The High Resolution Light Microscopic Autoradiographic Localization of 5-HT1 Binding Sites in Rat Brain

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January 1987

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master's in Science.

DEDICATION

This thesis is dedicated to:
Natalie Kemp
In tribute to the
friendship we share.

ABSTRACT

A computer-assisted quantification and fine topographical analysis of the distribution of 5-HT1 binding sites in the rat brain was conducted on frozen intact 20 micron sections incubated with 3H-5HT and processed for wet autoradiography. Prior to undertaking this study it was necessary to: 1) establish conditions for optimizing the binding of 3H-5HT and the morphological preservation of the tissue 2) demonstrate that 3H-5HT could be effectively crosslinked to its specific binding sites 3) validate the distribution of 5-HT1 binding sites as seen on sections processed by wet autoradiography, by comparison to film autoradiographs of adjacent sections.

This high resolution approach permitted the obtention of densitometric data in discrete brain regions. Densities of 5-HT1 binding sites were correlated to the data obtained from the literature pertaining to the densities of 5-HT axonal varicosities and the frequencies of 5-HT synaptic contacts established by 5-HT axon terminals in a number of brain areas. A unique relationship apparently did not exist between the densities of 5-HT1 binding sites and the 5-HT innervation or synaptic frequencies in the brain.

A reversible decrease in 3H-5HT binding was observed in several brain regions, 24 hours after the injection of colchicine into the medial forebrain bundle, which contains the main fascicles of ascending 5-HT axons. This decrease correlated with the density of 5-HT innervation, suggesting that colchicine may have interrupted the transport of 5-HT autoreceptors in these neurons.

RESUMÉ

Ce travail décrit la mise au point d'une méthode radioautographique à haute résolution applicable à la localisation en microscopie photonique, et éventuellement électronique, des récepteurs de la sérotonine (5-HT) de type 5-HT1 dans le système nerveux central.

Une série de travaux préliminaires a d'abord permis: 1) d'établir les conditions d'incubation de coupes de cerveau congelé pré- ou non fixé favorables à une liaison optimale de la sérotonine tritiée (3H-5HT) 2) de démontrer que les molécules de 3H-5HT pouvaient être fixées de façon covalente sur, ou à proximité immédiate de leurs sites récepteurs sous l'action du glutaraldéhyde 3) de déterminer que le "rendement" de cette réaction de pontage par le glutaraldéhyde était proportionnel dans diverses régions du cerveau. Cette méthode a ensuite été appliquée à l'analyse densitométrique de la distribution régionale et intra-régionale des sites marqués dans le système nerveux central. La comparaison des résultats avec les données disponibles dans la littérature concernant la densité des terminaisons à 5-HT dans les mêmes régions et la fréquence avec laquelle celles-ci établissent des contacts synaptiques suggère: 1) qu'il n'ya pas de lien direct entre la densité des récepteurs 5-HT1 et celle des terminaisons à 5-HT dans les régions étudiées, et 2) que la densité des récepteurs 5-HT1 est indépendante de la fréquence avec laquelle les fibres à 5-HT établissent des contacts synaptiques.

Nous avons également démontré que l'injection intra-tissulaire de colchicine, un bloqueur du transport axonal, à proximité du faisceau prosencéphalique médian s'accompagne d'une diminution réversible des sites 5-HT1 dans plusieurs régions du cerveau, en amont comme en aval du site d'injection. Les baisses enregistrées sont directement en corrélation avec les densités d'innervation à 5-HT de ces régions, ce qui suggère qu'elles pourraient résulter d'une interruption de la synthèse et/ou du transport axonal d'autorécepteurs 5-HT1.

ACKNOWLEDGEMENTS

I would like to express my gratitude to the many people who assisted me in the realization of this thesis. In particular, I am indebted to my advisor, Professor Alain Beaudet, for his patience and insight in supervising every aspect of this project.

Next I thank Dr. Emmanuel Moyse and Kathleen Leonard who taught me most of the technical aspects of this research.

I also extend thanks to the members of my advisory committee, Professor Marika Quik and Professor Allan Sherwin for their interest in this thesis.

