step biosynthetic pathway in which the substrate is initially hydroxylated and subsequently decarboxylated (Figure 1). The hydroxylation reaction is catalyzed by l-tryptophan-5-monooxygenase (conventionally named tryptophan hydroxylase), and requires the cofactors molecular oxygen and l-erythro-tetrahydrobiopterin (Figure 2): one atom of molecular oxygen is used in the 5-hydroxylation of tryptophan to form 5-HTP, and the other is reduced to water; electrons are donated by tetrahydrobiopterin, and the unstable product quinonoid dihydropterin is regenerated immediately to the reduced form by NAD(P)H-linked quinonoid dihydropteridine reductase (Kaufman, 1974). Conversion of 5-HTP to 5-HT occurs rapidly because the enzyme aromatic l-α-amino acid decarboxylase is present in far greater amounts than is tryptophan hydroxylase (Ichiyama et al., 1968), and for this reason tryptophan hydroxylation, rather than 5-HTP decarboxylation, is presumed to be the rate-limiting step in 5-HT biosynthesis. Indeed, only trace amounts of 5-HTP are measurable in brain tissue (Ashcroft et al., 1965; Moir and Eccleston, 1968). The affinity of aromatic amino acid decarboxylase, a pyridoxal-5'-phosphate dependent enzyme, for 5-HTP is at least one hundred-fold greater than for

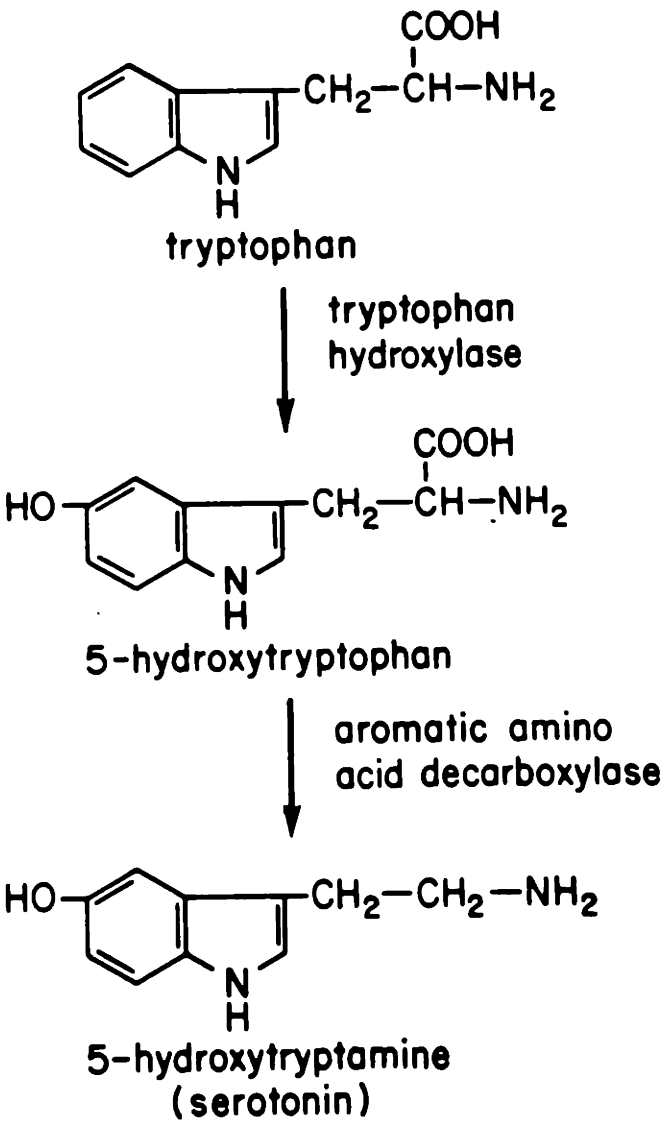

Figure 1. Biosynthesis of serotonin

(reproduced from Boadle-Biber, 1982*a*)

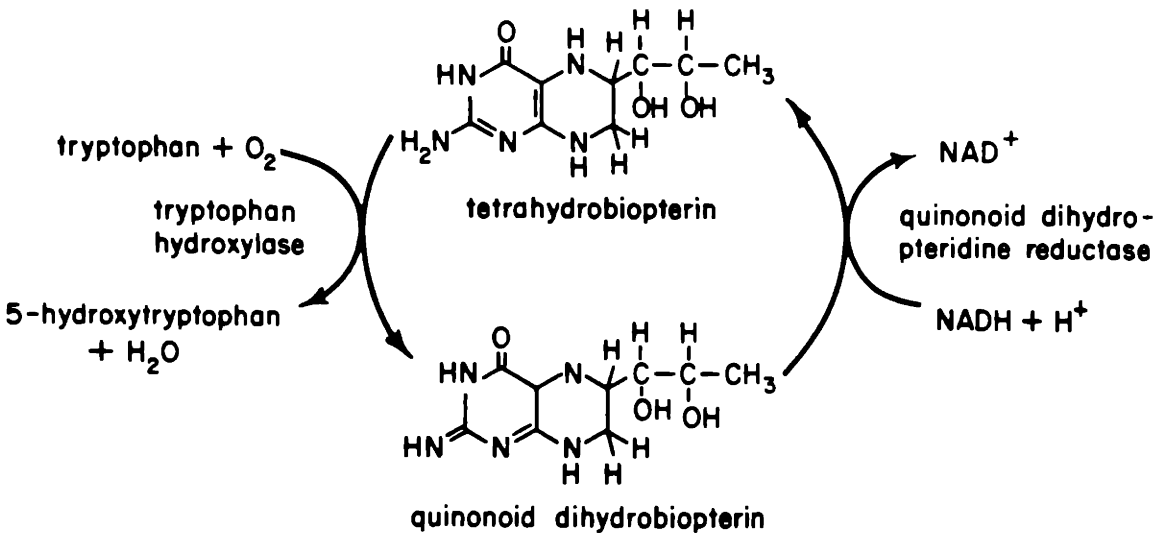

Figure 2. Regeneration of tetrahydrobiopterin

(reproduced from Boadle-Biber, 1982*a*)

tryptophan, and thus 5-HTP is preferentially decarboxylated in serotoninergic neurons (Ichiyama et al., 1968). Within catecholaminergic neurons, however, it is apparently this same enzyme which converts 3,4 dihydroxyphenylalanine to dopamine (DA) (Christenson et al., 1972; Hokfelt et al., 1973).

Purified tryptophan hydroxylase is a tetramer of identical subunits each having a molecular weight of 55,000 - 60,000 (Tong and Kaufman, 1975; Youdim et al., 1975; Nagata and Fujisawa, 1982; Cash et al., 1985). A single cDNA clone for tryptophan hydroxylase has been isolated from both rat and rabbit pineal gland, and each is highly homologous to phenylalanine and tyrosine hydroxylases (Darmon et al., 1986; Grennet et al., 1987). More recently, Darmon et al. (1988) obtained two full-length cDNA clones from a rat pineal gland library. These clones contain the same coding sequence for a 51,000 dalton protein, but differ by the length of their 3' untranslated region. Both mRNA species corresponding to these cDNA clones generate tryptophan hydroxylase enzymatic activity in a rabbit reticulocyte system. The functional significance of divergent tryptophan hydroxylase clones is unknown, though it was suggested that the 3' extension may play a role in the translation efficiency and/or stability of the corresponding mRNAs.

Tryptophan hydroxylase partially purified from mammalian brain displays approximate K_m values of 50 μM for l-tryptophan, 30 μM for tetrahydrobiopterin, and 2.5% for oxygen (Friedman et al., 1972; Kaufman, 1974; Kuhn et al., 1980). Endogenous levels in rat brain of tryptophan are 25 - 50 μM (Parfitt and Grahame-Smith, 1973), of tetrahydrobiopterin are 1 - 3 μM (Kuhn et al., 1980; Mandell et al., 1980) and of oxygen are 5% (Jamison and van den Brenk, 1965). Comparing these endogenous concentrations with their respective K_m values for tryptophan hydroxylase suggests that tryptophan and tetrahydrobiopterin are present in the

brain at substantially subsaturating levels and may thus each limit the rate at which 5-HT is synthesized *in situ*.

Although brain levels of tetrahydrobiopterin are only one-thirtieth to one-third its estimated K_m value for tryptophan hydroxylase, it appears unlikely that 5-HT synthesis is physiologically regulated by changes in the level of this cofactor. Tryptophan hydroxylase activity in brain homogenates is unchanged by the addition of exogenous reduced biopterin (Green and Sawyer, 1966; Grahame-Smith, 1967). A small increase (20 - 25%) in 5-HT synthesis does occur after intraventricular injection of tetrahydrobiopterin, but only when a dose is given which raises whole brain tetrahydrobiopterin levels by two hundred-fold (i.e., well beyond the physiologic range) (Miwa et al., 1985). Furthermore, pharmacologically reducing the cofactor pool by 50% and simultaneously inhibiting its *de novo* synthesis (from guanosine triphosphate) by 99% fails to alter the rate of 5-HT synthesis *in vivo* (Sherman and Gal, 1978). These investigators suggested that the low endogenous levels of tetrahydrobiopterin relative to its K_m value for tryptophan hydroxylase does not limit 5-HT synthesis because the regenerating activity of dihydropteridine reductase occurs at a rate which far exceeds that of tryptophan hydroxylase (and tyrosine hydroxylase). The apparent lack of a regulatory influence of physiologic changes in tetrahydrobiopterin availability on 5-HT synthesis contrasts to its potentially significant role in the molecular processes which underlie the acceleration in 5-HT synthesis secondary to tryptophan hydroxylase activation. The activation of tryptophan hydroxylase which follows various experimental manipulations (i.e., exposing the enzyme present in a crude extract or a partially purified preparation to detergents, phospholipids, calcium-activated protease, or trypsin) has been attributed to an increase in the enzyme's affinity for its biopterin cofactor, and sometimes also to an increase in its affinity for

tryptophan (for review, see Hamon et al., 1984; Boadle-Biber, 1982*a*). This suggests that under certain conditions serotoninergic neurons may utilize the presence of low levels of tetrahydrobiopterin to regulate the kinetic changes in tryptophan hydroyxlase activity.

Changes in the degree of saturation of tryptophan hydroxylase with its amino acid substrate, in contrast to that of its cofactor, probably does cause parallel changes in 5-HT synthesis. Intraperitoneal administration of tryptophan to rats, in a dose which produces a rise in plasma tryptophan levels within the physiologic range, causes proportionate increases in brain 5-HT levels (Fernstrom and Wurtman, 1971*a*). Administering a pharmacologic dose of tryptophan to rats increases brain tryptophan to levels which approach full saturation of tryptophan hydroxylase, and thus yield successively smaller increments in 5-HT synthesis (Weber and Horita, 1965; Fernstrom and Wurtman, 1971*a*; Grahame-Smith, 1971). Conversely, reducing brain tryptophan levels, by placing rats on a tryptophan-deficient diet, lowers brain 5-HT levels (Fernstrom and Wurtman, 1971*c*; Fernstrom and Hirsch, 1975; Messing et al., 1976).

1.3.2 Neuronal Activity and Serotonin Synthesis

The relationship between tryptophan availability and 5-HT synthesis is observed during periods of neuronal quiescence and depolarization, and thus is apparently not highly dependent on the firing rate of serotoninergic neurons. Resting brain synaptosomes (Karobath, 1972; Collard et al., 1982) and slices (Nagatsu et al., 1983) increase their rate of 5-HT synthesis proportionate to the concentration of tryptophan provided in the incubation medium. *In vivo* administration of certain pharmacological agents [i.e., MAO inhibitor; chlorimipramine; 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT)] which suppress the impulse flow of

serotoninergic midbrain raphe neurons (Aghajanian et al., 1970; Sheard et al., 1972; De Montigny et al., 1984) fails to attenuate the enhancement in 5-HT synthesis caused by elevating brain tryptophan levels by administering the amino acid or by consuming certain foods (Jacoby et al., 1975; Tian et al., 1989).

Electrical stimulation of serotoninergic perikarya in the midbrain raphe nuclei increases tryptophan hydroxylase activity in rat forebrain (Eccleston et al., 1970; Shields and Eccleston, 1972; Herr et al., 1975) and spinal cord (Bourgoin et al., 1980). The stimulation-induced increase in 5-HT synthesis is not associated with a detectable elevation in tissue tryptophan levels, but has been attributed to a kinetic change in tryptophan hydroxylase activity. Assay of tryptophan hydroxylase activity in soluble extracts prepared from brain tissue depolarized *in vivo* or *in vitro* reveals an increase in the V_{max} of the enzyme, with no change in its affinities for tryptophan or biopterin (Hamon et al., 1979; Boadle-Biber et al., 1986). Significantly, the rate of 5-HT synthesis by activated tryptophan hydroxylase is still sensitive to changes in tryptophan availability: In depolarized brain slices, the rate of 5-HT synthesis varies with the concentration of tryptophan in the incubation medium (Elks et al., 1979*b*; Auerbach and Lipton, 1985).

1.3.3 Phosphorylation-Induced Activation of Tryptophan Hydroxylase

The depolarization-induced activation of tryptophan hydroxylase is likely to involve a conformational change in the molecule resulting from its phosphorylation by a Ca^{2+}-dependent process (Hamon et al., 1978; Kuhn et al., 1978). Experimental manipulations which promote the influx of extracellular Ca^{2+} into brain slices (e.g., depolarization, calcium ionophores, ouabain, Na-free medium) activate tryptophan hydroxylase in soluble extracts

(Boadle-Biber, 1979), and render the enzyme significantly less sensitive to phosphorylating conditions as compared to enzyme from untreated slices (Hamon et al., 1979).

Initial attempts to characterize the molecular mechanisms mediating the activation of tryptophan hydroxlyase addressed the possible involvement of phosphorylation by cyclic adenosine monophosphate (cAMP)-dependent protein kinase. Several studies demonstrated an increase in tryptophan hydroxylase activity in soluble extracts prepared from brain slices pretreated with drugs that activate cAMP-dependent mechanisms: The addition of dibutyryl cAMP (Hamon et al., 1973; Boadle-Biber, 1980; Nagatsu et al., 1983), xanthine phosphodiesterase inhibitors (Boadle-Biber, 1982*b*), or forskolin (Garber and Makman, 1987) to the incubation medium activated soluble tryptophan hydroxylase activity. However, other agents that activate cAMP-dependent mechanisms (cholera toxin, adenosine, 2-chloroadenosine, 8-bromo-cAMP, nonxanthine phosphodiesterase inhibitors) failed to alter tryptophan hydroxylase activity (Boadle-Biber, 1982*b*). Futhermore, direct addition of dibutyryl cAMP, cAMP-dependent protein kinase, or the catalytic subunit of cAMP-dependent protein kinase to the enzyme assay medium had no effect on tryptophan hydroxylase activity (Kuhn et al., 1978; Lysz and Sze, 1978; Boadle-Biber, 1982*b*). Boadle-Biber (1982*b*) suggested that the ability of some agents that activate cAMP-dependent processes to enhance tryptophan hydroxylase activity may be mediated by an elevation in intracellular Ca^{2+} levels caused by release of the ion from intracellular stores. This hypothesis has not, however, been tested rigorously and has been challenged recently by the observation that the addition of 8-thiomethyl-cAMP to the assay medium of soluble tryptophan hydroxylase stimulates its activity (by 20%; Garber and Makman, 1987). This latter result reinvokes the possibility that a cAMP-dependent process may be directly involved in small alterations in tryptophan

hydroxylase activity independent of an effect on intracellular Ca^{2+} levels. However, the sum of the evidence does not support the hypothesis that the activation of tryptophan hydroxylase is mediated by it being phosphorylated by cAMP-dependent protein kinase, but it does not preclude the potential involvement of cAMP-dependent mechanisms in other covalent or noncovalent modifications of tryptophan hydroxylase.

The Ca^{2+}-dependent activation of tryptophan hydroxylase is probably mediated by the activity of brain specific Ca^{2+}/calmodulin-dependent protein kinase II (Yamauchi and Fujisawa, 1979; Yamauchi et al., 1981; Yamauchi and Fujisawa, 1983). An elevation in the Ca^{2+} concentration in serotoninergic nerve terminals, secondary to the depolarization-induced activation of voltage-sensitive Ca^{2+} channels in the plasma membrane, is likely to be a prerequisite to the kinase II-dependent activation of tryptophan hydroxylase (Cohen, 1985). Activation of soluble tryptophan hydroxylase *in vitro* requires the presence of ATP, Mg^{2+}, calmodulin, micromolar Ca^{2+}, and importantly, the 70 kDa (dimer of two identical 35 kDa subunits) brain 14-3-3 activator protein (Yamauchi et al., 1981; Ichimura et al., 1987). The *in vitro* reaction proceeds in two steps: phosphorylation of the hydroxylase by the kinase, and then activation of the phosphorylated hydroxylase by brain 14-3-3 protein (Yamauchi and Fujisawa, 1981). Maximal activation of tryptophan hydroxylase requires the incorporation of at least 1 mole of phosphate per mole of tetrameric native enzyme (Ehret et al., 1989); three serine residues within each 444-amino acid subunit of tryptophan hydroxylase are probable phosphorylation sites of Ca^{2+}/calmodulin-dependent protein kinase II (Darmon et al., 1988). Activation of tryptophan hydroxylase by Ca^{2+}/calmodulin-dependent protein kinase II induces maximally a two-fold increase in the rate of conversion of tryptophan to 5-HTP (Yamauchi

and Fujisawa, 1979; Yamauchi and Fujisawa, 1981; Ehret et al., 1989). To date, the kinetics of this enzyme activation appear to be untested.

1.3.4 Tryptophan Availability and Serotonin Synthesis

Tryptophan is an essential amino acid, composing 1 - 2% of a typical protein (Cohn, 1943), and thus the availability of tryptophan to the brain *in vivo* is dependent on food intake. Circulating tryptophan, and all other large neutral amino acids (LNAAs) have access to the brain by becoming associated with a specific transporter which facilitates their diffusion across the blood-brain barrier (BBB) (Oldendorf, 1971; Richter and Wainer, 1971). This carrier has an affinity for each of the LNAAs which approximates its concentration in the plasma (i.e., approximate tryptophan K_m value and its plasma concentration are 0.19 mM and 0.1 mM, respectively), and is saturated at physiologic plasma levels of the LNAAs (V_{max} approximately 30 mmol min^{-1} g^{-1}) (Banos et al., 1973; Pardridge and Oldendorf, 1975). Thus, tryptophan (and each of the LNAAs) must compete with the other LNAAs for transport across the BBB. Changes in brain tryptophan levels are predicted by fluctuations in the plasma tryptophan ratio, which is defined as the ratio of the plasma tryptophan concentration to the summed concentrations of the other LNAAs (Fernstrom and Wurtman, 1972*a*; Fernstrom and Faller, 1978). The correlation between brain tryptophan levels and the plasma tryptophan ratio is not improved by taking into consideration that 80 - 90% of circulating tryptophan is bound to the plasma protein albumin (Madras et al., 1974). This finding is compatible with the observed higher affinity of tryptophan for the BBB transporter than for albumin (K_m values for BBB transporter and albumin are 0.19 mM and 0.29 mM, respectively); moreover, exogenous albumin fails to significantly reduce BBB transport of

tryptophan *in vivo* (Pardridge, 1979). Thus, the BBB transporter effectively strips the amino acid off albumin as the blood passes through the cerebral capillaries.

Tryptophan in the brain's extracellular space enters into brain cells by utilizing one or more transport mechanisms located at the plasma membrane (Blasberg and Lajtha, 1965; Grahame-Smith and Parfitt, 1970; Kiely and Sourkes, 1972). Studies utilizing cultured cell lines (C6 glial and 3T3 fibrobrasts; Bauman et al., 1974) and brain synaptosomes (Parfitt and Grahame-Smith, 1973; Bauman et al., 1974; Mandell and Knapp, 1977) have revealed a high-affinity tryptophan uptake component (K_m 10 - 50 μM) distinguishable from a low-affinity component (K_m 0.3 - 1 mM). A high-affinity uptake process for tryptophan has not been observed in brain slices, however, possibly because its detection is obscured by the application of tryptophan concentrations which saturate the high-affinity process, or by the existence of multiple overlapping tryptophan uptake mechanisms (Sershen and Lajtha, 1979). The tryptophan concentration in rat brain extracellular space (assuming it is approximated by levels in rat cerebrospinal fluid and *in vivo* brain microdialysates) is 0.5 - 3 μM (Sarna et al., 1983; Hutson et al., 1985; Martin and Marsden, 1985; Anderson et al., 1987). If a high-affinity uptake process does exist in rat brain, then the influx of extracellular tryptophan *in vivo* is probably largely mediated by this transporter.

Similar to that observed at the BBB, tryptophan's influx into brain cells is competitively inhibited by other LNAAs (Parfitt and Grahame-Smith, 1973; Vahvelainen and Oja, 1975; Lahdesmaki and Hannus, 1977). The functional significance of a second competitive uptake process for LNAAs is unknown. This plasma membrane uptake process is not likely to limit the rate at which changes in the plasma tryptophan ratio are reflected in the tryptophan concentration in brain cells because the maximal rate of tryptophan transport across the BBB

is several-fold slower than across the brain cell membrane (Pardridge, 1977). However, competitive kinetics of tryptophan uptake at the plasma membrane may contribute to more accurately reproducing the plasma LNAA patterns intracellularly.

The processes mediating tryptophan's uptake into serotoninergic neurons apparently do not differ from those operating in other brain cells (i.e., non-serotoninergic neurons, glia). Synaptosomes prepared from rats with midbrain raphe lesions have a markedly reduced (75%) ability to accumulate [^{3}H]5-HT (3 - 10 nM), but show no change in [^{3}H]tryptophan uptake (0.1 - 100 μM) (Kuhar et al., 1972*b*). However, it is possible that serotoninergic neurons do have proportionately more tryptophan uptake carriers than do non-serotoninergic brain cells. The tryptophan concentration in the rat hypothalamus, a region densely innervated by serotoninergic neurons (see Section 2.2), is apparently as much as two-fold higher than in other brain regions (Knott and Curzon, 1974; MacKenzie and Trulson, 1978), and the initial rate of tryptophan uptake into brain slices of hypothalamus is higher than into slices prepared from other regions (Denizeau and Sourkes, 1977). [A similar suggestion has been made for catecholaminergic neurons, i.e., that they have a relatively high density of tyrosine uptake macromolecules (Morre et al., 1980; Morre and Wurtman, 1981).] If this were the case, serotoninergic neurons may be unusually well-suited to sense changes in the concentration of extracellular tryptophan relative to other LNAAs, and to respond by modulating their rate of neurotransmitter synthesis.

Physiologic fluctuations in brain tryptophan levels are driven by the consumption of foods which vary with respect to their relative proportions of carbohydrate and protein: The plasma tryptophan ratio, and thus brain tryptophan levels, increase in rats following consumption of a carbohydrate-rich, insulin-secreting meal (Fernstrom and Wurtman, 1971*b*;

Fernstrom and Faller, 1978; Yokogoshi and Wurtman, 1986). The plasma tryptophan ratio probably increases because circulating insulin causes a greater fall in plasma levels of the competing LNAAs, by enhancing their uptake into skeletal muscle, than of albumin-bound tryptophan (Fernstrom and Wurtman, 1972*b*). The carbohydrate-induced elevation in brain tryptophan levels accelerates brain 5-HT synthesis (Colmenares et al., 1975), and thereby causes its levels to rise (Fernstrom and Wurtman, 1971*b*; Colmenares et al., 1975; Teff and Young, 1988). In contrast, consuming a protein-rich meal either reduces (Yokogoshi and Wurtman, 1986; Teff et al., 1989) or fails to increase (Fernstrom and Faller, 1978) the plasma tryptophan ratio because the rise in plasma levels of the other LNAAs is proportionately greater than the rise in plasma tryptophan levels (i.e., tryptophan is a scarce amino acid in dietary protein). Reductions in the plasma tryptophan ratio reduce brain tryptophan levels, and thereby cause 5-HT levels to fall (Fernstrom and Wurtman, 1972*a*; Teff and Young, 1988).

1.3.5 Feedback Regulation of Serotonin Synthesis

It is possible that tryptophan hydroxylase activity is feedback-regulated by levels of brain 5-HT utilizing mechanisms similar to those demonstrated to regulate tyrosine hydroxylase activity (i.e., intraneuronal endproduct inhibition; autoreceptor-mediated inhibition) (Udenfriend, 1966; Demarest and Moore, 1979). Intraperitoneal administration of a MAO inhibitor to rats, in a dose which raises brain 5-HT levels two- to three-fold, decreases (by approximately 40%) the rate of conversion of [^{3}H]tryptophan to [^{3}H]5-HT within the first 2 hours after treatment (Macon et al., 1971; Hamon et al., 1973). These observations were initially interpreted as indicative of an intraneuronal negative feedback mechanism regulating tryptophan hydroxylase activity. However, from 3 to 72 hours after MAO inhibition, brain

5-HT levels remain elevated but no change in the rate of tryptophan hydroxylation is observed (Lin et al., 1969*a*; Millard and Gal, 1971; Bloom and Costa, 1971). This is in contrast to the tonic inhibition of brain tyrosine hydroxylase activity during prolonged elevations in brain catecholamine levels (Lin et al., 1969*a*; Bloom and Costa, 1971). Furthermore, partially purified tryptophan hydroxylase activity is not significantly inhibited by adding to the *in vitro* assay 0.1 mM 5-HT (Jequier et al., 1969; Kaufman, 1974), a concentration which is about twenty-times higher than that produced intraneuronally by MAO inhibition. Thus, tryptophan hydroxylase activity appears not to be inhibited directly by high intraneuronal levels of 5-HT. The transient inhibition in 5-HT synthesis observed shortly after MAO inhibition may be due, in part, to transsynaptic changes secondary to elevated catecholamine levels.

Experiments *in vitro* have demonstrated that 5-HT synthesis is reduced in brain slices (Hamon et al., 1973) and synaptosomes (Karobath, 1972) by incubating them in medium containing exogenous 5-HT (1 - 100 μM). This effect is probably not due, as originally suggested, to direct inhibition of tryptophan hydroxylase activity resulting from an elevation in intraneuronal 5-HT levels secondary to enhanced uptake of 5-HT, but more likely results from a receptor-mediated process which couples extracellular 5-HT levels to 5-HT synthesis. Presynaptic 5-HT receptors located on serotoninergic neurons (autoreceptors) may regulate tryptophan hydroxylase activity in a manner similar to the inhibition of 5-HT release (Cerrito and Raiteri, 1979; Gohert and Weinheimer, 1979) and the inhibition of serotoninergic nerve firing (Aghajanian et al., 1972; Haigler and Aghajanian, 1974) observed when synaptic 5-HT levels are elevated. The existence of synthesis-modulating autoreceptors is vaguely suggested by some pharmacological experiments demonstrating effects of serotoninergic drugs with

known affinities for 5-HT autoreceptors on 5-HT synthesis, but has not been as clearly delineated as the nerve terminal release-modulating (classified pharmacologically as the 5-HT_{1B} receptor subtype) nor the somatodendritic nerve firing-modulating (5-HT_{1A} receptor subtype) autoreceptors (Verge et al., 1985; Maura et al., 1986; Raiteri et al., 1986; Sprouse and Aghajanian, 1987): The 5-HT autoreceptor agonist LSD (Langer and Moret, 1982) decreases brain 5-HT synthesis *in vivo* (Lin et al., 1969*b*; Schubert et al., 1970; Pettibone and Pflueger, 1984). This effect of LSD may result primarily from autoreceptor-mediated inhibition of nerve firing (Aghajanian et al., 1972), but may also be due to direct activation of synthesis-modulating autoreceptors. Methiothepin, a potent release-modulating autoreceptor antagonist (Cerrito and Raitero, 1979; Baumann and Waldmeier, 1981) suppresses the LSD-induced inhibition of hypothalamic 5-HT synthesis (Pettibone and Pflueger, 1984). Quipazine, a 5-HT autoreceptor antagonist in forebrain nerve terminals (Martin and Sanders-Bush, 1982) increases 5-HT synthesis in depolarized brain slices (Hamon et al., 1984).

The putative existence of 5-HT synthesis-modulating autoreceptors raises the possibility that the transient inhibition of tryptophan hydroxylase associated with MAO inhibition, as described above, may result from enhanced synaptic levels of 5-HT. Indeed, inhibition of MAO activity does increase spontaneous (El Hawary et al., 1967; Eccleston et al., 1969; Holman and Vogt, 1972) and depolarization-induced (Auerbach and Lipton, 1985) 5-HT release. Future studies are required to address whether autoreceptors coupled to tryptophan hydroxylase activity actually do exist, and the mechanism by which this may occur.

1.4 Fate of Newly-Synthesized Serotonin

1.4.1 Serotonin Metabolism

Serotonin is synthesized in the cytoplasm, and is therefore susceptible to deamination by MAO attached tightly to the mitochondrial outer membrane (Schnaitman et al., 1967). The deaminated product of 5-HT, 5-hydroxyindoleacetaldehyde, is largely oxidized to form the major metabolite of 5-HT, 5-HIAA (Lane and Aprison, 1978). MAO exists in at least two forms in a variety of tissues, including brain (Johnston, 1968; Kim and D'Iorio, 1968). These isoenzymes have been distinguished by their differential sensitivity to inhibitors: MAO-A is inhibited by clorgyline, and MAO-B is inhibited by deprenyl (Yang and Neff, 1974; Ekstedt, 1976). Utilizing these inhibitors, it has been revealed that brain 5-HT is a preferential substrate of MAO-A *in vitro*, but can also be metabolized by MAO-B, albeit at ten-fold higher K_m and lower V_{max} values (Mitra and Guha, 1980; Fowler and Tipton, 1982).

The physiologically relevant issue, however, is the relative proportions of the MAO isoenzymes contained in serotoninergic neurons. Immunohistochemical and enzymatic studies have shown that levels of the MAO isoenzymes are unevenly distributed throughout the brain (Saavedra et al., 1976; Hirano et al., 1978; Pintar et al., 1983; Westlund et al., 1985). Significantly, brain regions containing a high density of serotoninergic perikarya and nerve terminals are not consistently matched by a relatively high proportion of the MAO-A isoenzyme (Williams et al., 1975; Suzuki et al., 1977; Kim et al., 1979; Levitt et al., 1982; Westlund et al., 1985). This apparent 'mismatch' in some brain regions probably contributes to the range of ratios of 5-HIAA to 5-HT levels measured in various brain regions (Knott and

Curzon, 1974; MacKenzie and Trulson, 1978), and to regulating the availability of releasable 5-HT.

1.4.2 Serotonin Storage

Serotonin which escapes degradation by MAO may be stored intraneuronally. A fraction of newly-synthesized, or reuptaken, 5-HT may be taken up into synaptic vesicles by a Mg^{2+}/ATP-dependent process; the active uptake of [^{3}H]5-HT into synaptic vesicles prepared from rat brain displays saturable kinetics with a K_m value of 1.25×10^{-7} M (Halaris and DeMet, 1978). Within rat brain synaptic vesicles, 5-HT may complex to a serotonin binding protein (SBP) with high-affinity ($K_d = 10^{-8}$ M; Tamir and Huang, 1974). That SBP is four-fold more concentrated in synaptosomal synaptic vesicles than in synaptosomal cytosol suggests that this protein may be involved in the storage of 5-HT, particularly in synaptic vesicles (Tamir and Gershon, 1979).

Immunocytochemical studies consistently confirm the association of 5-HT with synaptic vesicles in nerve terminals of the central nervous system (Pelletier et al., 1981; Maley and Elde, 1982; Pasik et al., 1982; Ruda et al., 1982; Maxwell et al., 1983). The 5-HT immunoreactive product is sometimes also noted to be present in the axoplasm (distinct from that associated with mitochondria) (Ruda et al., 1982). Although the latter observation may simply reflect the localization of newly-synthesized or reuptaken 5-HT, or diffusion of the reaction product, other biochemical evidence suggests that 5-HT may also be stored in a nonvesicular cytoplasmic compartment. The supernatant fraction of synaptosomes prepared from rat brain contains significant concentrations of 5-HT (Maynert et al., 1964; Halaris and Freedman, 1977). In addition, a soluble actin-like protein which binds 5-HT with high-affinity

($K_d = 10^{-5}$ M) has been partially purified from rat brain synaptosomes (Small and Wurtman, 1984). A small fraction of exogenous [^{3}H]5-HT taken up by rat pheochromocytoma (PC12) cells is associated with this soluble actin-like protein (Small and Wurtman, 1985*b*), and the kinetics of this association appear to be regulated by divalent cations, including Ca^{2+} (Small and Wurtman, 1985*a*).

The probable existence of multiple storage compartments for 5-HT is often used to support the idea of a functional distinction between cytoplasmic and vesicular 5-HT. Biochemical studies have demonstrated a biphasic loss of newly-synthesized [^{3}H]5-HT from depolarized brain tissue (Shields and Eccleston, 1972; Shields and Eccleston, 1973); and the specific activity of released radioactivity is higher than that of the tissue itself (Shields and Eccleston, 1973; Elks et al., 1979*b*). These findings suggest that newly-synthesized 5-HT is preferentially released. Pharmacological studies have suggested that certain drugs (i.e., LSD, p-chloroamphetamine, fenfluramine, quipazine) act preferentially on separate 5-HT pools to induce changes in 5-HT levels and release (Mennini et al., 1981; Kleven et al., 1983; Kuhn et al., 1985). Cumulatively, these findings have been interpreted as supporting the existence of a small 'functional' pool (possibly cytoplasmic) which is sensitive to the acute demands of serotoninergic neurons, while a larger 'reserve' pool (possibly vesicular) is largely unavailable for immediate release (see Kuhn et al., 1986). In contradistinction, the likelihood that 5-HT is stored primarily in synaptic vesicles often invokes the classic exocytotic mechanism of neurotransmitter release (see Sanders-Bush and Martin, 1982). The criteria for exocytotic release have not, however, been met for 5-HT (see Kuhn et al., 1986). Attempts to integrate these seemingly conflicting hypotheses has led to the delineation of various models requiring a

dynamic exchange between two 5-HT compartments (Tracqui et al., 1983; Auerbach and Lipton, 1985; Kuhn et al., 1986).

Though it is likely that 5-HT is stored in multiple subcellular compartments, a functional relationship has been demonstrated between total brain 5-HT levels and particular behaviors thought to be mediated by serotoninergic neurons. Increasing brain 5-HT levels in rats by administering tryptophan intraperitoneally decreases their locomotor activity (Taylor, 1976), food intake (Latham and Blundell, 1979), and frequency of haloperidol-induced head movements (Kozell et al., 1987); tryptophan administration has also been reported to induce sedation (Stewart et al., 1976) and to potentiate the monosynaptic reflex (Anderson and Shibuya, 1966; Barai and Roberts, 1973). Rats which raise their brain tryptophan levels, and thereby brain 5-HT levels, by consuming a carbohydrate-rich meal (Fernstrom and Wurtman, 1971*b*) eat less carbohydrate, relative to protein, at the subsequent meal (Wurtman et al., 1983). Unphysiologic elevations in rat brain 5-HT levels, induced by inhibiting MAO and administering tryptophan, cause gross hyperactivity and hyperautonomia (Grahame-Smith, 1971). Tryptophan given orally to humans reportedly increases daytime drowsiness (Smith and Prockup, 1962; Greenwood et al., 1975), decreases nighttime sleep latency (Hartman and Spinweber, 1979), 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).

Experimental treatments which reduce rat brain 5-HT levels (by placing them on a tryptophan-deficient diet; making chemical lesion of serotoninergic neurons by use of 5,7-dihydroxytryptamine; making electrolytic lesion of the medial forebrain bundle or selective raphe nuclei; or inhibiting tryptophan hydroxylase with p-chlorophenylalanine) increase their

pain sensitivity (Harvey and Lints, 1965; Harvey et al., 1975; Lytle et al., 1975), motor activity (Kostowski et al., 1968; Jacobs et al., 1975; Marsden and Curzon, 1976), reactivity to novel stimuli (Srebro and Lorens, 1975; Walters et al., 1979), and food intake (Hoebel et al., 1978). Reducing rat brain 5-HT levels also decreases the frequency of apomorphine-induced stereotypy (Sahakian et al., 1979), and facilitates certain aggressive behaviors (Gibbons et al., 1979; Kantak et al., 1980; Kantak et al., 1981). Each of these behavioral changes is reversed if brain 5-HT levels are elevated by directly administering a 5-HT precursor (Lytle et al., 1975; Marsden and Curzon, 1976; Hoebel et al., 1978; Sahakian et al., 1979) or by dietary manipulation (Lytle et al., 1975; Gibbons et al., 1979; Walters et al., 1979; Kantak et al., 1980; Kantak et al., 1981).

1.4.3 Serotonin Release

5-HT is released spontaneously from forebrain nerve terminals *in vivo* (Feldberg and Myers, 1966; Portig and Vogt, 1969; Ashkenazi et al., 1973); this rate is enhanced by electrically stimulating their perikarya in the midbrain raphe nuclei (Eccleston et al., 1969; Holman and Vogt, 1972; Chiueh and Moore, 1976). Release of 5-HT *in vivo* is sensitive to the presence of extracellular Ca^{2+} (Hery et al., 1979; Yaksh and Tyce, 1980; Kalen et al., 1988). The influx of extracellular Ca^{2+} through voltage-sensitive Ca^{2+} channels is triggered by invading action potentials originating from the somatodendritic region, and is thought to be required for the activation of subcellular processes involved in neurotransmitter release (for review, see Augustine et al., 1987). Evoked, but not spontaneous, 5-HT release *in vitro* is Ca^{2+}-dependent (Mulder et al., 1975; Elks et al., 1979*a*; Gohert, 1980). This distinction suggests that spontaneous release *in vitro* is regulated by factors other than the influx of Ca^{2+};

receptor-mediated events occurring at nerve terminals may be largely insensitive to the extracellular Ca^{2+} concentration, but may contribute to spontaneous 5-HT release *in vitro* and *in vivo*.

5-HT release from nerve terminals appears to be frequency-dependent. Decreasing the impulse flow along serotoninergic neurons by administering a 5-HT_{1A} autoreceptor agonist (i.e., LSD or 8-OH-DPAT) reduces 5-HT release in the forebrain (Gallager and Aghajanian, 1975; Sharp et al., 1989*a*; Sharp et al., 1989*b*). Increasing the frequency of electrical stimulation delivered to serotoninergic raphe nuclei *in vivo* (0.5 - 20 Hz; Holman and Vogt, 1972; Sharp et al., 1989*b*) or to *in vitro* brain slices (0.1 - 10 Hz; Gohert, 1980; Baumann and Waldmeier, 1981) causes incremental changes in the total amounts of 5-HT released; but the amounts of 5-HT released per pulse are inversely related to stimulation frequency. This latter observation may reflect a negative-feedback process regulated by nerve terminal release-modulating autoreceptors. The observed proportionality between stimulation frequency and total 5-HT release suggests that at stronger depolarizations the activity of release-modulating autoreceptors may be increased secondary to elevated synaptic 5-HT levels.

The presence in nerve terminals of release-modulating autoreceptors has been suggested by *in vitro* studies (utilizing slices or synaptosomes of rat hypothalamus, cerebral cortex, spinal cord, cerebellum and hippocampus) demonstrating a decrease in evoked [^{3}H]5-HT release in the presence of unlabelled 5-HT (10^{-8} - 10^{-6} M), an effect which is blocked by the 5-HT_{1B} receptor antagonist methiothepin and is mimicked by the 5-HT_{1B} receptor agonist RU-24969 (Cerrito and Raiteri, 1979; Gohert and Weinheimer, 1979; Monroe and Smith, 1985; Bonanno et al., 1986; Maura et al., 1986). The existence of nerve terminal release-modulating autoreceptors has been further supported by *in vivo* microdialysis studies which

report a reduction in extracellular 5-HT after injection of the presumed 5-HT_{1B} receptor agonist RU-24969 (Brazell et al., 1965; Sharp et al., 1989*a*); this effect is thought to be related directly to its activation of nerve terminal release-modulating autoreceptors, and not indirectly to somatodendritic impulse-modulating autoreceptors (Sharp et al., 1989*a*)

Activity of the nerve terminal autoreceptor has been reported to modulate the evoked, Ca^{2+}-dependent release of [^{3}H]5-HT (Gohert, 1980; Langer and Moret, 1982). Kinetic analysis of the dependence of [^{3}H]5-HT efflux from slices of rat cerebral cortex on the Ca^{2+} concentration present during membrane depolarization (induced by a high K^+ medium) reveals an increase in the K_m value in the presence of unlabeled 5-HT, and a decrease in the presence of the serotoninergic receptor antagonist, methiothepin (Gohert, 1980). These findings suggest that the activity of the autoreceptor may modulate 5-HT release by effecting the Ca^{2+} affinity of the subcellular elements involved in the release process.

Nerve terminal release-modulating autoreceptors in rat cerebral cortex (Middlemiss, 1984; Engel et al., 1986), hippocampus (Maura et al., 1986) and cerebellum (Bonanno et al., 1986; Raiteri et al., 1986) have been pharmacologically characterized as being of the 5-HT_{1B} subtype. 5-HT_{1B} receptors in homogenates of rat substantia nigra, a brain region particularly enriched in this receptor subtype (Pazos and Palacios, 1985), are negatively coupled to adenylate cyclase (Bouhelal et al., 1988). If the 5-HT_{1B} release-modulating autoreceptor is also linked to this second messenger, then changes in cAMP levels may initiate the altered Ca^{2+} sensitivity of 5-HT release.

The functional 5-HT_{1B} autoreceptor may be pharmacologically distinct from the 5-HT_{1B} binding site identified autoradiographically in rat brain. Chemical lesion of serotoninergic neurons by intracerebral injection of 5,7-dihydroxytryptamine markedly reduces 5-HT uptake

but variously renders 5-HT_{1B} binding sites unchanged in many brain regions (Verge et al., 1986); reduced (by 37%) in the substantia nigra (Verge et al., 1986); and increased (by 56%) in cerebral cortex (Blurton and Wood, 1986). Thus 5-HT_{1B} binding sites are probably not exclusively presynaptic, and they may not be highly correlated with the release-modulating 5-HT_{1B} properties.

1.4.3a Relationship Between Serotonin Synthesis and Release

The rate at which the brain synthesizes 5-HT *in vivo* varies with changes in brain tryptophan levels because tryptophan hydroxylase in unsaturated with its substrate (see Section 1.3.1). Experimental manipulations which alter the rate of 5-HT synthesis are correlated with changes in particular behaviors (see Section 1.4.2). These observations suggest that changes in the rate of 5-HT synthesis are coupled to changes in serotoninergic neurotransmission. Evidence supporting a physiologic coupling between an acceleration in 5-HT synthesis, induced by increasing brain tryptophan levels, and 5-HT release from nerve terminals has been presented by some experiments performed *in vivo*.