I am also grateful to Beverley Lindsay and Boyana Andric for their excellent secretarial assistance and to Lynda Mainville for proof-reading the thesis and teaching me the finer points of rat surgery. Finally, I thank Medical Artist Anne Fréchette and Photographer Mr. C. Hodge.

CLAIM FOR ORIGINAL WORK

This study describes the development of a high resolution wet autoradiographic method for visualizing 5-HT1 binding sites in frozen brain sections incubated with 3H-5HT. The successful application of this method made possible the completion of quantitative and fine topographical analyses of the distribution of 5-HT1 binding sites in rat brain, at the light microscopic level. 5-HT1 binding site densities recorded in several discrete brain regions were correlated with data obtained from the literature pertaining to both the density of 5-HT axon terminals and the frequency with which 5-HT axon terminals form synaptic contacts in those same regions. The results of this analysis provide original evidence that a unique relationship does not exist between the distribution of 5-HT1 binding sites and that of putative endogenous 5-HT release sites in the brain.

Evidence was also presented which suggests that 3H-5HT labelled binding sites may be transported by a colchicine-sensitive mechanism in fibers of 5-HT containing neurons passing through the medial forebrain bundle.

The results documented herein represent original contributions to knowledge of the cellular localization of 5-HT1 binding sites and their relationships with the 5-HT containing neurons of the brain.

TABLE OF CONTENTS

		Page
I.	Introduction	
A.	Anatomical Considerations	1
i.	Distribution of serotoninergic perikarya	1
ii.	Projections of the serotoninergic neuronal system	2
iii.	Ultrastructural characteristics of serotoninergic neurons	4
B.	Functional Considerations	5
i.	Serotonin biosynthesis	5
ii.	Multiple binding sites for serotonin	6
iii.	Characteristics of 5-HT binding sites	8
C.	Receptor Autoradiography	13
i.	Historical perspective	13
ii.	Autoradiographic visualization of 5-HT1 binding sites	14
D.	Receptor Transport Studies	14
i.	Macromolecular axonal transport	14
ii.	Colchicine-related changes in the regional distribution of 5-HT autoreceptors: A paradigm for studying the axonal transport of 5-HT autoreceptors	16
II.	Methods and Materials	
A.	Animal Maintenance	17
B.	Histological Techniques	17
i.	Prefixed vs fresh tissue	17
ii.	Tissue extraction and storage	18
iii.	Preparation of slides	18
iv.	Preparation of sections	18
C.	In Vitro Labelling of 5-HT1 Binding Sites	19
i.	Basic protocol	19
ii.	Establishment of optimal binding conditions	19
iii.	Optimizing morphological preservation	20
D.	Measurement of Total Bound Radioactivity	21
E.	Crosslinking of 3H-5HT Molecules	21
F.	Autoradiographic Processing	22
i.	Film autoradiography	22
ii.	Light microscopic autoradiography	23
iii.	Neuroanatomical staining	23
iv.	Wet vs film autoradiography	24
G.	Densitometric Analyses	24
i.	Preparation of standards and plotting of standard curves	24
ii.	Distributional analyses	25

Table of Contents (continued) Page

H. Receptor Transport Studies 26
i. Animal subjects and treatment 26
ii. Identification of the injection site 26
iii. Injection protocol 26
iv. Tissue processing 27
v. Quantitative distributional analysis 27

III. Results

A. Methodology 28
i. Prefixed vs fresh tissue 28
ii. Timecourse of 3H-5HT binding 28
iii. Tissue preincubation 28
iv. Buffer osmolarity and supplementation 28
v. Effects of histological processing on bound 3H-5HT 29
vi. Effects of light microscopic processing on the distribution of 3H-5HT binding 29

B. Distributional Analyses of 5-HT1 Binding Sites 30
i. Regional distribution 30
ii. Light microscopic localization of 5-HT1 binding sites 33
iii. Correlation of 5-HT1 site densities with the 5-HT innervation of discrete areas in rat brain 33

C. Effects of Colchicine Injection on the Distribution of 3H-5HT Binding 34

IV. Discussion

A. Methodology 35
i. Tissue Prefixation 35
ii. Specificity of 3H-5HT binding 35
iii. Kinetics of 3H-5HT binding 36
iv. Tissue preincubation 36
v. Incubation media 37
vi. The effects of histological processing on the retention of bound 3H-5HT 38