The amounts of 5-HT released spontaneously into media perfusing the rat lateral ventricle (Ternaux et al., 1976) or the ependymal surface of the cat caudate nucleus (Ternaux et al., 1977) increase two- to three-fold following tryptophan administration (100 mg/kg i.p.). Extracellular 5-hydroxyindole levels, as monitored by *in vivo* voltammetry, in the rat hippocampus increase dose-dependently after tryptophan administration (50 or 100 mg/kg i.p., Joseph and Kennett, 1981). In contrast, tryptophan administration failed to increase the voltammetric signal in the rat striatum (100 mg/kg i.p., Marsden et al., 1979; 150 mg/kg p.o., De Simoni et al., 1987*b*). This signal, however, reflects not only extracellular 5-HT, but also

5-HIAA and possibly other interfering substances (i.e., uric and ascorbic acids; Joseph and Kennett, 1981; Baumann and Waldmeier, 1984). A modified voltammetric method, which selectively detects extracellular 5-HT, has been used to demonstrate a tryptophan-induced (75 mg/kg i.p.) elevation (by 25%) in 5-HT release in the rat striatum (Broderick and Jacoby, 1988). Even more recently, a dose-dependent enhancement (40 - 150%) in extracellular 5-HT levels was measured by *in vivo* microdialysis in the frontal cortex of freely moving rats following injection of the amino acid (50, 100 or 200 mg/kg i.p., Carboni et al., 1989).

5-HT released from activated neurons is apparently also sensitive to changes in brain tryptophan levels. Immobilization stress enhances 5-hydroxyindole release in the rat hippocampus in a manner more pronounced than that elicited by intraperitoneal administration of tryptophan; and this stress-induced effect is dependent on the increased availability of tryptophan to the brain (Joseph and Kennett, 1983). Tryptophan administration (150 mg/kg p.o.) potentiates evoked 5-hydroxyindole release induced by electrical stimulation of the dorsal raphe nucleus (De Simoni et al., 1987*b*). Similarly, the addition of tryptophan (50 μM) to the medium perfusing the rat nucleus accumbens prevents the decrements in evoked 5-HT release (induced locally by elevating the extracellular K^+ concentration to 35 mM) which occur with successive periods of stimulation (Guan and McBride, 1987).

Cumulatively, these *in vivo* experiments suggest that accelerating brain 5-HT synthesis by elevating brain tryptophan levels causes a parallel increase in the amounts of 5-HT released spontaneously and with neuronal firing. Significantly however, administering a pharmacological dose of tryptophan (i.e., 50 - 200 mg/kg) or providing a high concentration of extracellular tryptophan (50 μM) produces elevations in brain tryptophan levels beyond the physiologic range. For example, intraperitoneal administration of tryptophan at the lowest

dose used in the experiments described above, 50 mg/kg, elevates brain tryptophan levels approximately four-fold (Grahame-Smith, 1971; MacKenzie and Trulson, 1977), an increase which is much greater than the two-fold variation in brain tryptophan levels which occurs after the consumption of certain meals (Fernstrom and Wurtman, 1971*b*; Fernstrom et al., 1973), diurnally (Morgan et al., 1975; Hery et al., 1977), with exercise (Chaouloff et al., 1985) or after particular stressors (Knott et al., 1973; Kennett and Joseph, 1981; Culman et al., 1984). Furthermore, injecting 50 mg/kg of tryptophan elevates rat brain 5-HT levels by no more than lower tryptophan doses (Fernstrom and Wurtman, 1971*a*); this suggests that the concentration of brain tryptophan produced by a 50 mg/kg dose approaches that which saturates tryptophan hydroxylase and/or the 5-HT storage capacity of its neurons.

In vitro studies have similarly yielded results which are confounded by the experimental methods applied. A dose-dependent enhancement (26 - 100%) in 5-HT released from rat hippocampal slices during membrane depolarization, but not under basal conditions, has been demonstrated with the addition of tryptophan (2 or 10 μM) to the incubation medium (Auerbach and Lipton, 1985). In this study, slice membranes were depolarized by elevating the extracellular K^+ concentration to 60 mM, a concentration which these authors showed to evoke the release of 5-HT in a Ca^{2+}-independent manner. In addition, the incubation medium contained a 5-HT reuptake inhibitor (to allow measurement of released 5-HT), and a release-modulating autoreceptor antagonist. The latter drug may have obviated a physiologically relevant mechanism controlling 5-HT release.

Another *in vitro* study, utilizing rat whole-brain slices, observed a dose-dependent increase in spontaneous and electrically-evoked 5-HT release with increases in the medium

tryptophan concentration (0, 2, 10 or 100 μM) in the presence, but not in the absence, of a MAO inhibitor (Elks et al., 1979*b*). The tryptophan-induced enhancement in extracellular 5-HT in the presence of an MAO inhibitor is likely to have resulted from leakage of intracellular 5-HT secondary to an unregulated elevation in tissue 5-HT levels similar to that observed *in vivo* (Grahame-Smith, 1971). The failure to observe an effect of tryptophan supplementation on 5-HT release in the absence of a MAO inhibitor may involve the delivery of electrical pulses, applied to evoke neurotransmitter release, at a frequency (100 Hz) which far exceeds that occurring *in vivo* in serotoninergic dorsal raphe cell bodies (0.1 - 6 Hz; McGinty and Harper, 1976; Trulson and Jacobs, 1979).

1.4.3b Reuptake of Released Serotonin

Synaptic 5-HT, like other biogenic amines, is inactivated by being recaptured into nerve endings (Bogdanski et al., 1970; Iverson, 1970). This recaptured 5-HT may be deaminated by intraneuronal MAO or stored for future release (Reinhard and Wurtman, 1977). The transport of 5-HT from the extra- to intra-cellular space is mediated by a carrier whose activity has been reported to be dependent on the asymmetric distribution of Na^+ and K^+ across the plasma membrane (Bogdanski et al., 1968, Tissari et al., 1969). Kinetic analysis of this transport process in rat brain synaptosomes has demonstrated that the affinity of the carrier for 5-HT is increased in the presence of increasing Na^+ concentrations (10 - 75 mM), and is decreased by elevating the K^+ concentration (6 - 100 mM at 50 mM Na^+) (Bogdanski et al., 1970). These kinetic properties of the carrier suggest that during neuronal depolarization the action of synaptic 5-HT may be potentiated as a consequence of a transient suppression in 5-HT reuptake: A decrease in the extracellular Na^+ concentration in the vicinity of the carrier may

decrease its affinity for 5-HT; with subsequent repolarization, as Na^+ is extruded by Na^+-K^+ATPase, synaptic 5-HT may bind with high-affinity to the Na^+-complexed carrier; transport of the tripartite complex to the inner membrane surface exposes the carrier to a relatively high K^+ concentration, thus favoring dissociation of 5-HT.

Kinetic analysis of the uptake of [^{3}H]5-HT into brain slices (Shaskan and Snyder, 1970) and synaptosomes (Ross, 1982) reveals at least two transport processes, one with high-affinity for 5-HT (K_m approximately 0.1 μM), and another with a relatively low-affinity (K_m approximately 50 μM). Inhibition studies with catecholamines suggest that the low-affinity process reflects 5-HT uptake into catecholaminergic neurons, whereas the high-affinity process represents its selective uptake into serotoninergic neurons (Shaskan and Snyder, 1970). This proposal is compatible with the results of lesion studies which differentially destroy catecholaminergic or serotoninergic neurons: 1) If the medium incubating synaptosomes prepared from 6-hydroxydopamine-treated or control rats contains a low concentration of [^{3}H]5-HT (0.05 μM), then the addition of unlabeled norepinephrine (5 μM) to the medium does not inhibit [^{3}H]5-HT uptake; however, at a higher concentration of [^{3}H]5-HT (10 μM), uptake of the amine is markedly reduced in 6-hydroxydopamine-treated tissue, and norepinephrine fails to substantially reduce [^{3}H]5-HT uptake as it does in control tissue (Iverson, 1970). 2) A substantial reduction in [^{3}H]5-HT uptake into synaptosomes prepared from rats with midbrain raphe lesions is observed at low concentrations of the amine ($\leq$ 0.05 μM), and this effect is attenuated at higher concentrations ($\geq$ 0.1 μM) (Kuhar et al., 1972*b*).

1.5. Research Objectives

The purpose of this research was to test the hypothesis that there exists a physiologic coupling between brain tryptophan availability and 5-HT release. I intended to address *in vitro* whether experimentally-induced changes in brain tryptophan levels, within the physiologic range, modulate the rate of 5-HT release. The specific goals of this project were as follows:

1. Develop an *in vitro* experimental system, utilizing superfused rat hypothalamic slices, to monitor reliably the amounts of endogenous 5-HT released spontaneously and evoked by low-frequency electrical field-stimulation.
2. Characterize this newly-developed *in vitro* experimental system with respect to:
    a. Time-courses of 5-HT release and 5-HIAA efflux under basal conditions and periods of electrical field-stimulation;
    b. Dependence of 5-HT release on extracellular Ca^{2+} and activity of voltage-sensitive Na^{+} channels (i.e., tetrodotoxin-sensitivity);
    c. Integrity of hypothalamic slices as assessed by their retention of tissue lactate dehydrogenase (LDH) activity, and time-course of the ratio of tissue protein to DNA levels;
    d. Time-course of endogenous tryptophan concentrations in superfusion medium;
    e. Time-courses of tissue tryptophan, 5-HT and 5-HIAA levels with superfusion, and the effects of adding tryptophan to the superfusion medium; determine whether these time-courses are modified by exposure to fluoxetine and to periods of electrical field-stimulation.

3. Apply this newly-developed *in vitro* experimental system to examine the effects on 5-HT release (and tissue 5-HT levels) of:

   a. Increasing tissue tryptophan levels, by superfusing the slices in medium supplemented with a low tryptophan concentration (1, 2, 5 or 10 μM);

   b. Decreasing tissue tryptophan levels, by superfusing the slices in medium supplemented with another LNAA which is likely to compete with tryptophan uptake and enhance its efflux;

   c. Varying frequencies of electrical field-stimulation; and the interaction with tryptophan availability.

2. MATERIALS AND METHODS

2.1 Animal Care

Male Sprague-Dawley rats, received from Charles River Breeding Laboratories weighing approximately 150 g, were housed in pairs for 4 - 7 days prior to sacrifice. During this time, they were exposed to 12 hours of light daily (0700 - 1900), and provided *ad libitum* rat chow (Charles River Rat, Mouse and Hamster Formula, 26.3% protein) and tap water. Rats weighing 180 - 250 g were sacrified by decapitation between 1000 - 1200 hours.

2.2 Slice Preparation

Each brain was rapidly removed from the skull and immersed in ice-chilled physiologic medium (previously gassed with 95% O_2/5% CO_2) of the following composition (in mM): NaCl 130, KCl 3.5, $CaCl_2$ 1.3, $MgSO_4$ 1.5, NaH_2PO_4 1, $NaHCO_3$ 25, d-glucose 10. The chilled brain was placed on filter paper with the ventral surface facing up, and a hypothalamic block (5 x 5 x 2 mm) was dissected out and then bissected along the third ventricle. During dissection, the tissue was kept moist with chilled medium. The demi-hypothalami were cut simultaneously into 300 μm coronal slices using a McIlwain tissue slicer, and then submerged into chilled medium contained in a petri dish. Each slice was isolated sequentially using a fine sable brush and placed alternately into one of two glass tubes containing chilled medium. For many experiments, some slices (approximately every fifth) were retained, frozen over dry ice and stored at -70°C until assayed biochemically for initial slice levels. The hypothalamic slices in each tube were transferred, using a 1 ml pipetman with its tip cut to widen the opening, into parallel superfusion chambers (0.75 ml

each). The amount of time lapsed from decapitation to placing the slices in the superfusion chambers was approximately 10 minutes. The chambers were constructed as described by Milner and Wurtman (1984), though modified by the placement of a nylon disc on both bottom electrodes. The chambers were in a water bath of 36 - 37°C and were delivered (0.6 ml/min) pre-warmed medium by a peristaltic pump (Rabbit; Rainin Instrument Co., Woburn, MA). The medium was continuously gassed with 95% O_2/5% CO_2 to achieve pH 7.4. Each experiment used the hypothalamus from one or two rats; thus each chamber received the equivalent of one or two demi-hypothalami. The slices were equilibrated by superfusion for 50 minutes from the time that all of the tissue was in the chambers.

2.3 Release Experiments

2.3.1 Experimental Design

In experiments designed to examine the relationships between tryptophan availability and 5-HT release, the superfusion medium contained fluoxetine hydrochloride (2 μM) (provided by Eli Lilly Laboratories, Indianapolis, IN) 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 evoked 5-HT release was recovered.] Each of the parallel superfusion chambers contained the equivalent of two demi-hypothalami. The medium superfusing the slices in one of the two chambers was supplemented with tryptophan (1 - 10 μM); leucine (100 μM); tryptophan (2 μM) plus leucine (100 μM); or histidine (10 μM) (all l-amino acids were purchased from Sigma Chemical Co., St. Louis, MO). Slices in the other chamber were superfused with amino acid-unsupplemented medium, and thus

served as control. Successive 5-minute fractions were collected (FC-80K Fractionator; Gilson Medical Electronics, Inc., Middleton, WI) for 80 minutes, between 50 and 130 minutes from the onset of superfusion. [Due to limitations in the stimulation apparatus, the slices within only one chamber could be electrically field-stimulated at a given point in time. In order to compensate for this inadequacy, the start of fraction collection from the parallel chambers was staggered by 5 minutes.] Each fraction of superfusion medium was collected into a glass tube containing 100 µl of 7 mM ascorbate and 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). Once collected, each fraction was mixed by vortex and stored in the dark, on ice, until undergoing further processing (within 1 - 2 hours). The slices were electrically field-stimulated for three periods of 4.7 minutes each starting at 60, 85, and 110 minutes from the onset of superfusion. [The delivery of electrical pulses to the parallel chamber was staggered by 5 minutes.] Electrical field-stimulation was induced by delivery of bipolar square-wave pulses (5 Hz, 2 ms, 100 mA/cm^2) using a stimulator (S88; Grass Instruments Co., Quincy, MA) in series with a 12 V relay; pulses were monitored by an oscilloscope (V-212; Hitachi Denshi Ltd., Tokyo, Japan). At the end of each experiment (i.e., after 130 minutes of superfusion), the slices were removed from their chambers, quickly rinsed with distilled water, frozen over dry ice, and stored at -70°C until subsequent biochemical assays.

In experiments addressing the Ca^{2+}-dependence of 5-HT release, the slices in one of the two chambers were exposed to Ca^{2+}-free medium, prepared without $CaCl_2$ and containing EGTA (1 mM; Sigma), from the onset of superfusion until 85 minutes; from 85 to 135 minutes the slices were superfused with the usual physiologic medium (1.3 mM $CaCl_2$).

Slices in the other chamber were superfused with physiologic medium throughout. Fractions were collected every 5 minutes between 50 and 85 minutes, and between 100 and 135 minutes. Electrical field-stimulation (using the same parameters as above) was delivered starting at 65 minutes (S1) and again at 115 minutes (S2).

To test whether 5-HT release from the slices required the activation of voltage-sensitive Na^+ channels, the effect of the voltage-sensitive Na^+ channel inhibitor tetrodotoxin (TTX, 1 μM; Sigma) on 5-HT release was monitored. Slices in the two chambers were superfused initially with physiologic medium; between 30 and 85 minutes, 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 one hour (85 - 145 minutes) with physiologic medium, and then superfused at the usual rate (0.6 ml/min) for the remainder of the experiment. Fractions were collected at 5-minute intervals between 50 and 85 minutes and between 145 and 180 minutes. The slices were electrically field-stimulated (parameters as above) beginning at 65 minutes (S1) and 160 minutes (S2).

In experiments addressing the interaction between tryptophan availability and the frequency of membrane depolarization in controlling evoked 5-HT release, each superfusion chamber contained the equivalent of a demi-hypothalamus. The medium superfusing the slices of one of the parallel chambers was supplemented with tryptophan (2 μM); the other slices were superfused with amino acid-unsupplemented medium (control). Ten-minute fractions were collected over a 70-minute period (between 50 and 120 minutes) beginning at 50, 60, 75, 85, 100 and 110 minutes from the onset of superfusion. The slices were electrically field-stimulated for three periods of 4 minutes each, starting at 60, 85, 110 minutes. Therefore, each fraction containing the 5-HT released by electrical field-stimulation

was preceded by a fraction containing 5-HT released spontaneously. Electrical field-stimulation was induced as described above, though the frequency of the stimulator was set at either 1, 3 or 5 Hz, which delivers 240, 720 or 1200 pulses, respectively, over a constant (4 minute) period of electrical field-stimulation.

In control experiments designed to assess the specificity of the tryptophan-induced increase of 5-HT release, the effect of supplementing the medium with tryptophan (2 μM) on DA release from the slices was monitored. Slices from two demi-hypothalami were in both chambers and were superfused (0.5 ml/min) with physiologic medium containing the DA reuptake inhibitor nomifensine maleate (10 μM; provided by Hoechst-Roussel Pharmaceuticals Inc., North Somerville, New Jersey) (Hunt et al., 1974). At 80 minutes from the onset of superfusion, the medium superfusing one of the two chambers was replaced with the same medium to which tryptophan (2 μM) had been added. Five-minute fractions were collected from 50 to 80 minutes and then from 95 to 125 minutes. The slices were electrically field-stimulated (4 Hz, 2 ms, 100 mA/cm^2) for 4-minute periods beginning at 60 minutes (S1) and at 105 minutes (S2). These fractions were collected into a solution containing: 50 μl of 10 mM Na_2EDTA, 100 μl of 10 mM NaS_2O_5, and the internal standard 3,4-dihydroxybenylamine hydrobromide (DHBA; Sigma). [Separate experiments were conducted to assure that under these modified conditions tryptophan supplementation still increased 5-HT release.]

2.3.2 Biochemical Analysis

2.3.2a 5-Hydroxyindoles in Superfusion Medium

The 5-hydroxyindoles 5-HT, 5-HIAA and the two internal standards (5-HT-CH_3 and 5-HICA) were extracted from superfusion medium by passing the fraction through a preparative reverse-phase column. These columns were prepared by loading dry C_{18} sorbent (40 μm; Analytichem International, Harbor City, CA) into glass wool-plugged Pasteur pipettes (9 inch). The 5-minute (3.1 ml) and 10-minute (6.1 ml) fractions were extracted through columns containing 100 and 200 mg of the sorbent, respectively. The columns were first 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 (usually 80 μl and 160 μl in 3.1 ml and 6.1 ml of superfusion medium, respectively). After a sample passed through its column, the aqueous phase in a 100 mg column was displaced by 125 μl of 70% methanol/30% acetic acid, and the aqueous phase in a 200 mg column by 200 μl of this organic solution. The 5-hydroxyindoles were eluted into Eppendorf tubes with 300 μl of this methanol/acetic acid solution, and the solvent was evaporated under a stream of nitrogen. 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 limit of sensitivity for the 5-HT assay was approximately 0.3 pmol (50 pg).

The reconstituted samples (45 of 50 μl) were injected (712; Water Intelligent Systems Program; Milford, MA) over a reverse-phase C_{18} column (5 μm, 25 cm; Beckman Instruments Co., San Ramon, CA). The mobile phase was pumped (110A; Altex Scientific

Inc., Berkeley, CA) over this column at a rate of 1.2 ml/min, and was of the following composition (in mM): NaH_2PO_4 200, Na_2EDTA 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 (LC-4A; Bioanalytical Systems, Inc., West Lafeyette, IN) at 2 nA/V when the potential of the glassy carbon electrode was set at 0.55 V against the Ag/AgCl reference electrode.

2.3.2b Tissue Indole Levels

Frozen tissue samples retrieved before and after slice superfusion were homogenized by ultrasonication in 0.3 ml and 1 ml, respectively, of 0.2 M $HClO_4$ containing 0.5 mM ascorbate and the two internal standards (5-HT-CH_3 and 5-HICA). The homogenates were centrifuged (35,000 g; 10 min); the tissue supernatants were transferred into Eppendorf tubes and recentrifuged by microcentrifuge. Aliquots (50 µl in duplicate) of these supernatants were assayed for tissue indole (tryptophan, 5-HT, 5-HIAA) levels using the same chromatographic system as described in Section 2.3.2a, though the applied potential was set at 0.85 V, in order to detect tryptophan in the samples, and the sensitivity at 5 nA/V. 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.

2.3.2c Dopamine in Superfusion Medium

DA and DHBA were extracted from fractions of superfusion medium (2.65 ml) utilizing alumina columns. These columns were prepared by loading dry, activated alumina (50 mg) into glass wool-plugged Pasteur pipettes (9 inch). Each column was pretreated with 1 ml of 50 mM Tris HCl (pH 8.6). Just before loading a sample, 1 ml of 2 M Tris HCl (pH 8.6) was added to it, and mixed by vortex. Following passage of a sample, the column was washed with 0.5 ml of distilled water (Millipore Corp., Bedford, MA), and then with 100 µl of 0.5 M acetic acid. DA was eluted from the column, and into Eppendorf tubes, with 550 µl of 0.5 M acetic acid. These eluates were dried by lyophilization or under nitrogen. The dried samples were reconstituted in 60 µl of 0.1 M HCl. Samples (50 of 60 µl) were assayed by high-performance liquid chromatography with electrochemical detection as described in Section 2.3.2a, though the mobile phase was of the following composition (in mM): NaH_2PO_4 100, Na_2EDTA 0.1, octyl sodium sulfate 0.05, with 4% methanol (vol/vol) and having a final pH of 4.4.

2.4 Time-Course of Tryptophan Concentrations in Superfusion Medium

The concentrations of endogenous tryptophan in the superfusion medium were monitored during slice superfusion and with electrical field-stimulation. Each superfusion chamber contained the equivalent of two demi-hypothalami and was superfused (0.6 ml/min) with amino acid-unsupplemented medium. Slices in one of the parallel chambers were electrically field-stimulated (4.7 min, 5 Hz, 2 ms, 100 mA/cm^2) starting at 60, 85, and 110 minutes; slices in the other chamber were just superfused throughout. Fractions (15 sec, 150 µl) of superfusion medium were collected from the latter chamber starting at 5, 15, 30, 60, 90

and 120 minutes; fractions (15 sec, 150 µl) from the electrically field-stimulated chamber were collected starting at 5, 15, 30, 60, 65, 85, 90, 110 and 115 minutes. Ten microliters of 0.75 mM ascorbate containing the internal standard 5-HICA were added to each fraction (final concentration of ascorbate was 0.5 mM). The samples were stored on ice until assay (usually within 20 minutes). Aliquots (50 µl) of these samples were assayed by high-performance liquid chromatography with electrochemical detection using the same conditions as described in Section 2.3.2a, though the applied potential was set at 0.9 V.

2.5 Time-Course of Tissue Contents

2.5.1 Tissue Indole Levels

The levels of tissue indoles (tryptophan, 5-HT, 5-HIAA) were monitored over a 120-minute period during superfusion with medium to which exogenous tryptophan (2 µM) was or was not added. Slices (approximately 6 per time-point) were retrieved after 0, 5, 15, 30, 60, 90 and 120 minutes of superfusion. These slices were quickly rinsed with distilled water, blotted, frozen over dry ice and stored at -70^{o}C until subsequent assays. Frozen tissue samples were homogenized by ultrasonication in 300 µl of 0.2 M $HClO_4$ containing 0.5 mM ascorbate and the two internal standards (5-HT-CH_3 and 5-HICA). These homogenates were further processed and assayed biochemically (for tryptophan, 5-HT, 5-HIAA and protein) as described in Section 2.3.2b.

The effects on tissue indole levels of adding fluoxetine (2 µM) to the superfusion medium, and exposing the slices to periods of electrical field-stimulation (4.7 min, 5 Hz, 2

ms, 100 mA/cm^2) were tested. Slices were retrieved after 0, 30, 65, 90 and 115 minutes of superfusion, and handled as described above.

2.5.2 Ratio of Tissue Protein to DNA Levels

The ratio of protein to DNA levels in the slices was determined in order to assess the appropriateness of using tissue protein levels to normalize the data, and the degree to which protein breakdown might contribute to free tryptophan levels. This ratio was monitored over a 120-minute superfusion period. Slices in one of the parallel chambers were electrically field-stimulated (4.7 min, 5 Hz, 2 ms, 100 mA/cm^2) beginning after 60, 85 and 110 minutes of superfusion, and were retrieved (approximately 6 slices per time-point) after 0, 30, 60, 65, 85, 90, 110 and 115 minutes. Slices in the other chamber were just superfused throughout, and were retrieved after 0, 30, 60, 90, and 120 minutes. The tissues were rinsed, blotted and frozen until biochemical analysis.

Each frozen tissue sample was homogenized by ultrasonication in 250 µl of distilled water, and the homogenate was divided into two aliquots of equal volume. These homogenates were assayed for their contents of DNA, by the fluorimetric method of Labarca and Paigen (1980), and for protein.

2.5.3 Tissue Lactate Dehydrogenase Activity

The retention of tissue lactate dehydrogenase (LDH) activity was used as an index of the integrity of slice membranes. Slices in one of the parallel chambers were electrically field-stimulated (4.7 min, 5 Hz, 2 ms, 100 mA/cm^2) beginning at 60, 85 and 110 minutes from the onset of superfusion, and slices in the other chamber were just superfused. Tissues were

retrieved just after slicing and after 120 minutes of superfusion; they were frozen until biochemical analysis. Frozen tissue samples obtained before and after superfusion were homogenized by ultrasonication in 0.6 ml and 1 ml, respectively, of distilled water. An aliquot (100 µl) from each homogenate was taken, and diluted 1:1 with distilled water, for LDH assay. LDH activity was assayed spectrophotometrically using a Sigma Diagnostics kit (Procedure No. 228-UV). The remainder of each homogenate, 0.5 ml and 0.9 ml, was acidified by the addition of 100 µl of 1.2 M or 2 M $HClO_4$, respectively (for a final concentration of $HClO_4$ of 0.2 M). These samples were mixed by vortex, centrifuged, and assayed for their tissue protein contents.

2.6 Data Analysis

The 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 tissue tryptophan; 5-HICA for 5-HIAA and medium tryptophan), by the following formula:

$$\text{Corrected PH of indole (mm)} = \text{PH of indole (mm)} \times \frac{\text{PH of IS in standard (mm)}}{\text{PH of IS in sample (mm)}} \times \frac{\text{total volume of sample (µl)}}{\text{total volume of assayed sample (µl)}}$$

where PH = peak height and IS = internal standard.

These peak height values were converted to amounts of indole in the sample using 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 pmol 5-HT or 5-HIAA/g protein/min for rates of release; nmol tryptophan, 5-HT or 5-HIAA/g protein for levels within slices; and μmol tryptophan/L/g protein for concentrations in superfusion medium.

For each experiment monitoring the release of 5-hydroxyindoles from hypothalamic slices superfused with physiologic medium (amino acid-supplemented or unsupplemented), calculations were made of the average rates of 5-HT release during the four rest periods (spontaneous 5-HT release) and the three periods of electrical field-stimulation (evoked 5-HT release); the rate of 5-HIAA efflux was taken as the average across the 80-minute collection period. The fraction of intracellular 5-HT released per minute, spontaneously or with electrical field-stimulation, was calculated as follows:

Fraction of 5-HT released (%) per minute spontaneously or evoked =

$$\frac{\text{average rate of 5-HT release (pmol/g protein/min)}}{\text{final tissue 5-HT levels (nmol/g protein)}} \times 100$$

The fraction of intracellular 5-HT released over the 80-minute collection period was calculated as follows:

Total fraction of 5-HT released spontaneously (%) =

$$\frac{\text{average rate of spontaneous 5-HT release (pmol/g protein/min)} \times 50 \text{ min}}{\text{final tissue 5-HT levels (nmol/g protein)}} \times 100$$

and total fraction of evoked 5-HT release (%) =

$$\frac{\text{average rate of evoked 5-HT release (pmol/g protein/min)} \times 30 \text{ min}}{\text{final tissue 5-HT levels (nmol/g protein)}} \times 100$$

The Ca^{2+}-dependence and TTX-sensitivity of spontaneous 5-HT release were assessed by comparing the average rates of 5-HT release from slices superfused with Ca^{2+}-free or TTX-containing medium versus release occurring in the presence of physiologic medium. The Ca^{2+}-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 physiologic media. Restoration of evoked 5-HT release was determined by comparing the amounts of 5-HT released by the slices in the two chambers due to S2.

For experiments addressing the effect of tryptophan availability on 5-HT release evoked by varying frequencies of membrane depolarization, the amounts of 5-HT released spontaneously in the fraction preceding the electrical field-stimulation were substrated from the amounts released with the stimulation, thus yielding a more accurate measure of release actually elicited by the stimulation. Calculations were then made of the amounts of 5-HT released per pulse (pmol/g protein/pulse) and the total amounts of 5-HT release (pmol/g protein) evoked by each period of stimulation and collected within a 10-minute fraction.

For experiments examining the effect of tryptophan on DA release, the amounts of DA recorded were corrected for their recovery using the internal standard DHBA. The amounts of DA released (pmol/g protein/min) from the slices in a chamber during the two periods of electrical field-stimulation (S1 and S2) and their preceding rest periods were calculated and compared to yield ratios of basal DA release (B2/B1; amounts of DA released prior to S2 relative to that released prior to S1) and of evoked DA release (E2/E1; amounts of DA released by S2 relative to S1).

Values are expressed as means ± s.e.m. or as mean percents ± s.e.m. Raw data were evaluated appropriately by the two-tailed Student's t-test applied to independent groups (with equal or unequal variances as determined by the F-test for equality of two variances) or to paired groups, and by analysis of variance (one-way or two-way) followed by the Duncan's multiple range post-hoc test. Statistically significant differences are noted when the probability value was less than 0.05.

3. RESULTS AND DISCUSSION

3.1 Characterization of the In Vitro System

3.1.1 5-Hydroxyindole Release

Hypothalamic slices superfused with physiologic medium unsupplemented with amino acids released 5-HT spontaneously at a rate of 49 ± 4 pmol/g protein/min (N = 8; Figure 3A). This rate increased to 295 ± 14 pmol/g protein/min (N = 8) for 10 minutes with each of the three periods of electrical field-stimulation (4.7 min, 5 Hz, 2 ms, 100 mA/cm^2), after which 5-HT release returned to its basal rate. Based on this profile of 5-HT release, evoked 5-HT release was considered to be collected in three pairs of 5-minute fractions (60 - 65, 65 - 70; 85 - 90, 90 - 95; and 110 - 115, 115 - 120 min); spontaneous 5-HT release was collected in the remaining ten fractions. The fraction of 5-HT stored intraneuronally and released spontaneously was 0.06 ± 0.01% per minute (N = 8); that fraction evoked with electrical field-stimulation was 0.34 ± 0.02% per minute (N = 8). These values represent 2.84 ± 0.29% and 10.18 ± 0.57% released spontaneously and with depolarization, respectively, over the 80-minute collection period. There was a time-dependent decline in evoked 5-HT release [$F(2,21) = 3.52$, $p < 0.05$], with the amount released by the third stimulation period significantly less than that released by the first. The rate of 5-HIAA efflux was 639 ± 31 pmol/g protein/min (N = 8) across the 80-minute collection period, which included transient post-stimulation elevations (Figure 3B). The molar ratio of 5-HT to 5-HIAA in the superfusion medium when the slices were at rest was approximately 0.08; with electrical field-stimulation this ratio rose to approximately 0.45. These values were reliably obtained despite

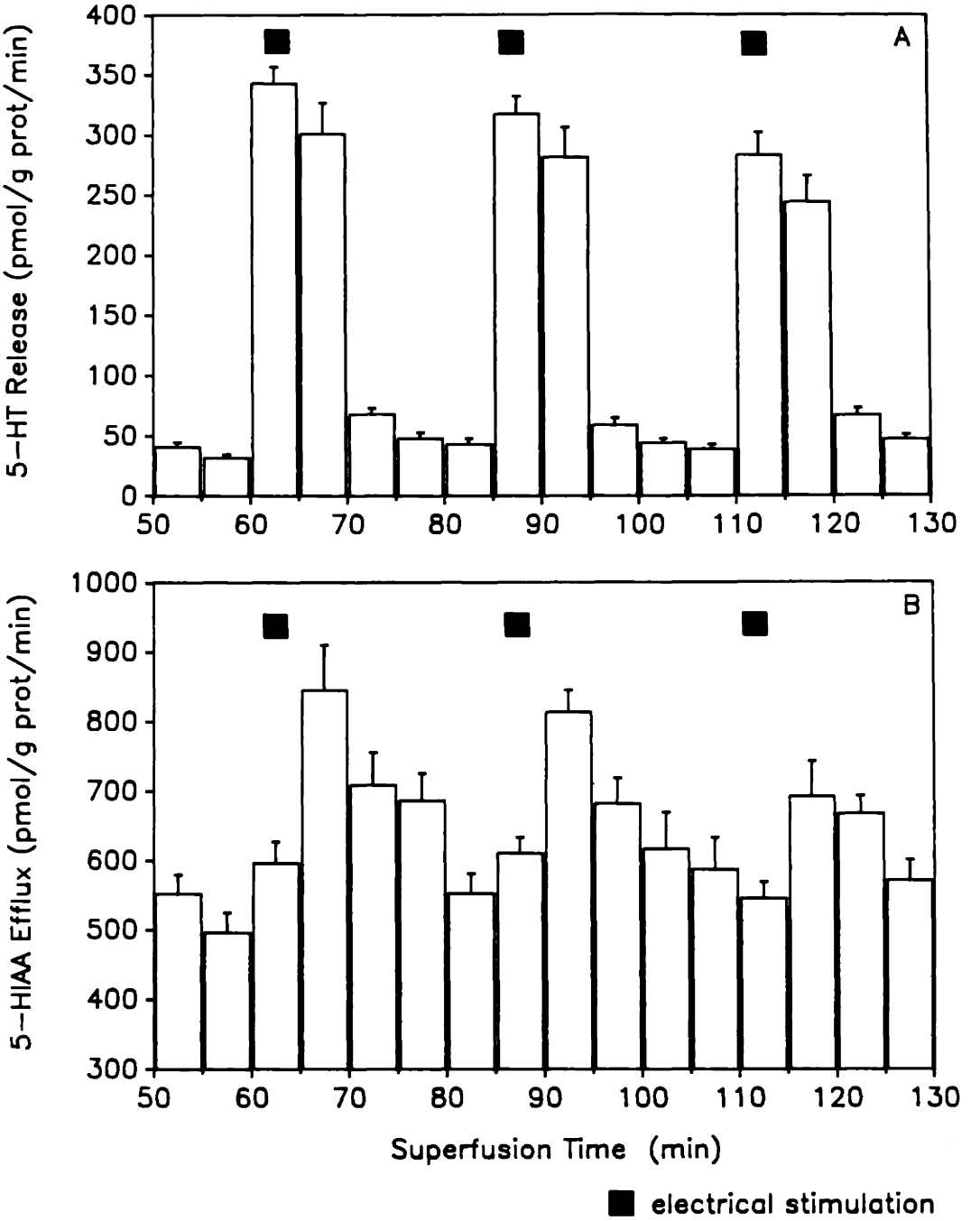

Figure 3. Time-courses of 5-HT release (A) and 5-HIAA efflux (B) from rat hypothalamic slices superfused with medium containing fluoxetine (2 μM). Successive 5-minute fractions of superfusion medium were collected after an initial equilibration period, and the amounts of 5-HT and 5-HIAA release were monitored. The slices were electrically field-stimulated (4.7 min, 5 Hz, 2 ms, 100 mA/cm^2) for three periods. Data are expressed as means ± s.e.m. for N = 8 separate experiments.

the imperfect timing (i.e., serial slice preparation and their introduction into chambers; staggered start of fraction collection).

The electrically-evoked release of 5-HT from the hypothalamic slices was dependent on the presence of Ca^{2+} in the superfusion medium (Figure 4). Slices superfused with Ca^{2+}-free medium containing EGTA (1 mM) released only 18 ± 2% (N = 9) as much 5-HT with electrical field-stimulation as control slices ($p < 0.01$). In contrast, basal 5-HT release was unaffected by the lower Ca^{2+} concentration (physiologic medium: 43 ± 3 compared to Ca^{2+}-free medium: 35 ± 4 pmol/g protein/min; N = 7). When slices which had been exposed to Ca^{2+}-free medium were subsequently superfused with physiologic medium (Ca^{2+} concentration = 1.3 mM), the electrically-evoked release of 5-HT returned to control.

Evoked 5-HT release was sensitive to the activity of voltage-dependent Na^{+} channels (Figure 5). Inhibiting their activity with TTX (1 μM) caused a 60 ± 4% reduction (N = 3, $p < 0.05$) in 5-HT release with electrical field-stimulation, again without changing basal 5-HT release (physiologic medium: 39 ± 2 compared to TTX-containing medium: 38 ± 4 pmol/g protein/min; N = 3). The rate of evoked 5-HT release was partially recovered, to 80 ± 2% of control, after the slices were rapidly superfused for one hour with physiologic medium.

3.1.2 Tryptophan Concentrations in Superfusion Medium

The unsupplemented medium superfused under control conditions actually contained endogenous tryptophan in concentrations of 0.06 - 0.12 μM; its concentration varied with time [$F(5,6) = 4.99$, $p < 0.01$], peaking 15 - 30 minutes after the onset of superfusion, and then declining gradually over the next 90 minutes (Figure 6). This time-course was not affected by delivery of three periods of electrical field-stimulation.

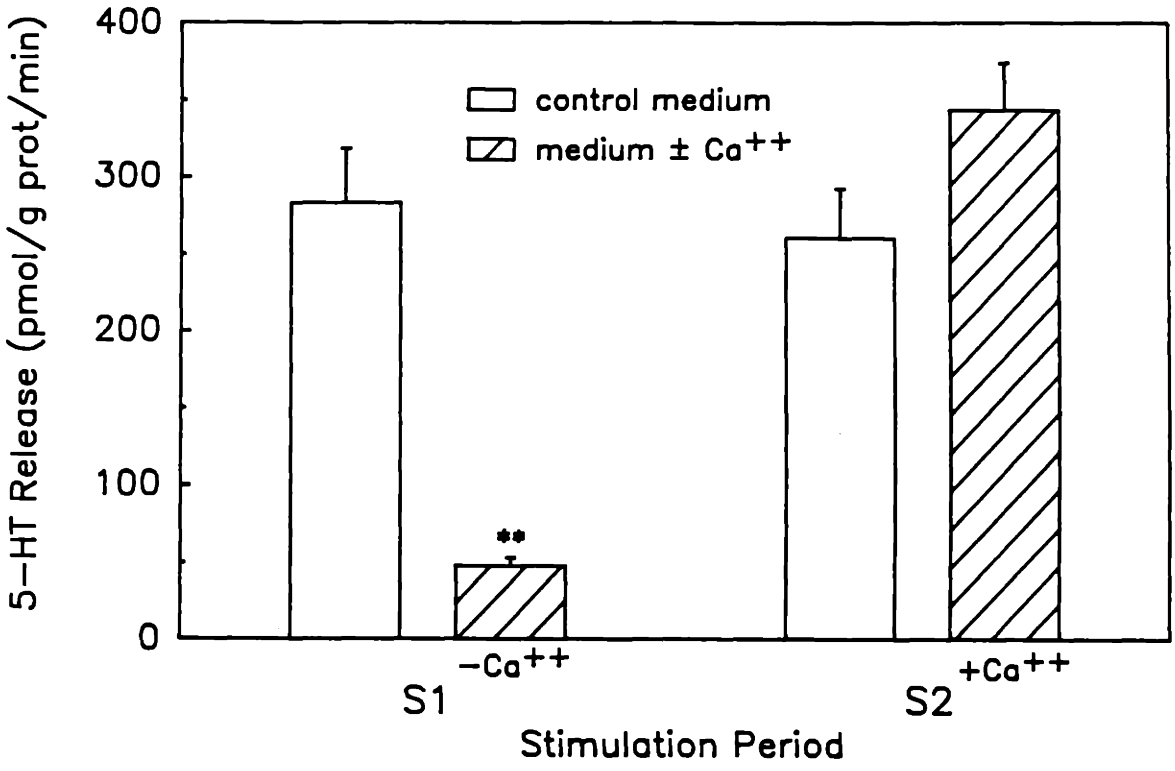

Figure 4. Ca^{2+}-dependence of electrically-evoked 5-HT release. Rat hypothalamic slices were initially superfused with either physiologic medium or Ca^{2+}-free medium containing 1 mM EGTA, and were delivered the first period of electrical field-stimulation (S1) under these conditons. Physiologic medium replaced the Ca^{2+}-free medium prior to the second period of electrical field-stimulation (S2). Values are group means ± s.e.m. for N = 9. ** $p < 0.01$ differs from control by the Student's t-test with unequal variances.