B. Distributional Analyses of 3H-5HT Binding 39

C. Colchicine-Related Changes in the Distribution of 5-HT1 Binding Sites 46

V. Bibliography 48

LIST OF TABLES

		Page
1.	Effects of Prefixation on 3H-5HT Binding	59
2.	Effects of Preincubation on 3H-5HT Binding	60
3.	Effects of Buffer Osmolarity and Supplementation of 3H-5HT Binding	61
4.	Effects of Fixation and Defatting on Bound 3H-5HT	62
5.	Regional Retention of Specifically Bound 3H-5HT After Postfixation and Delipidation	63
6.	Comparison of Densitometric Data Obtained from Film and Wet Autoradiographs	64
7.	Quantitative Distributional Analysis of 5-HT1 Binding Sites in Rat Brain	65
8.	Correlation of 5-HT1 Binding Site Densities with 5-HT Innervation Densities	69
9.	Correlation of 5-HT1 Binding Site Densities with Densities of 5-HT Synaptic Contacts	70
10.	Colchicine-Related Changes in the Regional Distribution of 5-HT1 Binding Sites	71
11.	Correlation Between Regional Changes in 3H-5HT Binding After Colchicine and 3H-Imipramine Binding	72

LIST OF FIGURES

		Page
1.	Standard curve for tritium labelled sections	74
2.	Timecourse of 3H-5HT binding to intact frozen sections of rat midbrain	76
3.	Topographical distribution of 3H-5HT binding sites before and after histological processing	78
4.	Comparison of the topographical distribution of 3H-5HT binding as seen on film and wet autoradiographs of adjacent sections	80
5.	The distribution of 3H-5HT binding sites within selected brain regions seen in dipped sections	83
6.	Laminar labelling within the rat hippocampus seen in dipped sections	86
7.	The cellular localization of 3H-5HT binding sites in selected brain regions	88
8.	Saturation curve for 3H-5HT binding	91

I. INTRODUCTION

A. Anatomical Considerations

Serotonin was first identified biochemically in the central nervous system by Twarog and Page (1953)[113]. The subsequent demonstration of a reduction in brain serotonin resulting from a lesion of the median forebrain bundle provided an initial indication that the indolaminergic pool was associated with a particular neuronal system[48]. Confirming evidence came with the advent of fluorescence histochemistry which allowed for the localization of 5-HT-containing specific cell groups in the brain[35]. Through the use of this technique, the distribution of serotoninergic cell bodies was depicted as being confined to the raphe and reticular systems of the brainstem[30]. These neurons have been classified by Dahlstrom and Fuxe (1964) into nine major groups designated B1 to B9[30]. This classical scheme has recently been extended to include a tenth neuronal group that was localized in the hypothalamus by autoradiographic[12] and immunocytochemical[39] methods.

The projections of the serotoninergic system have been traced using several technical approaches[26,83,109,114]. Emerging from these studies was a description that established the widespread connections of the serotoninergic cell groups with all regions of the central nervous system.

i. Distribution of serotoninergic perikarya

The nine cell groups described in the rat by Dahlstrom and Fuxe (1964) extend along the midline from the caudal medulla to the rostral mesencephalon[30].

Medullary serotoninergic groups B1, B2, and B3 can be identified within the raphe nuclei pallidus, obscurus and magnus, respectively[30]. The B1 serotoninergic group is ventromedially placed in the caudal medulla underlying the B2 group. Emerging as a rostral prolongation of B1 is the B3 group: a large body of neurons that stretches towards the caudal pons. The ventral groups (B1 and B3) produce lateral extensions that penetrate the reticular formation of the medulla; B1 extends into the nucleus paragigantocellularis lateralis and B3 extends around the emergence of the 12th nerve into nucleus gigantocellularis[30].

In the pons, groups B4 and B6 are found medially within the periventricular grey; the latter placed rostral to the former[30]. Group B5 corresponds to nucleus raphe pontis. Serotoninergic perikarya are also found in the oral part of the pontine reticular nucleus[30].