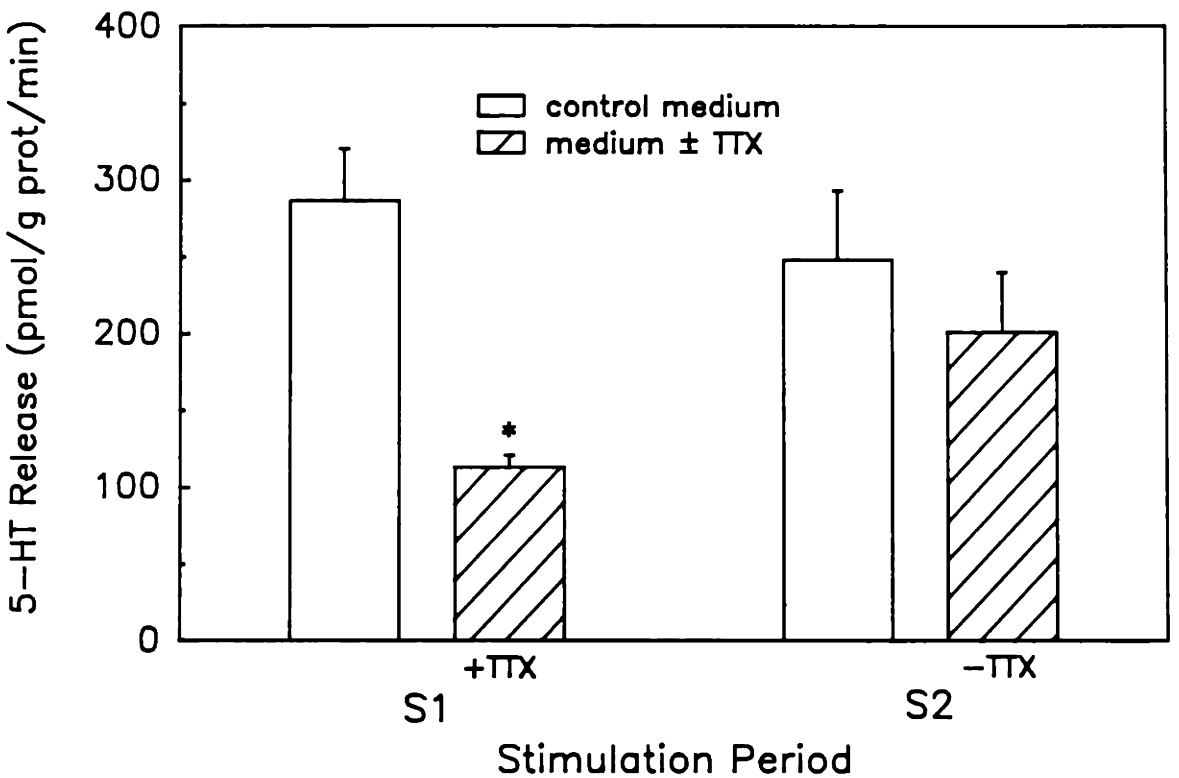

Figure 5. TTX-sensitivity of electrically-evoked 5-HT release. Rat hypothalamic slices were initially superfused with physiologic medium; the medium superfusing one of the two parallel chambers was replaced with TTX-containing (1 μM) medium prior to delivery of the first period of electrical field-stimulation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

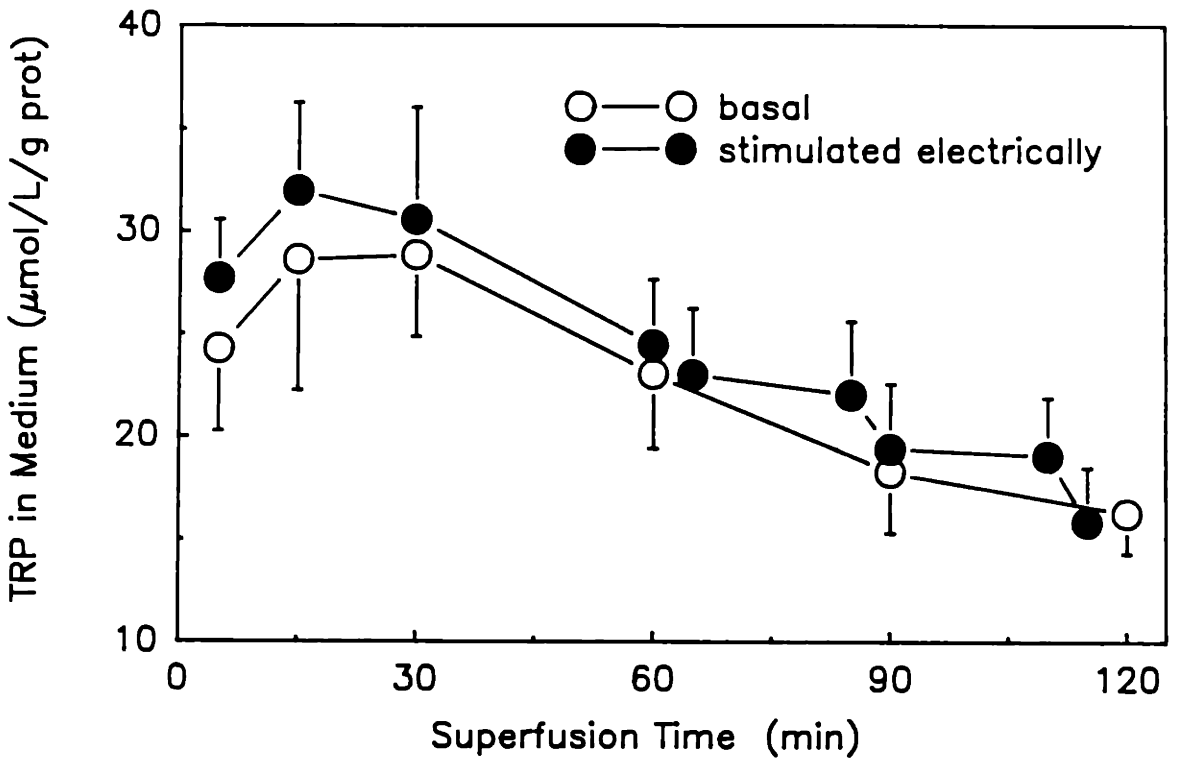

Figure 6. Time-course of the actual tryptophan (trp) concentration in the control medium with superfusion and electrical field-stimulation of rat hypothalamic slices. The slices in one of the parallel chambers were electrically field-stimulated (4.7 min, 5 Hz, 2 ms, 100 mA/cm^2) for three periods starting at 60, 85 and 110 minutes from the onset of superfusion. Values are group means ± s.e.m. Two-way analysis of variance detected a significant effect of time [$F(5,36) = 4.99$, $p < 0.01$] but not treatment.

3.1.3 Tissue Indole Levels

Levels of tryptophan, 5-HT and 5-HIAA in hypothalamic slices prior to superfusion (t = 0) were 195.9 ± 7.0, 71.4 ± 2.0 and 45.7 ± 3.0 nmol/g protein (N = 5), respectively. [These levels were not different from indole levels measured in hypothalamic tissue taken immediately after decapitation.] Slice tryptophan levels rose in association with the onset of superfusion in unsupplemented medium [$F(6,55) = 16.94$, $p < 0.01$], and then declined gradually (Figure 7A). Superfusing the slices in medium supplemented with tryptophan (2 μM) elevated slice tryptophan levels above those in control slices [$F(1,55) = 116.79$, $p < 0.01$]. The magnitude of this tryptophan-induced elevation in slice tryptophan levels was relatively constant after 30 minutes of superfusion. Slice 5-HT levels also rose with superfusion in unsupplemented medium [$F(6,50) = 18.21$, $p < 0.01$], with peak levels occurring after those of tryptophan (Figure 7B). Slice 5-HT levels were relatively stable after 30 minutes of superfusion. Superfusing the slices with tryptophan-supplemented (2 μM) medium produced an additional increment in slice 5-HT levels [$F(1,50) = 7.88$, $p < 0.01$] which was relatively constant by 60 minutes of superfusion. Tissue 5-HIAA levels fell precipitously with the onset of superfusion [$F(6,32) = 131.17$, $p < 0.01$], and remained low thereafter (Figure 7C). Tissue 5-HIAA levels were not affected by the addition of tryptophan (2 μM) to the superfusion medium.

The time-courses of tissue tryptophan and 5-HT levels with superfusion were not modified by exposing the slices to three periods of electrical field-stimulation (N = 3 - 4; Figure 8), nor by the addition of fluoxetine (2 μM) to the medium superfusing electrically field-stimulated slices (N = 3 - 4; Figures 9). Furthermore, supplementing the fluoxetine-containing medium with tryptophan (2 μM) elevated tissue tryptophan and 5-HT levels

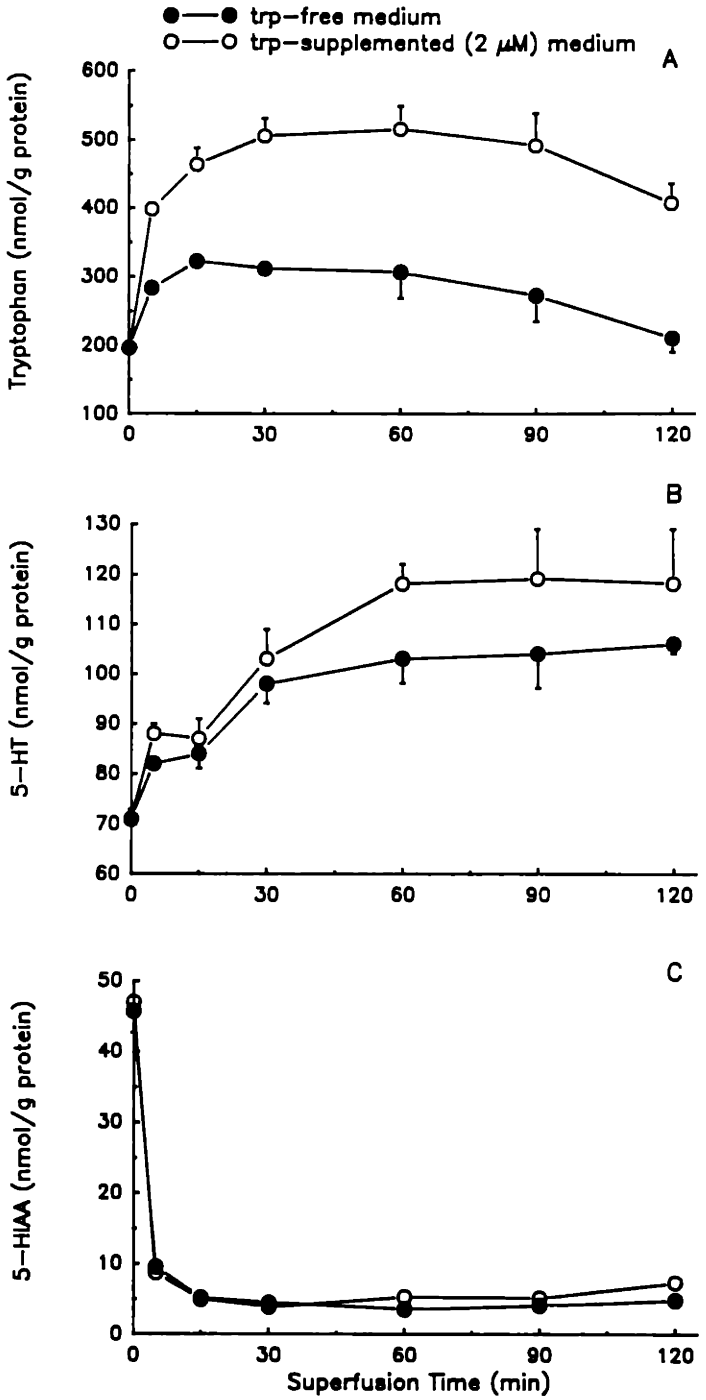

Figure 7. Time-courses of tissue tryptophan (A), 5-HT (B) and 5-HIAA (C) levels with superfusion in tryptophan-free or tryptophan-supplemented (2 μM) medium. Slices (6 per time-point) were retrieved over 120 minutes of superfusion. There were time-dependent changes in tissue levels of tryptophan [$F(6,55) = 16.94$, $p < 0.01$], 5-HT [$F(6,50) = 18.21$, $p < 0.01$] and 5-HIAA [$F(6,32) = 131.17$, $p < 0.01$] with superfusion in tryptophan-free medium. Superfusing the slices with tryptophan-supplemented medium elevated tissue levels of tryptophan [$F(1,55) = 116.79$, $p < 0.01$] and 5-HT [$F(1,50) = 7.88$, $p < 0.01$], but not 5-HIAA, above those in control slices.

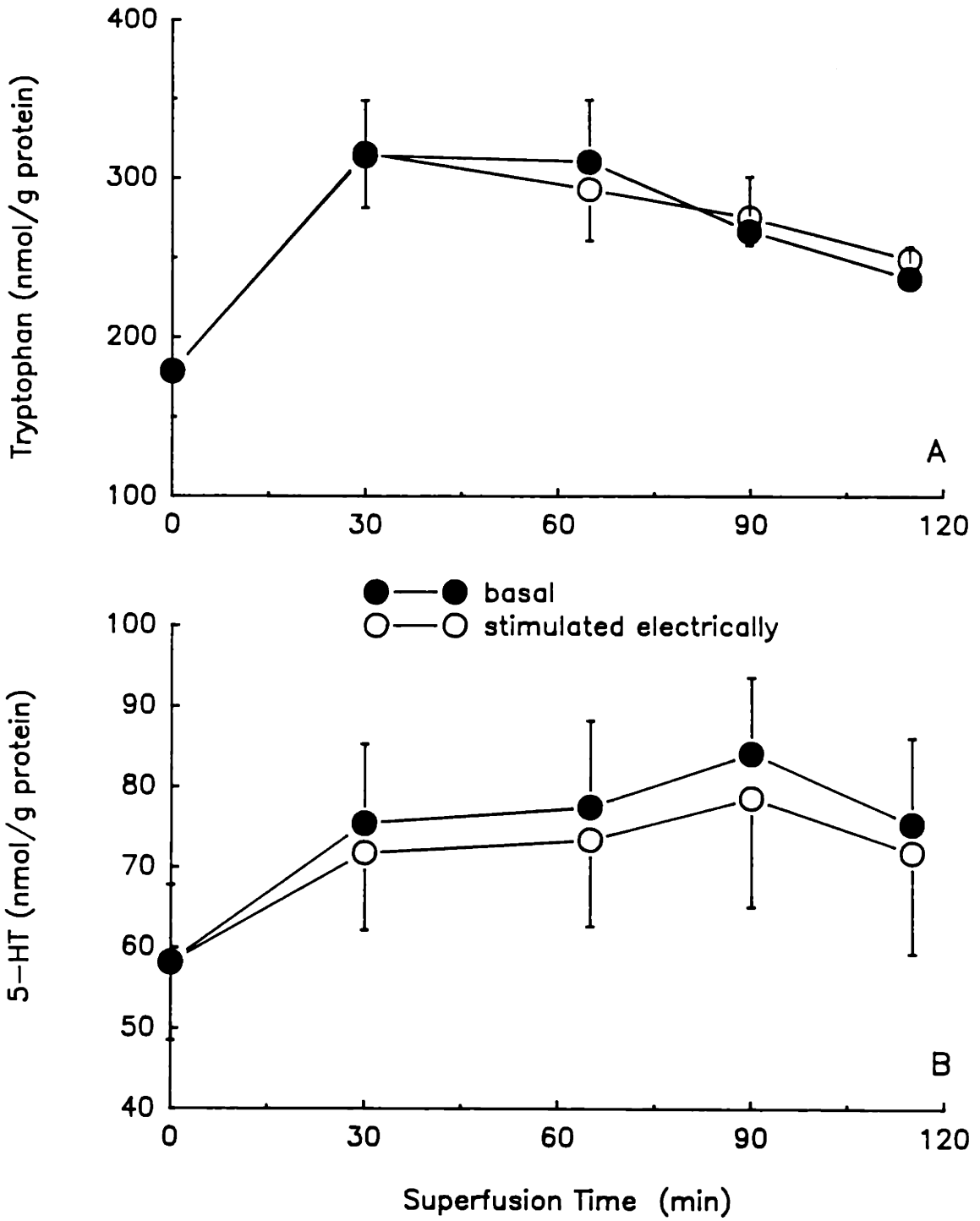

Figure 8. Time-courses of tissue tryptophan (A) and 5-HT (B) levels in superfused slices with or without exposure to periods of electrical field-stimulation. Slices in one of the parallel chambers were electrically field-stimulated (4.7 min, 5 Hz, 2 ms, 100 mA/cm^2) starting at 60, 85 and 110 minutes from the onset of superfusion. Slices (6 per time-point) were retrieved from both chambers after 0, 30, 65, 85 and 115 minutes. Electrically field-stimulating the slices did not affect the time-courses of tissue indole levels found with superfusion alone. Data are group means ± s.e.m. for N = 3 - 4 at each time-point.

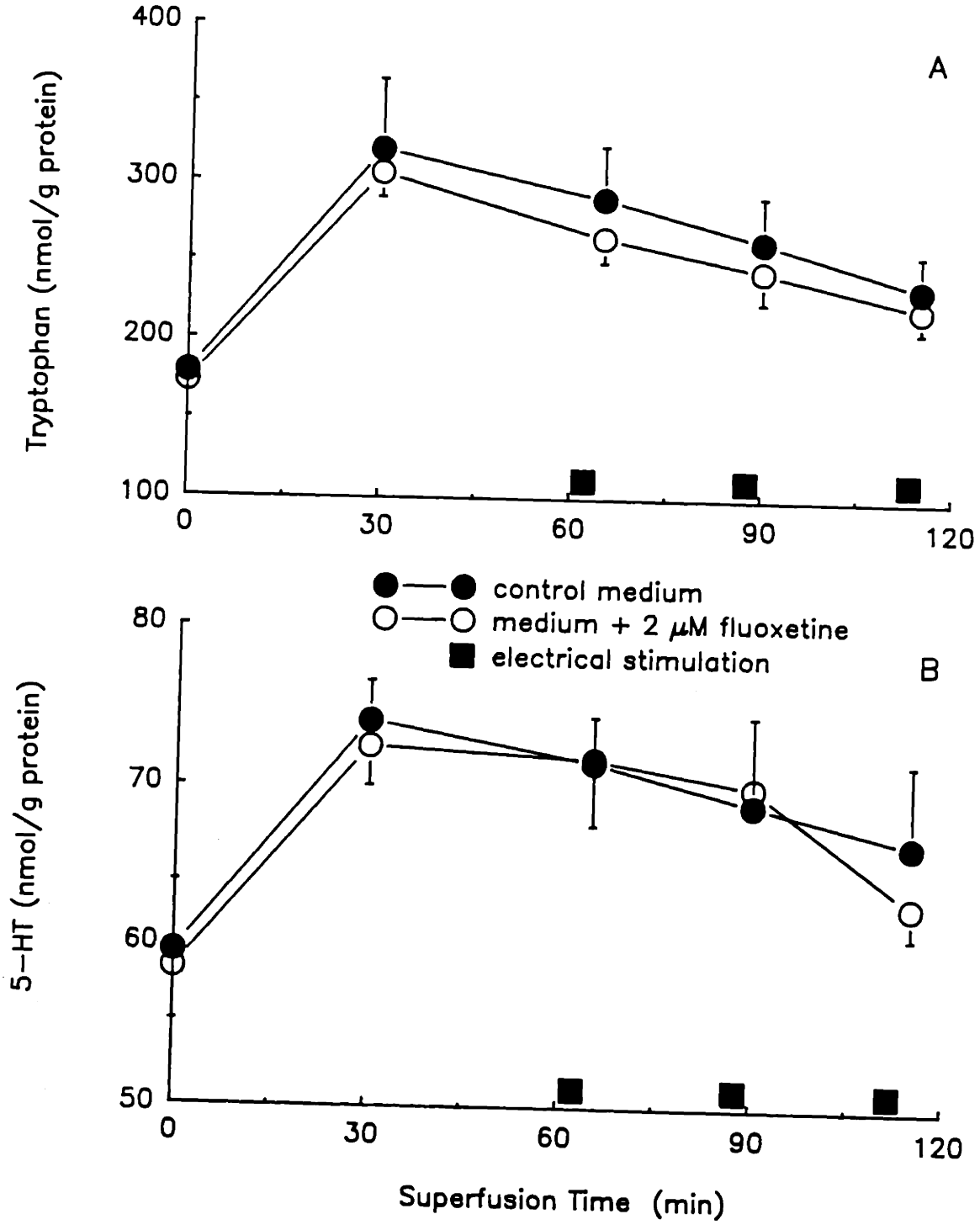

Figure 9. Time-courses of tissue tryptophan (A) and 5-HT (B) levels in electrically field-stimulated slices superfused with control medium or this medium to which fluoxetine (2 μM) was added. Slice stimulation and retrieval were as described in the legend of Figure 8. The addition of fluoxetine to the superfusion medium did not affect the time-courses of tissue indole levels. Data are group means ± s.e.m. for N = 3 - 4 at each time-point.

in stimulated slices (N = 4; Figure 10) as observed with resting slices superfused with fluoxetine-free medium (Figure 7).

3.1.4 Ratio of Tissue Protein to DNA Levels

The ratio of slice protein to DNA levels was relatively unchanged over the 120-minute superfusion period (N = 4; Figure 11A), and was unaffected by the delivery of three periods of electrical field-stimulation (N = 4; Figure 11B).

3.1.5 Tissue Lactate Dehydrogenase Activity

Slice LDH activity prior to superfusion was 350 ± 32 μmol NADH/min/g protein (N = 3); this activity was unchanged with 120 minutes of superfusion (318 ± 40 μmol NADH/min/g protein), as well as after three periods of electrical field-stimulation (333 ± 41 μmol NADH/min/g protein).

3.1.6 Discussion

These data describe a reliable *in vitro* experimental system, utilizing superfused rat hypothalamic slices, for monitoring the release of endogenous 5-HT during rest and with low-frequency electrical field-stimulation. This system utilizes a novel method for extracting 5-HT (and its principal metabolite 5-HIAA) which allows for the measurement of small amounts of 5-HT released from brain slices into relatively large volumes of superfusion medium. Coupled with the sensitivity of the assay system (i.e., high-performance liquid chromatography with electrochemical detection), this method permits the detection of small and transient changes in the release of endogenous 5-HT.

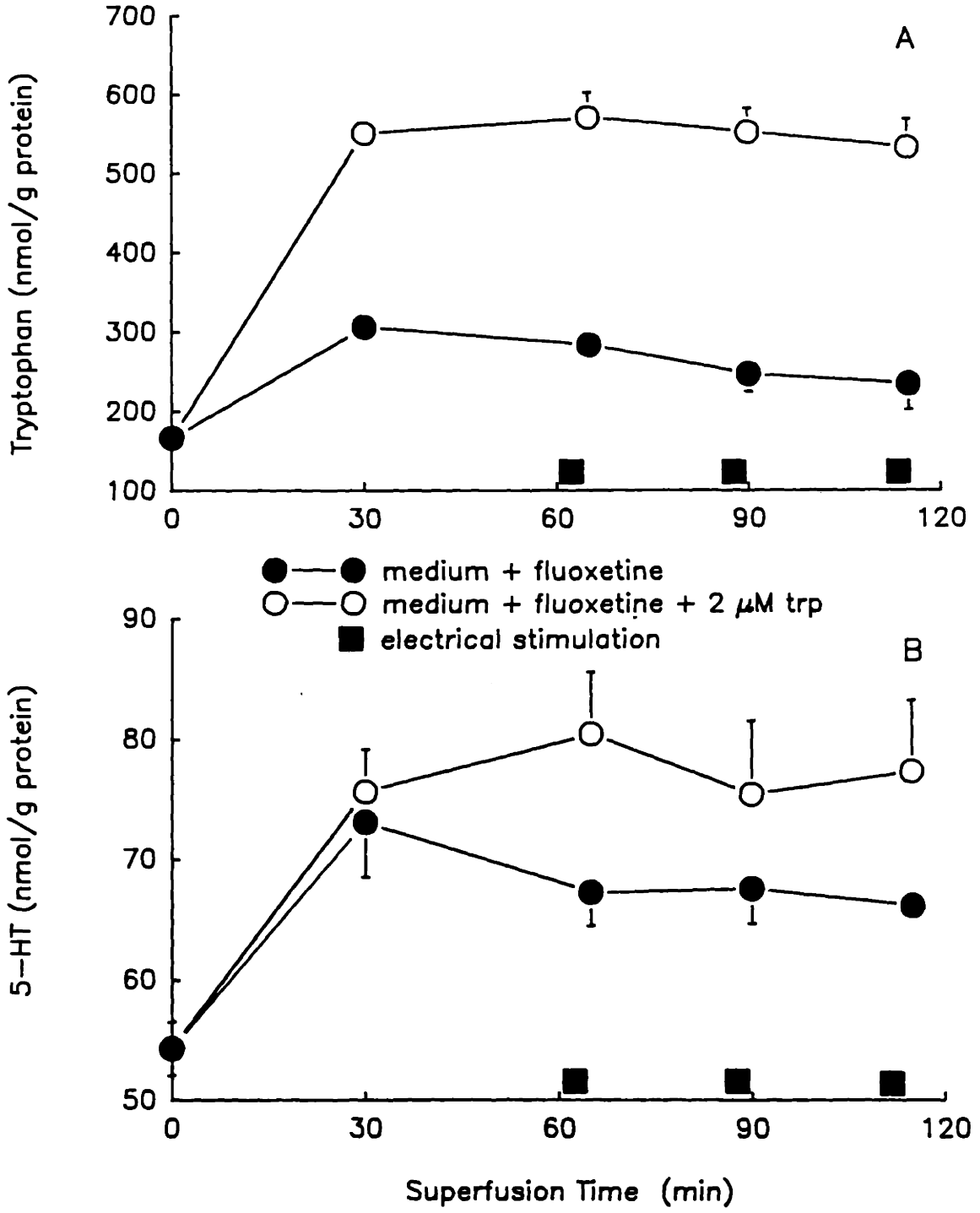

Figure 10. Time-courses of tissue tryptophan (A) and 5-HT (B) levels in electrically field-stimulated slices superfused with fluoxetine-containing medium which did or did not contain additional tryptophan (2 μM; trp). Slice stimulation and retrieval were as described in the legend of Figure 8. Superfusing the slices with medium containing supplemental tryptophan elevated tissue levels of tryptophan [$F(1,29) = 221.25$, $p < 0.01$] and 5-HT [$F(1,30) = 7.47$, $p < 0.05$] above those in control slices.

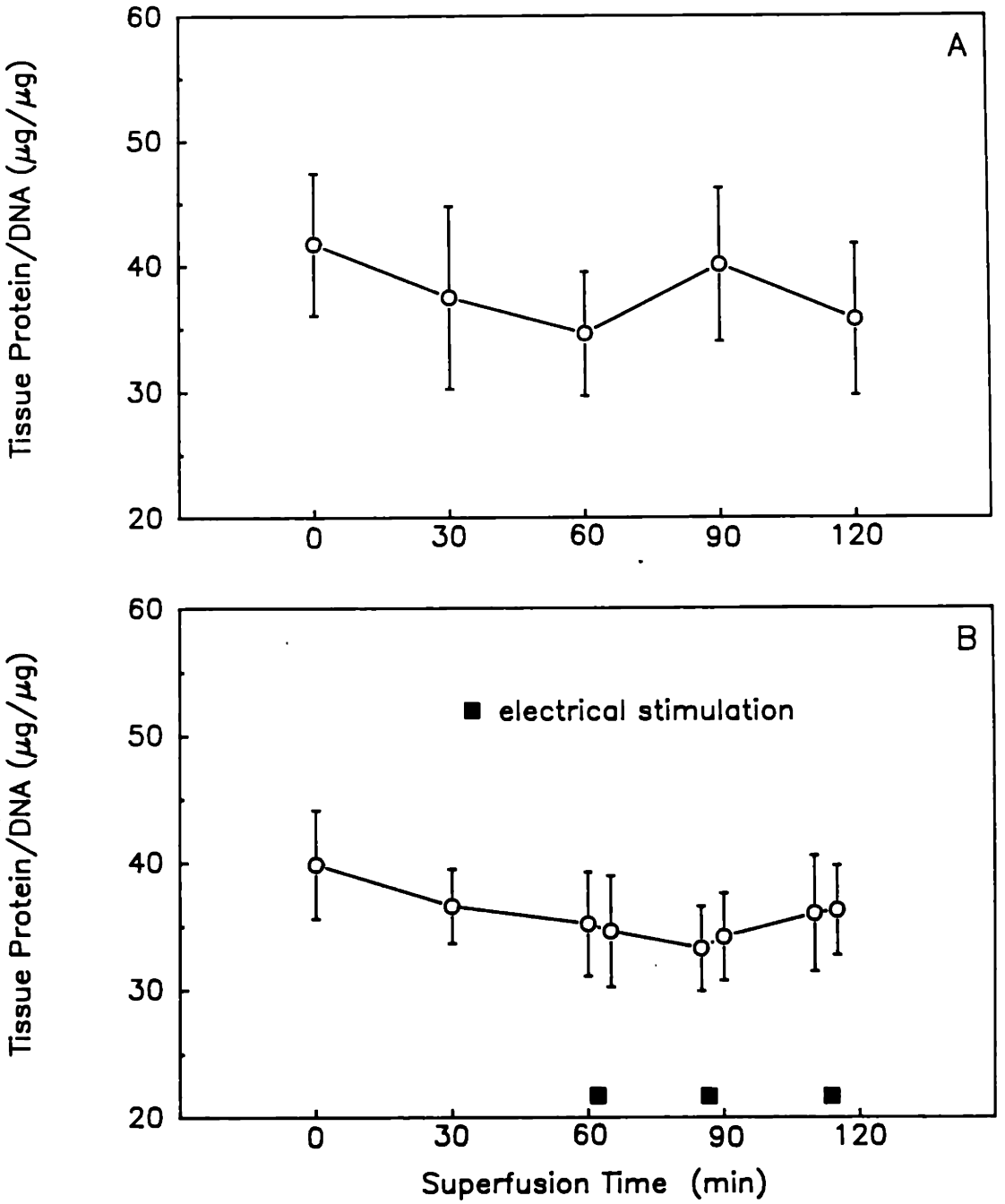

Figure 11. Time-courses of the ratio of slice protein to DNA levels with superfusion (A) and with three periods of electrical field-stimulation (4.7 min, 5 Hz, 2 ms, 100 mA/cm^2) (B). Slices (6 per time-point) were retrieved over 120 minutes of superfusion. Neither superfusion or electrical field-stimulation significantly altered the ratio of protein to DNA levels in the slices. Data are expressed as group means ± s.e.m. for N = 4 at each time-point.

This experimental system is an apparent improvement over other *in vitro* systems which frequently impose experimental conditions which may disturb 5-HT dynamics. For example, membrane depolarization is commonly induced by elevating the extracellular potassium concentration, though this procedure is effective for prolonged periods (Haycock et al., 1978), and may thus release more neurotransmitter than the amounts liberated by phasic physiologic stimuli. The slices were electrically field-stimulated here at a frequency (5 Hz) within the range recorded *in vivo* (0.1 - 6.0 Hz; McGinty and Harper, 1976; Trulson and Jacobs, 1979), and may have thus permitted 5-HT to be released in amounts similar to those occurring *in situ*. This experimental system monitors the actual amounts of 5-HT released from the slices, rather than the efflux of radiolabeled 5-HT (either previously taken up or converted intraneuronally from labeled tryptophan). Though the latter methodology is extremely sensitive, interpretation of such data may be confounded by the possible existence of multiple 5-HT pools with varying specific activities (Shields and Eccleston, 1973; Elk et al., 1979*b*; Mennini et al., 1981; Kleven et al., 1983; Kuhn et al., 1985). Furthermore, the continuous superfusion of brain slices, as applied in the present study, may minimize the possible deleterious effects of prolonged incubations, such as cellular damage caused by exposing them to anaerobic conditions (Lipton, 1985) and the induction of feedback changes secondary to high extracellular levels of released substances.

Spontaneous 5-HT release was relatively stable over the 80-minute collection period (Figure 3A). The relatively low proportion of 5-HT to 5-HIAA released from the slices under basal conditions (in the presence of a concentration of fluoxetine which maximally inhibited 5-HT reuptake; data not shown) suggests that a substantial portion of extracellular 5-HIAA in the hypothalamus is derived from 5-HT which has been metabolized intraneuronally prior to

being released. This assessment corroborates with earlier *in vivo* reports demonstrating that fluoxetine treatment reduces 5-HIAA levels in whole-brain (Reinhard and Wurtman, 1977) and in discrete hypothalamic nuclei (Lookingland et al., 1986) by only 10 - 40%. The relative insensitivity of brain 5-HIAA levels to 5-HT reuptake inhibition may partly be attributed to MAO deamination subsequent to low-affinity uptake of synaptic 5-HT into nonserotoninergic cells, and in the present study to exposure of extracellular 5-HT to MAO externalized due to slice preparation. It is also likely that serotoninergic neurons regulate their levels of intraneuronal 5-HT by tonic activity of the degradative enzyme.

Electrically field-stimulating the slices (4.7 min, 5 Hz, 2 ms, 100 mA/cm^2) induced an increase in 5-HT release which persisted for 10 minutes (two consecutive fractions), as well as a delayed increase in 5-HIAA efflux (Figure 3). That the levels of 5-HT in the superfusion medium were elevated in a fraction collected after the termination of electrical field-stimulation probably resulted, in part, from a lag (estimated 1 - 2 minutes) between the release of 5-HT and its collection; this lag was due to diffusional impedences and to chamber/tubing volume. This prolonged enhancement in 5-HT release with electrical field-stimulation is reminiscent of the persistent activation of tryptophan hydroxylase which follows the termination of electrical stimulation to the dorsal raphe nucleus *in vivo* (Eccleston et al., 1970; Herr et al., 1975; Boadle-Biber et al., 1986). Furthermore, a delayed and prolonged increase in forebrain 5-HIAA efflux has been demonstrated previously, by *in vivo* voltammetry, to follow raphe stimulation (De Simoni et al., 1987*a*; De Simoni et al., 1987*b*). Thus, our data suggest that during periods of membrane depolarization the activation of tryptophan hydroxylase causes an increase in 5-HT synthesis which contributes to an enhancement in 5-HT release; for a period of time after the cessation of stimulation, the rates of 5-HT synthesis and release

may still be accelerated, but a greater proportion of newly-synthesized 5-HT is metabolized intracellularly prior to storage or release, and thus causes an increase in 5-HIAA efflux which is delayed with respect to the period of stimulation. The amounts of 5-HT released by depolarization decreased slightly over the experimental period, paralleling a decline in tryptophan levels in the medium (Figure 6) and in the tissue (Figure 7A). This parallelism is compatible with the view that tryptophan availability regulates 5-HT synthesis by affecting the saturation of tryptophan hydroxylase with its amino acid substrate (Fernstrom and Wurtman, 1971*a*), and thereby modifies 5-HT release (Ternaux et al., 1976; Auerbach and Lipton, 1985; Guan and McBride, 1987; Broderick and Jacoby, 1988; Carboni et al., 1989).

Small fractions of intracellular 5-HT were released spontaneously ($0.06 \pm 0.01\%$ per minute and $2.84 \pm 0.29\%$ totally) and with electrical field-stimulation ($0.34 \pm 0.02\%$ per minute and $10.18 \pm 0.57\%$ totally). It is very difficult to compare these values with those of [^{3}H]5-HT efflux from other *in vitro* studies since the specific activity of medium and tissue 5-HT are most often not reported. The fractional rates of endogenous 5-HT release measured here are in the range of those reported by others: They are approximately four-times higher than those from incubated whole-brain slices (calculated from Elks et al., 1979*b*) and hippocampal slices (calculated from Auerbach and Lipton, 1985), and approximately ten-times lower than those from superfused midbrain slices (Waller and Richter, 1980). However, direct comparisons will be avoided because of the substantial differences in the experimental conditions applied among these studies. A study from this laboratory, which applied similar experimental conditions to measure endogenous DA release from superfused striatal slices (Milner and Wurtman, 1984), reported the same fractional rate of spontaneous DA release ($0.06 \pm 0.01\%$ per minute) as found here for 5-HT, although the fractional rate of electrically-

evoked (90 sec, 20 Hz, 2 ms, 60 mA) DA release was approximately three-fold greater (10.0 ± 0.06% over 10 minutes) than that of electrically-evoked (4.7 min, 5 Hz, 2 ms, 100 mA/cm^2) 5-HT release. While a direct comparison between fractional rates of evoked DA and 5-HT release is difficult, given that the patterns of electrical field-stimulation applied in these two studies are similar to those occurring *in vivo* for the respective neuronal pathways [i.e., nigrostriatal for DA (Grace and Bunney, 1980) and raphe-hypothalamic for 5-HT], the observed low fractional rates of 5-HT release are compatible with the view that 5-HT is released tonically from nerve terminals to exert a 'hormone-like' influence on brain function (see Bloom, 1981).

Though 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 serotoninergic autoreceptors on hypothalamic nerve terminals (Cerrito and Raiteri, 1979) and thus suppressed 5-HT release. Indeed, preliminary studies found that the addition of the nerve terminal autoreceptor antagonist methiothepin (Gohert, 1980) to the medium (in the presence of fluoxetine) potentiated spontaneous and electrically-evoked 5-HT release by about 80% (data not shown). Excessive activation of these autoreceptors, possibly resulting from fluoxetine superfusion, may have caused an underestimation of 5-HT release in relation to that occurring *in situ*.

The release of 5-HT evoked by electrical field-stimulation was more Ca^{2+}-dependent than TTX-sensitive (Figures 4 and 5). This suggests that the influx of Ca^{2+} caused directly by stimulation-induced activation of voltage-sensitive Ca^{2+} channels may be greater than that caused indirectly by Na^+ influx-induced membrane depolarization. Basal 5-HT release was not altered by the omission of Ca^{2+} from the superfusion medium nor by the addition of TTX

to the medium. This differential sensitivity of basal and evoked 5-HT release to Ca^{2+} ions and to TTX has been previously observed by others (Elks et al., 1979*a*; Gohert, 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 Ca^{2+} through voltage-sensitve Ca^{2+} channels.

The tryptophan concentration in control medium rose with the initiation of slice superfusion, and peaked at 15 - 30 minutes (Figure 6). This elevation may have resulted from the artifactual liberation of free amino acids secondary to protein breakdown associated with slice preparation, as well as from the physiologic flux of amino acids across brain cell membranes (Oja and Korpi, 1983). The subsequent time-dependent lowering of medium tryptophan concentrations probably reflects the continual dilution of this medium with fresh (tryptophan-free) medium and net amino acid uptake into slice cells. That the uptake of medium tryptophan into slice cells contributed to the time-dependent decline in extracellular tryptophan is supported by the parallel elevation in tissue tryptophan levels with superfusion (Figure 7A). This elevation in tissue tryptophan levels (approximately 100 nmol/g tissue protein) requires the degradation of only 5 μg of tissue protein [given that a typical protein is composed of 1 - 2% tryptophan (Cohn, 1943)]. The variabilities inherent in the protein and DNA assays used here prevented our detection of this relatively low level of protein degradation (Figure 11A). Since the ratio of slice protein to DNA levels did not change measurably over the experimental time period, the cellular damage resulting from slice preparation may have caused equivalent disruptions in the molecular structures of protein and DNA. [The relative constancy of this ratio also validated the use of tissue protein levels to normalize the data.] Slice preparation did not, however, render cellular membranes

permanently leaky to cytoplasmic proteins such as LDH, reflected by the fact that tissue LDH activity did not decline with 120 minutes of superfusion.

Slice tryptophan levels decreased during the second hour of superfusion. A similar time-course of tryptophan accumulation has been described in slices prepared from rat cerebral cortex (Kiely and Sourkes, 1972). This time-dependent reduction in tissue tryptophan levels may represent a net efflux of tryptophan from the slices and its conversion to 5-HT within serotoninergic nerve terminals. The latter hypothesis is compatible with the finding that tissue 5-HT levels rose after those of its precursor, and then remained stable thereafter (Figure 7B). 5-HT levels in hippocampal slices have similarly been shown to increase within 60 minutes of incubation and remain constant for the next 30 minutes (Auerbach and Lipton, 1980). The rapid decline in tissue 5-HIAA levels with the onset of superfusion probably reflects the efficient transport of this acid metabolite into the extracellular space. Indeed, the ratio of 5-HIAA levels in the tissue to those in the superfusion medium was approximately 0.01.

Exposing the superfused slices to conditions imposed during the release experiments (i.e., periods of electrical field-stimulation, fluoxetine) did not further modify medium tryptophan concentrations (Figure 6), tissue tryptophan and 5-HT levels (Figures 8 and 9), nor the integrity of the slices [as assessed by the ratio of tissue protein to DNA levels (Figure 11) and tissue LDH activity]. Furthermore, the addition of tryptophan to the superfusion medium potentiated the magnitude of the elevations in tissue tryptophan and 5-HT levels observed with superfusion alone, but did not alter the time-course of these changes (Figures 7A, 7B); these tryptophan-induced effects were also not modified in electrically field-stimulated slices superfused with fluoxetine-containing medium (Figure 10). These results suggest that the influence of tryptophan availability on slice indole levels (and, as to be described, on 5-HT

release) were probably related directly to effects of the amino acid-supplemented medium, and not indirectly to effects on tissue viability or cellular disposition. The stability of tissue 5-HT levels from 60 - 120 minutes even in electrically field-stimulated slices superfused with fluoxetine-containing medium probably occurred because the relatively small amounts of 5-HT released over this time period (approximately 13% of intracellular 5-HT) could be readily replenished with 5-HT newly-synthesized from the available pool of intracellular free tryptophan.

3.2 Application of the In Vitro System

3.2.1. Increasing Tryptophan Availability and Serotonin Release

Superfusing hypothalamic slices with medium supplemented with tryptophan (1 - 10 μM) produced dose-dependent increases in the rates of spontaneous and electrically-evoked 5-HT release (Table 1): Spontaneous 5-HT release was elevated by 8 ± 4%, 36 ± 8% ($p < 0.01$), 47 ± 9% ($p < 0.01$) and 67 ± 13% ($p < 0.01$) with the addition of 1, 2, 5 or 10 μM tryptophan to the superfusion medium, respectively (N = 8 - 11). Electrically-evoked 5-HT release was elevated by 5 ± 3%, 19 ± 4% ($p < 0.01$), 34 ± 6% ($p < 0.01$) and 59 ± 10% ($p < 0.01$) in the presence of these tryptophan concentrations (N = 9 - 12). The efflux of 5-HIAA also increased dose-dependently.

In control experiments designed to assess the specificity of this tryptophan-induced effect on 5-HT release, slices superfused with medium containing 10 μM of a different amino acid, histidine, released 5-HT spontaneously and with electrical field-stimulation at rates not

Table 1. Dose-response effects of increasing tryptophan availability on release of 5-hydroxyindoles from rat hypothalamic slices.

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

Rat hypothalamic slices were superfused with medium which contained either 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 (4.7 min, 5 Hz, 2 ms, 100 mA/cm^2), and 5-HIAA effluxed from the slices are expressed in pmol/g protein/min. Values in parentheses are the relative amounts, in percents, 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 ± s.e.m. for N = 8 - 12 pairs. * $p < 0.05$, ** $p < 0.01$ differs from control group by the Student's paired t-test.

different [101 ± 5% and 99 ± 3% of control, respectively (N = 4)] from those superfused with amino acid-unsupplemented medium. Furthermore, adding 2 μM tryptophan to the superfusion medium, while increasing the rates of 5-HT release, did not alter the rates of basal DA release [B2/B1 with unsupplemented medium: 1.03 ± 0.01 compared to tryptophan-supplemented medium: 0.97 ± 0.06 (N = 4)] nor electrically-evoked DA release [E2/E1 with unsupplemented medium: 0.93 ± 0.04 compared to tryptophan-supplemented medium: 0.89 ± 0.03 (N = 4)].

Tissue indole levels in the slices at the end of the 130-minute superfusion period (which included three periods of electrical field-stimulation) were elevated dose-dependently with tryptophan supplementation (1 - 10 μM). Tissue tryptophan levels increased linearly, by 40 ± 4% ($p < 0.01$), 105 ± 8% ($p < 0.01$), 223 ± 25% ($p < 0.01$) and 490 ± 30% ($p < 0.01$), with the addition of 1, 2, 5 or 10 μM tryptophan to the superfusion medium, respectively (N = 9 - 12; Figure 12A). Final tissue levels of 5-HT rose biphasically with increases in the medium tryptophan concentration: The addition of low tryptophan concentrations caused proportionately greater incremental changes in tissue 5-HT levels [1 μM: 8 ± 3% and 2 μM: 13 ± 3% ($p < 0.01$) over control] than those caused by adding higher tryptophan concentrations [5 μM: 19 ± 2% ($p < 0.01$) and 10 μM: 34 ± 4% ($p < 0.01$) over control] (N = 8 - 12; Figure 12B). The amount of 5-HIAA remaining in the slices following superfusion rose linearly with increases in the medium tryptophan concentration (N = 8 - 12; Figure 12C).

Superfusing rat hypothalamic slices with tryptophan-containing medium (1 - 10 μM) thus caused dose-dependent elevations in tissue 5-HT levels [$F(3,38) = 10.37$, $p < 0.01$] which were paralleled by changes in spontaneous 5-HT release [$F(3,36) = 8.35$, $p < 0.01$; Figure 13A] and evoked 5-HT release [$F(3,38) = 14.48$, $p < 0.01$; Figure 13B]. Though each increment in the

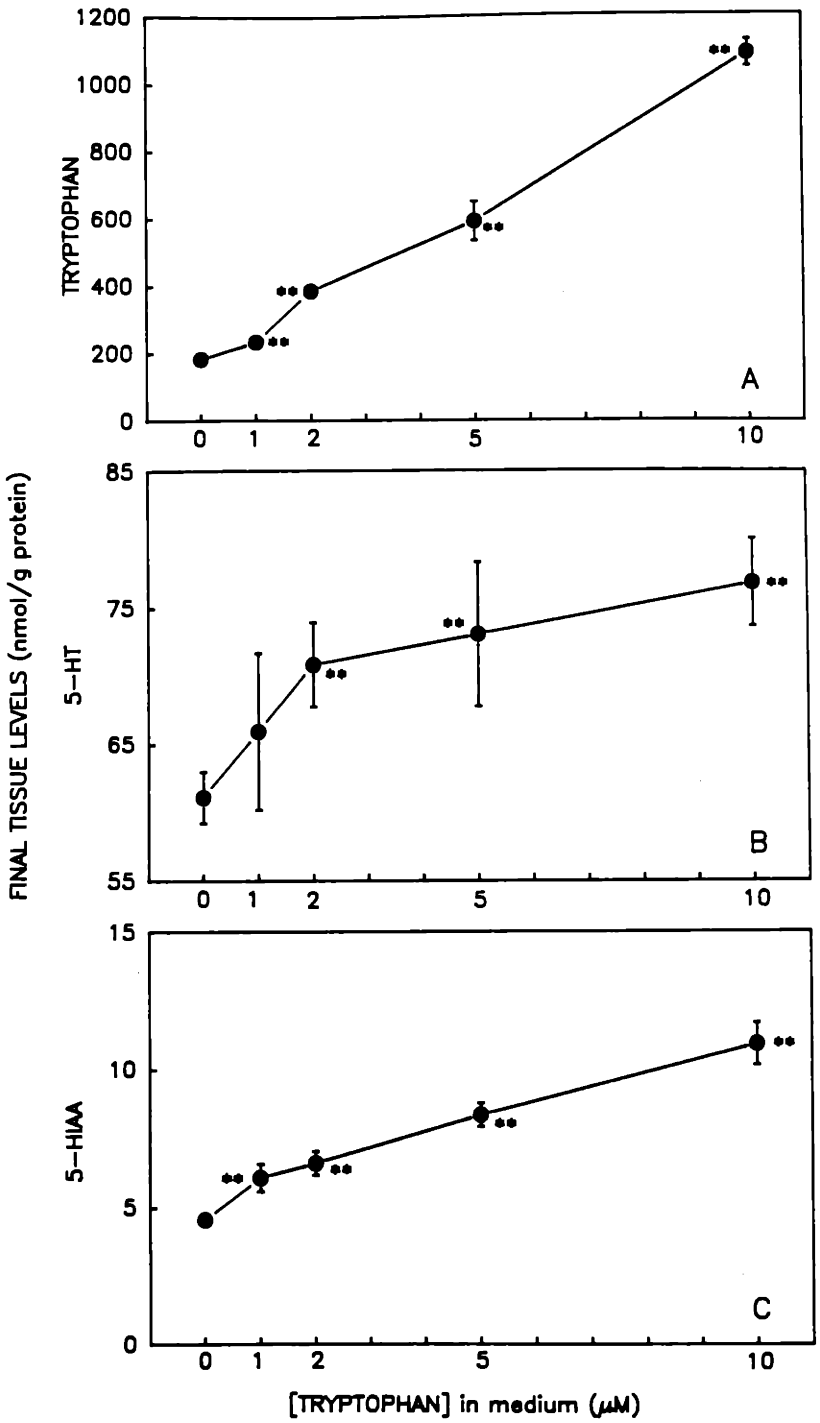

Figure 12. Dose-response effects of increasing tryptophan availability on final tissue levels of tryptophan (A), 5-HT (B) and 5-HIAA (C). Paired hypothalamic slices were superfused with medium which was either tryptophan (trp)-free or tryptophan-supplemented (1, 2, 5 or 10 μM). Slices were retrieved at the end of each release experiment (i.e., after 130 minutes of superfusion) and assayed for their contents of these indoles. Data are expressed as group means ± s.e.m. for N = 8 - 12 pairs. ** $p < 0.01$ differs from slices superfused with tryptophan-free medium by the Student's paired t-test.

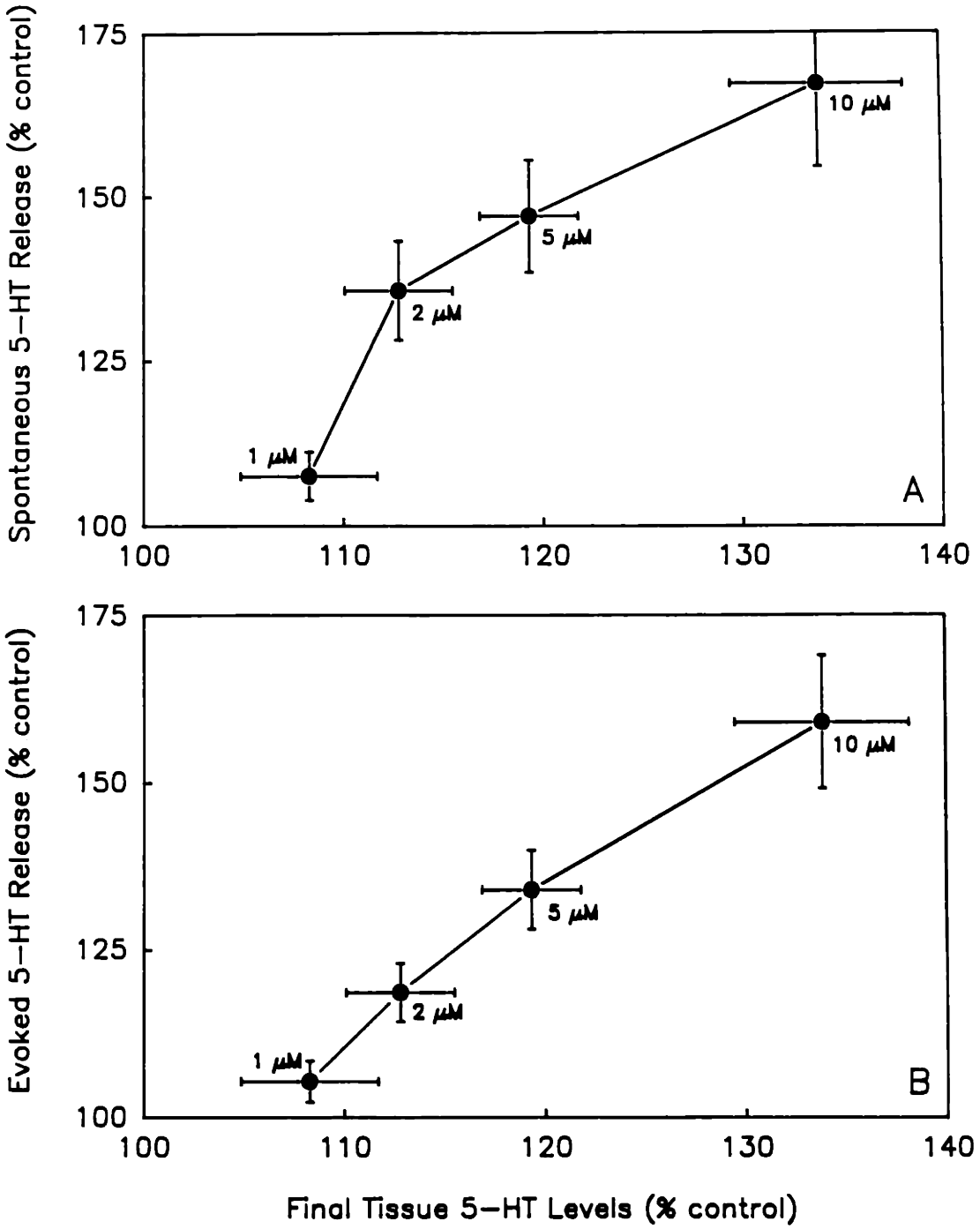

Figure 13. Dose-dependent relationships between changes in final tissue 5-HT levels and in spontaneous (A) and electrically-evoked (B) 5-HT release when slices were superfused with tryptophan-supplemented medium (1, 2, 5 or 10 μM) as compared to tryptophan-free medium (control). These are replottings of data from Figure 12B and Table 1. Data are expressed as mean percents ± s.e.m. for N = 8 - 12 pairs. Analyses of variance detected significantly different effects with levels of tryptophan supplementation for changes in 5-HT levels [$F(3,38) = 10.37$, $p < 0.01$], spontaneous 5-HT release [$F(3,36) = 8.35$, $p < 0.01$] and evoked 5-HT release [$F(3,38) = 14.48$, $p < 0.01$].

tryptophan concentration of the superfusion medium did not always produce a parallel significant increment in tissue 5-HT levels or in 5-HT release (spontaneous and evoked), a clear dose-dependence between medium tryptophan concentration and 5-HT (levels and release) was observed, as assessed by the Duncan's multiple range post-hoc test.

3.2.2 Decreasing Tryptophan Availability and Serotonin Release

Superfusing hypothalamic slices with medium containing 100 μM leucine decreased their spontaneous release of 5-HT to 72.6 ± 5.0% ($p < 0.05$) and their electrically-evoked release to 78.4 ± 3.0% ($p < 0.01$) of control (N = 4; Figure 14); the efflux of 5-HIAA was lowered to 70.3 ± 2.1% of control ($p < 0.01$). Confirming that reported in Section 3.2.1, superfusing the slices with medium containing 2 μM tryptophan resulted in increases in spontaneous and electrically-evoked 5-HT release to 138.1 ± 3.9% ($p < 0.01$) and to 127 ± 3.3% ($p < 0.01$) of control, respectively; 5-HIAA efflux was increased to 161.3 ± 9.0% ($p < 0.01$). Co-supplementing the medium with 100 μM leucine and 2 μM tryptophan suppressed the changes induced by either amino acid individually.

These decreases in 5-HT release caused by superfusing the slices with medium containing 100 μM leucine were associated with reductions in final tissue levels of tryptophan to 60.7 ± 1.0% ($p < 0.01$) and 5-HT to 88.7 ± 2.0% ($p < 0.05$) of those in control slices (N = 4; Figure 15). Conversely, supplementing the medium with 2 μM tryptophan elevated final tissue tryptophan and 5-HT levels to 183.1 ± 12.4% ($p < 0.01$) and 115.0 ± 5.5% ($p < 0.05$) of control, respectively. Co-supplementing the medium with both of these LNAAs resulted in final tissue tryptophan and 5-HT levels of 118.1 ± 10.5% and 97.7 ± 5.4% of control, respectively.

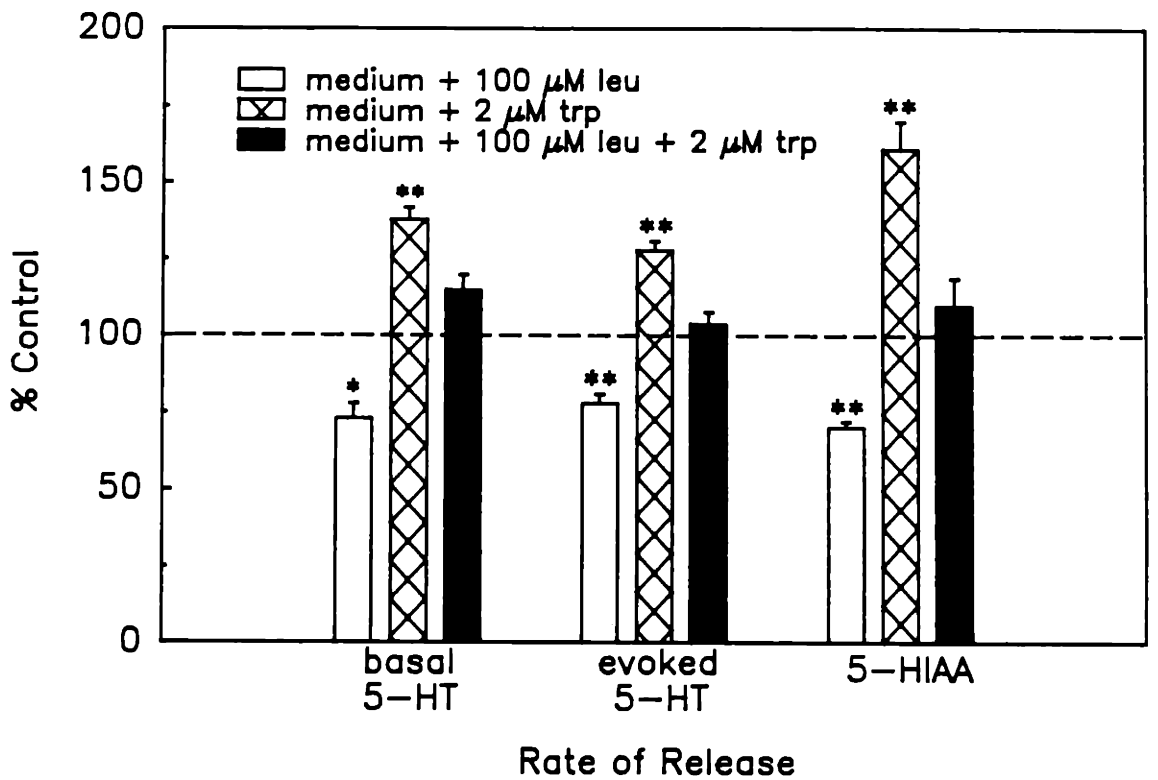

Figure 14. Effects on 5-hydroxyindole release of decreasing and increasing tryptophan availability. Paired hypothalamic slices were superfused with amino acid-unsupplemented medium (control) or medium to which the LNAAs leucine (100 μM; leu) and/or tryptophan (2 μM; trp) were added. Data are expressed as mean percents ± s.e.m., relative to control, for N = 4 pairs for each treatment. * $p < 0.05$, ** $p < 0.01$ differs from control by the Student's paired t-test.

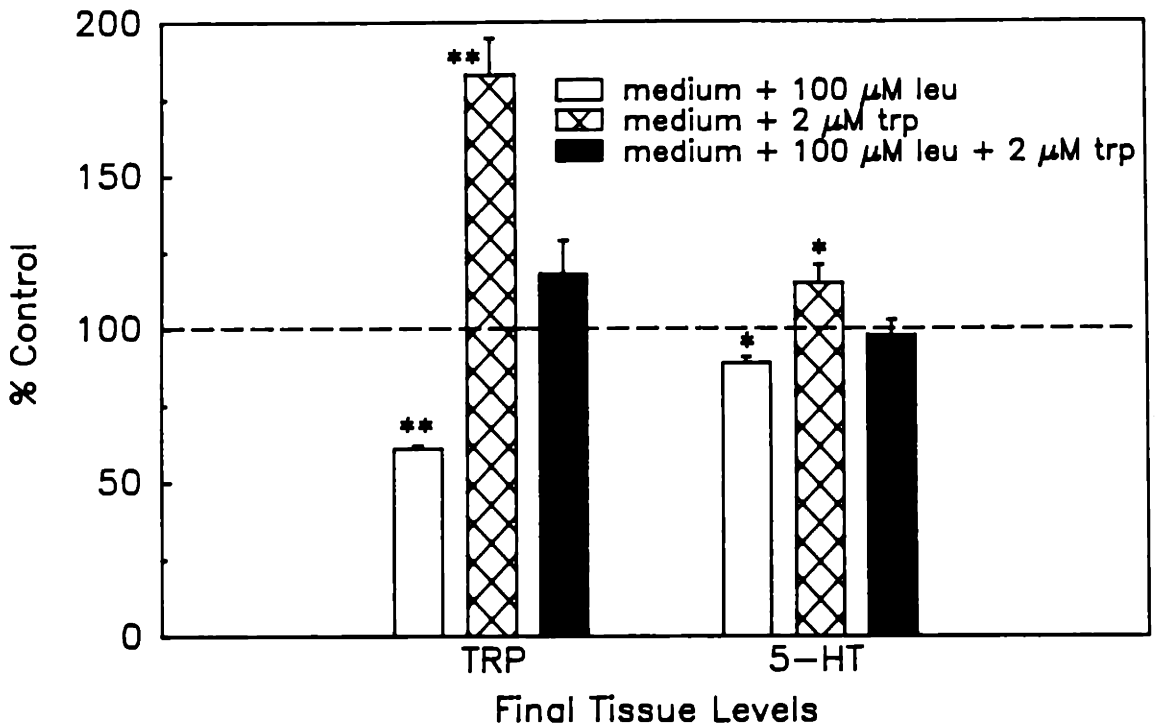

Figure 15. Effects on tissue indole levels of decreasing and increasing tryptophan availability. Paired hypothalamic slices were superfused with amino acid-unsupplemented medium (control) or medium to which the LNAAs leucine (100 μM; leu) and/or tryptophan (2 μM; trp) were added. Slices were retrieved at the end of each release experiment (i.e., after 130 minutes of superfusion) and assayed for their contents of tissue tryptophan and 5-HT. Data are expressed as mean percents ± s.e.m., relative to control, for N = 4 pairs for each treatment. * $p < 0.05$, ** $p < 0.01$ differs from control by the Student's paired t-test.

The changes in final tissue 5-HT levels and 5-HT release caused by supplementing the medium with these LNAAs were proportionate; therefore the LNAAs did not modify the fractional rates of 5-HT released spontaneously or with electrical field-stimulation (Table 2).

3.2.3 Interaction Between Tryptophan Availability and Frequency of Stimulation

The total amounts of 5-HT release evoked by depolarization increased pulse-dependently [$F(2,38) = 74.47$, $p < 0.01$] with increases in the frequency of electrical field-stimulation (1 Hz: 753 ± 69 pmol/g protein; 3 Hz: 1496 ± 87 pmol/g protein; 5 Hz: 2142 ± 154 pmol/g protein). However, the amounts of 5-HT released per pulse decreased with frequencies greater than 1 Hz [$F(2,38) = 24.41$, $p < 0.01$; Figure 16]. Superfusing the slices with medium containing 2 μM tryptophan augmented, by 21.4 ± 4.5%, the total amounts of evoked 5-HT release [$F(1,38) = 8.20$, $p < 0.01$] and the amounts of 5-HT released per pulse [$F(1,38) = 6.67$, $p < 0.05$]. The magnitude of the tryptophan-induced change in evoked 5-HT release was independent of the frequency of electrical field-stimulation.

3.2.4 Discussion

These studies demonstrate that altering the availability of tryptophan to rat hypothalamic slices produces changes in tissue 5-HT levels which are paralleled by proportionate changes in the rates of 5-HT released spontaneously and with electrical field-stimulation. Elevating slice tryptophan levels, by superfusing them with medium containing low concentrations of tryptophan, produced dose-dependent increases in spontaneous and evoked 5-HT release. The magnitude of the tryptophan-induced effect on evoked 5-HT release was independent of the

Table 2. Fractional rates of 5-HT release per minute from hypothalamic slices superfused with medium containing large neutral amino acids.

		Fractional Rates of 5-HT Release (%)	
LNAA		Basal	Evoked
Leu	-	0.06	0.36 ± 0.03
	+	0.05	0.32 ± 0.03
Trp	-	0.05	0.35 ± 0.04
	+	0.06	0.39 ± 0.03
Leu + Trp	-	0.06	0.37 ± 0.02
	+	0.07	0.39 ± 0.02

Rat hypothalamic slices were superfused with either control medium (-) or medium supplemented with 100 μM leucine (leu); 2 μM tryptophan (trp); or 100 μM leucine plus 2 μM tryptophan (+). The amounts of 5-HT released basally and with electrical field-stimulation (4.7 min, 5 Hz, 2 ms, 100 mA/cm^2) were monitored; tissue 5-HT levels were assayed in slices retrieved at the end of each experiment (i.e., after 130 minutes of superfusion). Fractional rates of 5-HT released (%) per minute were calculated. Values are mean percents ± s.e.m (where greater than 0) for N = 4 pairs for each experimental condition. The Student's paired t-test did not detect significant differences between fractional rates of basal or evoked 5-HT release.

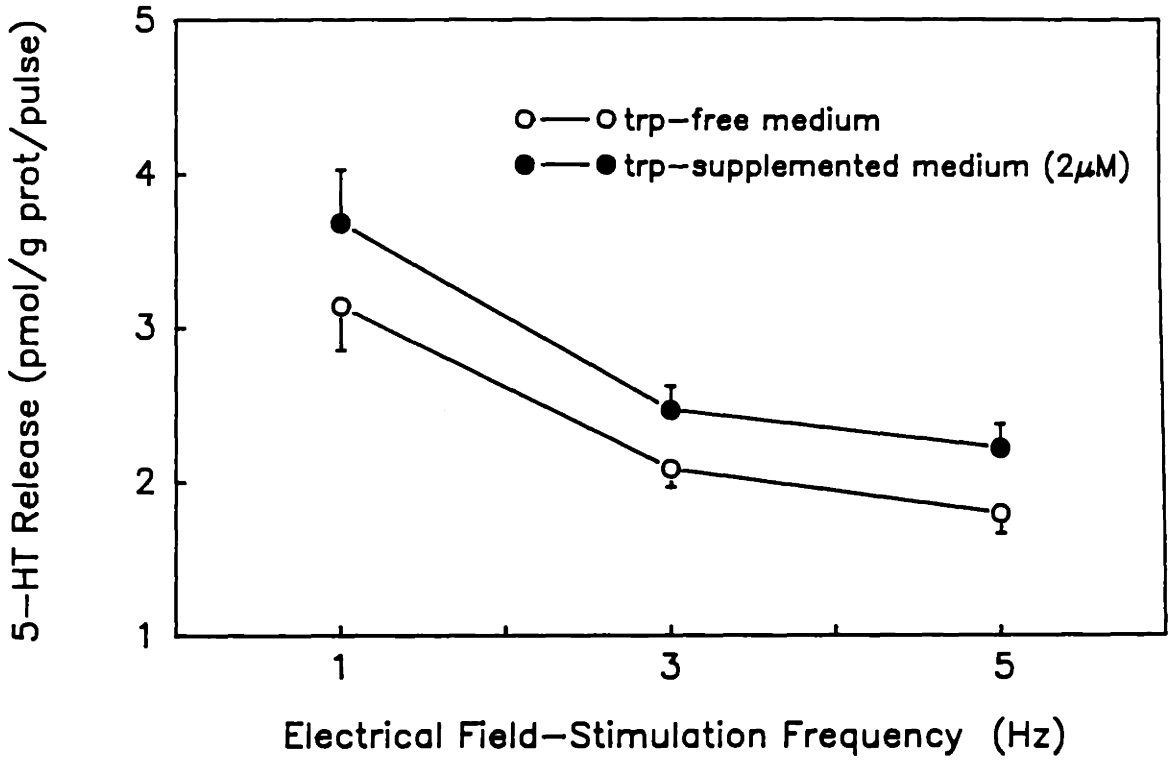

Figure 16. Effect of electrical field-stimulation frequency (1, 3 or 5 Hz) on the evoked release of 5-HT per pulse from slices superfused with tryptophan (trp)-free or tryptophan-supplemented (2 μM) medium. Data are expressed as group means ± s.e.m. Two-way analysis of variance detected a significant effect of tryptophan supplementation [$F(1,38) = 6.67$, $p < 0.05$] which was independent of the frequency of electrical field-stimulation; a significant effect of stimulation frequency was also detected [$F(2,38) = 24.41$, $p < 0.01$] with 5-HT release evoked by 1 Hz different from that evoked by 3 or 5 Hz, as assessed by the Duncan's multiple range post-hoc test.

frequency of electrical field-stimulation. Conversely, reducing slice tryptophan levels by superfusing them with a medium containing another LNAA decreased 5-HT release.

Adding exogenous tryptophan to the medium at concentrations within the range estimated to exist in rat brain extracellular space (0.5 - 3 μM; see Section 1.3.4) caused elevations in tissue tryptophan levels (Figure 12A) quantitatively similar to those which occur physiologically in rat brain after consumption of a carbohydrate-rich meal (Fernstrom and Wurtman, 1971*b*; Colmenares et al., 1975), with time of day (Morgan et al., 1975; Hery et al., 1977), with exercise (Chauloff et al., 1985; 1986) or after particular stressors (Knott et al., 1973; Kennett and Joseph, 1981; Culman et al., 1984; Dunn, 1988). The observed relationship between increased tryptophan availability and tissue 5-HT levels (Figure 12B) 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 medium containing 1 or 2 μM tryptophan, tissue 5-HT levels increased proportionately; however, superfusing the slices with 5 or 10 μM tryptophan caused smaller incremental rises in tissue 5-HT levels. Notably, this inflection in the curve relating medium tryptophan concentration to tissue 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 uniformly distributed throughout the tissue (Wurtman and Fernstrom, 1976).] Such levels were attained when the slices were superfused with 2 and 5 μM tryptophan, respectively. That tryptophan hydroxylase was unsaturated with tryptophan across the range of medium concentrations tested is suggested by the incremental changes in tissue

5-HIAA levels (Figure 12C) and 5-HIAA efflux (Table 1) even in the presence of the higher tryptophan concentrations.

Accelerating 5-HT synthesis, by superfusing the slices with supplemental tryptophan, enhanced dose-dependently the rates of 5-HT release and 5-HIAA efflux (Table 1). This study is distinguished from other *in vivo* and *in vitro* studies, which have also demonstrated an effect of increasing brain tryptophan availability on 5-HT release, by its application of experimental conditions perhaps more reflective of those occurring physiologically (see Section 1.4.3a): Tryptophan levels in the slices were varied within the physiologic range for brain tryptophan levels; the slices were depolarized by delivery of electrical pulses with a frequency similar to that occurring *in vivo*, and thereby may have permitted evoked 5-HT release to approximate that occurring *in situ*.

The changes in 5-HIAA efflux (in the presence of fluoxetine), produced by tryptophan-supplementation, were greater than those in 5-HT release (Table 1). This finding supports the earlier conclusion, based on observations of 5-hydroxyindole release from slices superfused with amino acid-unsupplemented medium, that a major fraction of hypothalamic extracellular 5-HIAA is derived from newly-synthesized 5-HT which is metabolized prior to being released (see Section 3.1.6); and extends it to suggest that the effect of elevating brain tryptophan levels on 5-HT release is dampened by the robust activity of MAO. Preliminary studies found that inhibiting MAO-A by pargyline (10 μM) increased the rate of spontaneous 5-HT release by three- to four-times more than the enhancement in evoked 5-HT release (data not shown). This observation suggests that intraneuronal metabolism of newly-synthesized 5-HT does limit the availability of releasable 5-HT, particularly of that released spontaneously. Teleologically,

these intracellular dynamics may serve to maintain 5-HT release within a range which discretely biases postsynaptic neuronal activity.

The tryptophan-induced increases in 5-HT release were proportionate to the elevations in final tissue levels of 5-HT (Figure 13). These data suggest a physiologic coupling between 5-HT synthesis and release; accelerating 5-HT synthesis increased the levels of intracellular 5-HT and thereby the amounts of 5-HT available for spontaneous and evoked release. This proposed coupling may explain why *in vivo* treatments (i.e., consumption of a carbohydrate-rich meal, food deprivation, immobilization stress, exercise) which increase brain tryptophan levels produce either only a small elevation (Fernstrom and Wurtman, 1971*b*; Curzon et al., 1973) or no change (Chaouloff et al., 1985; Knott et al., 1973; Kennett and Joseph, 1981) in 5-HT levels in postmortem brain tissue. A tight coupling between brain 5-HT synthesis and release would minimize resultant elevations in tissue 5-HT levels. This dynamic may be particularly operative in the hypothalamus since it is in this brain region where the greatest increase in 5-HTP accumulation (40%, in the presence of a decarboxylase inhibitor) and the lowest increase in 5-HT levels (4%) are produced by consuming a carbohydrate-rich meal (Colmenares et al., 1975).

A physiologic coupling between 5-HT synthesis and release is further supported by the parallel reductions in tissue tryptophan levels, produced by superfusing the slices with medium containing 100 μM leucine, and 5-HT release (Figures 14 and 15). [This concentration of leucine (100 μM) was chosen because it approximates the summed concentration of the LNAAs in cerebrospinal fluid (McGale et al., 1977; Applegarth et al., 1979; Fernstrom and Fernstrom, 1981).] This is apparently the first report to demonstrate directly an effect on spontaneous and evoked 5-HT release of reducing brain tryptophan availability. A few earlier

in vivo studies have shown indirectly that decreased availability of tryptophan to serotoninergic neurons is associated with reductions in 5-HT release: Guan and McBride (1987) demonstrated that the decrements in evoked 5-HT release from the rat nucleus accumbens, caused by repeatedly stimulating with a locally perfused high K^+ medium, were prevented by supplementing the medium with tryptophan (50 μM). Their finding implies that excessive depolarization of serotoninergic neurons depleted local tryptophan levels, and thereby limited the amounts of 5-HT available for evoked release. In a series of studies Kennett and Joseph demonstrated that brain tryptophan levels and hippocampal 5-hydroxyindole release, as monitored by *in vivo* voltammetry, rise following immobilization stress, and both of these effects are blocked by prior administration of valine (Kennett and Joseph, 1981; Joseph and Kennett, 1983). The results of Kennett and Joseph are comparable to the present finding of a suppression in the tryptophan-induced increase in 5-HT release with co-supplementation of the medium with leucine and tryptophan; however, in the present study the LNAA presumably acted at the plasma membrane of brain cells within the slice, while in their study the LNAA probably acted largely at the BBB to inhibit tryptophan transport. The novel aspects of the present findings are 1) the suppression of the baseline rates of 5-HT release (spontaneous and evoked) by high extracellular levels of a LNAA; 2) the direct association between reduced brain tryptophan levels and decreased 5-HT release; and 3) the demonstration of this relationship in an *in vitro* experimental system.

The percent changes in 5-HT release induced by superfusing the slices with LNAA-supplemented medium were greater than those in final tissue 5-HT levels (Figures 14 and 15). However, on a molar basis the changes in 5-HT release were approximately one-quarter of those in tissue 5-HT levels (and approximately one-seventh of those in 5-HIAA efflux). Thus

changes in the amounts of 5-HT released spontaneously and with low-frequency electrical field-stimulation were apparently minimized by more pronounced changes in tissue 5-HT levels (and in 5-HIAA efflux). This finding suggests that tissue stores of 5-HT may serve as a deposit for unreleased 5-HT (when synthesis exceeds immediate demands for release) and as a reservoir for releasable 5-HT (when synthesis contributes less to tissue stores than immediate demands). The amounts of 5-HT released from hypothalamic nerve terminals are likely to be proportionate to the level of intracellular 5-HT; this is reflected quantitatively by the invariant fractional rates of 5-HT release when the slices were superfused with medium which either raised, lowered or maintained tissue 5-HT levels (Table 2). The availability of tryptophan regulates tissue 5-HT levels, and thereby the pool of releasable 5-HT, probably by its effect on 5-HT synthesis. This hypothesis is compatible with earlier suggestions that newly-synthesized 5-HT is preferentially released (see Section 1.4.2), though these data do not provide substantive evidence that newly-synthesized 5-HT is functionally distinct from stored 5-HT. The capacity of serotoninergic neurons to regulate their neurotransmitter output relative to the level of neurotransmitter stored intracellularly may allow the brain to sense metabolic processes (e.g., food consumption, stress, exercise) which alter brain tryptophan levels, and to bias the activity of postsynaptic neurons involved in certain behaviors (see Section 1.4.2).

Studies by others, utilizing brain slices and synaptosomes, have demonstrated that other LNAAs inhibit tryptophan's influx competitively (Parfitt and Grahame-Smith, 1973; Vahvelainen and Oja, 1975; Lahdesmaki and Hannus, 1977) and facilitate its efflux by an exchange process (Grahame-Smith and Parfitt, 1970; Cohen and Lajtha, 1972; Parfitt and Grahame-Smith, 1973). That the LNAAs participate in mediating their bidirectional flux across plasma membranes of brain cells is supported by the findings that 1) superfusing slices

with medium to which 100 μM leucine was added reduced tissue tryptophan levels, compared to those superfused with control medium, and 2) extracellular leucine suppressed the elevation in tissue typtophan levels caused by supplementing the medium with 2 μM tryptophan. These data do not allow for speculating about whether leucine acted predominantly on tryptophan's influx or its efflux. However, since the concentration of extracellular tryptophan in these experiments (0.06 - 2.12 μM) was in the range to be uptaken mainly by the high-affinity process (K_m 10 - 50 μM; see Section 1.3.4), it is likely that leucine competed with tryptophan for uptake into the slices at these transport sites (like that which probably occurs *in vivo*). Tryptophan uptake into another *in vitro* preparation, C6 glial cells, is inhibited competitively by the LNAA phenylalanine at both the high- and low-affinity processes (Bauman et al., 1974).

This study demonstrates that varying the frequency of electrical field-stimulation applied to hypothalamic slices over a constant period of time (i.e., the number of electrical pulses was the independent variable) regulated the rate of 5-HT release. Other *in vivo* studies have demonstrated that 5-HT release is decreased by pharmacological agents (i.e., 5-HT_{1A} agonists) which reduce the impulse flow along serotoninergic neurons (Gallager and Aghajanian, 1975; Sharp et al., 1989*a*; Sharp et al., 1989*b*), and is increased by delivery of electrical pulses to the raphe perikarya to accelerate their impulse flow (Holman and Vogt, 1972; Sharp et al., 1989*b*). Collectively, these findings suggest that 5-HT release from brain nerve terminals is frequency-dependent.

Increasing the frequency of electrical field-stimulation applied to the hypothalamic slices increased pulse-dependently the total amounts of evoked 5-HT release, but decreased the amounts of 5-HT released per pulse (Figure 16). These seemingly paradoxical observations

have been previously reported *in vivo* (Holman and Vogt, 1972; calculated from Sharp et al., 1989*b*) and *in vitro* (Gohert, 1980; Baumann and Waldmeier, 1981), and are likely to have occurred here because of the activity of release-modulating serotoninergic autoreceptors present on hypothalamic nerve terminals (Cerrito and Raiteri, 1979). In support of this hypothesis, as mentioned in Section 3.1.6, preliminary experiments suggested functional activity of these receptors in the slices, and others have demonstrated that electrically field-stimulating rat cerebral cortex slices at a high frequency in the presence of the 5-HT autoreceptor antagonist, methiothepin, increases the efflux of [^{3}H]5-HT per pulse to the higher rate measured at a low frequency in the absence of the drug (Baumann and Waldmeier, 1981). Alternatively, the frequency of electrical field-stimulation may be inversely related to the incremental change induced in the activity of Ca^{2+}-mediated release processes (i.e., voltage-dependent Ca^{2+} channels and/or Ca^{2+}-dependent cytoskeletal motility). Whichever process is responsible for the pulse-dependent suppression in 5-HT release, this discussion highlights yet another means by which serotoninergic neurons apparently limit the dynamic range of 5-HT release.

The magnitude of the effect of supplemental tryptophan on 5-HT release was found to be invariant (an increase of $21.4 \pm 4.5\%$ with the addition of 2 μM tryptophan to the superfusion medium) across the frequency range tested. This result is consistent with other *in vivo* studies demonstrating that raising rat brain tryptophan levels, by administering the amino acid or by consuming a carbohydrate-rich meal, causes an acceleration in brain 5-HT synthesis which is resistant to various pharmacological treatments which suppress impulse flow along serotoninergic neurons (see Section 3.2). Furthermore, the availability of tryptophan apparently regulates 5-HT synthesis, and thus 5-HT release, both during neuronal quiescence

and depolarization. Thus, the final output of a serotoninergic neuron, reflected in the amounts of 5-HT released per unit time, is probably determined by processes occurring both at the somatodendritic region and at its nerve terminals. Events at the somatodendritic region regulate the pattern of neuronal firing, and thus influence the rate at which 5-HT molecules residing in nerve terminals are released. The amounts of 5-HT available for release from nerve terminals are regulated by the activity of tryptophan hydroxylase, and thus by the availability of tryptophan. Action potentials invading serotoninergic nerve terminals at a certain frequency are likely to release amounts of 5-HT proportionate to the level of intracellular 5-HT.

4. SUMMARY AND CONCLUSIONS

1. A novel *in vitro* experimental system, utilizing superfused rat hypothalamic slices, was developed to monitor reliably the release of endogenous 5-HT (and 5-HIAA) under basal conditions and with electrical field-stimulation. The novelty of this system is based principally on the development of a new method to extract these 5-hydroxyindoles from superfusion medium; combined with high-performance liquid chromatography with electrochemical detection, these substances were assayed with high sensitivity. Secondarily, the experimental conditions applied in these studies may have permitted the expression of 5-HT dynamics which reflect those occurring physiologically.

2. This study characterized 5-HT release from rat hypothalamic slices superfused with physiologic medium (which contained fluoxetine). The relatively low molar ratios of 5-HT to 5-HIAA in superfusion medium under basal conditions suggest that a major fate of newly-synthesized 5-HT is to be metabolized rather than released or stored; with depolarization of the slices this changes transiently, so that more of the newly-synthesized 5-HT is released. 5-HT released with electrical field-stimulation, but not spontaneously, was both Ca^{2+}-dependent and TTX-sensitive. The small fractions of intracellular 5-HT released spontaneously and with depolarization are compatible with the view that 5-HT exerts a tonic, 'hormone-like' influence on brain function.

3. Time-dependent changes in medium and tissue indole levels were characterized. The superfusion medium actually contained low concentrations of endogenous tryptophan which varied with time. This medium tryptophan probably derived largely from free amino acids liberated from cellular proteins broken down in association with slice preparation. Tryptophan and 5-HT levels in slices superfused with physiologic medium changed in parallel over the experimental period, and were not further modified by exposure to fluoxetine and to periods of electrical field-stimulation.

4. The integrity of the slices during the experimental period was suggested by the reliable changes described in Items 2 and 3 above, the relatively constant ratio of tissue protein to DNA levels, and the retention of tissue LDH activity.

5. Elevating tissue tryptophan levels within the physiologic range, by superfusing the slices with medium supplemented with low concentrations of tryptophan, increased dose-dependently spontaneous and electrically-evoked 5-HT release. Control experiments demonstrated that under these conditions the addition of tryptophan to the superfusion medium did not modify the release of another hypothalamic neurotransmitter, dopamine; and superfusing the slices with medium supplemented with a low concentration of another amino acid, histidine, did not modify 5-HT release. These results suggest that there exists a physiologic coupling between increases in 5-HT synthesis and 5-HT release from nerve terminals.

6. Reducing tissue tryptophan levels, by superfusing the slices with medium containing a high concentration of leucine, produced decreases in the amounts of 5-HT released spontaneously and with electrical field-stimulation. These decrements in tissue tryptophan levels and 5-HT release were restored by superfusing the slices with medium co-supplemented with leucine and tryptophan. These data support the existence of carrier(s) which mediate the bidirectional flux of LNAAs across the plasma membrane of brain cells. They further suggest that the resultant level of intracellular tryptophan regulates 5-HT synthesis and thereby 5-HT release.

7. The amounts of 5-HT released from slices spontaneously and with electrical field-stimulation were proportionate to tissue levels of 5-HT; the fractions of intracellular 5-HT released per minute were invariant under treatments which either raised, lowered or maintained slice 5-HT levels. Thus, the amounts of brain 5-HT released spontaneously and with depolarization per unit time are likely to be proportionate to the level of intracellular 5-HT.

8. The frequency of electrical field-stimulation was related directly to the total amounts of evoked 5-HT release, but related indirectly to the amounts of 5-HT released per pulse. The magnitude of the tryptophan-induced potentiation in evoked 5-HT release was independent of the frequency of electrical field-stimulation. These data suggest that the level of 5-HT available for release is dependent on the rate of 5-HT synthesis, and thus on tryptophan availability; the actual amounts of 5-HT released from hypothalamic nerve terminals are likely to be dependent on the pattern of depolarizing current.

5. SUGGESTIONS FOR FUTURE RESEARCH

1. Altering the availability of tryptophan to hypothalamic slices produced proportionate changes in tissue 5-HT levels and 5-HT release. This finding suggests that 5-HT synthesis regulates the level of intracellular 5-HT, and thereby the amounts of 5-HT available for release. The proposed coupling between the rates of 5-HT synthesis and release could be tested directly by simultaneously monitoring tryptophan hydroxylase activity, 5-HT release and final tissue 5-HT levels when the slices were superfused with exogenous tryptophan or other LNAAs.

2. The proposed coupling between 5-HT synthesis and release is compatible with other studies indicating preferential release of newly-synthesized 5-HT. This intracellular dynamic may be tested by superfusing the slices with trace amounts of [^{3}H]tryptophan, and then monitoring the spontaneous and electrically-evoked release of [^{3}H]5-HT as a fraction of endogenous 5-HT, as well as the specific activity of final tissue 5-HT levels.

3. The fractions of intracellular 5-HT released were invariant under conditions which caused parallel physiologic changes in tissue tryptophan and 5-HT levels. These data suggest that the size of the intracellular 5-HT pool, which depends on 5-HT synthesis, controls 5-HT release. A pharmacologic study may provide some insight regarding the relative degree of coupling among 5-HT release, 5-HT synthesis and stored 5-HT. Slices prepared from reserpine-treated and control rats could be superfused with tryptophan-supplemented medium. If the amounts of 5-HT released from reserpine-treated slices were less than

those from control, then the dynamic proposed in this study would be supported (i.e., 5-HT release is proportionate to tissue 5-HT levels); if 5-HT release was not different, then it may be more tightly coupled to synthesis than to tissue levels. A caveat for this proposed study is that the initial tryptophan concentration in serotoninergic nerve terminals may not be the same in reserpine-treated and control slices (because 5-HT synthesis may be accelerated in reserpine-treated tissue secondary to reduced tissue 5-HT levels), and thus the incremental change in 5-HT synthesis induced by tryptophan-supplementation may be different.

4. The magnitude of the tryptophan-induced enhancement in evoked 5-HT release was invariant across the range of stimulation frequencies tested (1 - 5 Hz) when the train length was 4 minutes. It would be interesting to further examine this relationship when slices were exposed to stimulation conditions which demand even more 5-HT to be released. This could be accomplished by delivering to the slices pulses at 5 Hz (still within the physiologic frequency range) with increasing train lengths (e.g., 5 - 60 minutes) when they were superfused with or without supplemental tryptophan.

5. The relationship between tryptophan availability and 5-HT release has been described in hypothalamic slices. Experiments, like those done in this study, could be conducted using slices prepared from other brain regions to determine the extent to which this relationship is manifest in other serotoninergic nerve terminals.

6. There is evidence that 5-HT release from forebrain nerve terminals is presynaptically modulated by other neuroactive substances [i.e., norepinephrine, dopamine, acetylcholine, GABA, glutamate, somatostatin, tackykinins (substance P and neuromedin K), enkephalins, prostaglandins, adenosine]. It would be of interest to examine the interaction between tryptophan availability and receptor-mediated event(s) in regulating 5-HT release.

7. Tryptophan availability has been shown here to modulate 5-HT release from hypothalamic slices. A critical piece of evidence towards solidifying the principle that tryptophan availability influences serotoninergic neurotransmission is to demonstrate that there are specific consequences to tryptophan-induced changes in 5-HT release. This could be approached either biochemically or electrophysiologically. The former approach could utilize the property that most 5-HT receptors are linked to particular second messenger signals (i.e., cAMP, phosphatidylinositol metabolites: inositol triphosphate and diacylglycerol). If tryptophan-induced changes in 5-HT release are paralleled by changes in serotoninergic neurotransmission, then the levels of one or more of these intracellular signals would also be expected to change (in the presence of selective pharmacological agents which block their reincorporation or breakdown). The latter electrophysiological approach could be addressed in an invertebrate neural system to enable intracellular recording from a large, identified neuron which receives serotoninergic innervation, such as the siphon motor neurons of the marine snail Aplysia. Preliminary experiments would need to be conducted to assure that under basal conditions tryptophan hydroxylase in sensory nerve terminals is unsaturated with tryptophan, and that tryptophan supplementation increased

5-HT release. Following these confirmations, the electrophysiologic effects of supplementing the perfusing medium with tryptophan would be tested.

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