Serotoninergic cell populations achieve maximal development in the mesencephalon[30]. Therein lie the groups designated B7, B8, and B9. The B7 group is found in the ventral aspect of the periaqueductal grey, incorporated into the nucleus raphe dorsalis and its lateral extensions[30]. Centered on the midline of the tegmentum is the B8 group, forming part of the nucleus raphe medianus (also referred to as nucleus centralis superior) and its rostral prolongation the nucleus linearis caudalis[30]. Stretching ventrolaterally from B8 along the dorsal border and between the fibers of the medial lemniscus is the B9 group[30]. In addition, serotoninergic neurons are dispersed in the mesencephalic reticular formation and in the interpeduncular nucleus[30].

The existence of a tenth serotoninergic cell group has now been established in the pars ventralis of the nucleus dorsomedialis hypothalami. This diencephalic population has been shown to concentrate 3H-5HT administered via ventricular infusion and has been immunocytochemically labelled with anti-5HT antiserum[12,39].

ii. Projections of the serotoninergic neuronal system

Pertinent information regarding the projections of the serotoninergic neuronal system has been accumulated by way of: 1) 5-HT histofluorescence techniques[30,114] 2) the light and electron microscopic detection of tryptophan hydroxylase or 5-hydroxytryptamine immunoreactivity[93] 3) tracing techniques dependent on the anterograde transport of horseradish peroxidase or labelled proteins[73] 4) and light microscopic autoradiographic studies following the intraventricular infusion of 3H-5HT[12,83].

Ascending projections

Ascending 5-HT fibers issue primarily from the mesencephalic raphe nuclei and are organized into two systems: a transtegmental system and a periventricular system[83]. These systems ascend separately sending forth several branches within the mesencephalon and diencephalon[83]. They eventually converge in the medial forebrain bundle (MFB) in conjunction with several limbic pathways[83]. Fibers transmitted in the MFB are organized into pathways that then reach distant territories of innervation.

The transtegmental system corresponds to the ventral serotoninergic system referred to by certain authors[5]. It originates mainly from the nucleus raphe dorsalis with a small contingent of fibers emanating from nucleus raphe medianus[83]. From

these midbrain nuclei it is conveyed to the ventral tegmental area by first coursing ventrally across the decussaton of the superior penduncles and then passing rostrally through the ventral tegmental decussation[83]. The transtegmental system branches to innervate the substantia nigra and ventral tegmental area ventrally, and the habenular, and the parafascicular nucleus of the thalamus dorsally; but the bulk of its fibers proceed rostrally towards the MFB[83]. After entering the MFB those fibers originating in nucleus raphe dorsalis project mainly to lateral forebrain areas (e.g. basal ganglia, amygdala, piriform cortex)[5]. In contrast, the contingent of fibers from nucleus raphe medianus are conveyed mainly to ventromedial targets (e.g. cingulate cortex, medial septum and hippocampus)[5]. The periventricular system emanates from the rostral pole of raphe nucleus dorsalis to closely follow the path taken by the dorsal longitudinal fasciculus of Schutz[83]. A majority of its fibers arch ventralward beneath the posterior commissure to reach the dorsal hypothalamus[83]. Along this path branches are emitted that impinge upon the tectum and subcommissural organ in addition to providing a dense 5-HT innervation to the periventricular grey matter of the midbrain and caudal diencephalon[83].

Descending pathways

For the most part, descending serotoninergic pathways originate from the medullary raphe nuclei (B1-B3)[26,114]. A minor contribution also arises from the nucleus raphe dorsalis and the serotoninergic neurons of the mesencepahlic and medullary reticular formations[26,114].

Within the brainstem, fibers are issued that innervate the solitary nucleus, dorsal motor nucleus of the vagus and the marginal and gelatinous layers of the spinal trigeminal nucleus[114]. The spinal cord receives an extensive serotoninergic input to all layers[114]. Axons descend in the medial part of the anterior funiculus and in the anterior part of the lateral funiculus to terminate in the ventral horn[114]. Others are conveyed in the dorsal part of the lateral funiculus to innervate the dorsal horn and sympathetic lateral column[26,114].

Cerebellar pathways

As evidenced by retrograde transport studies several raphe nuclei project to the cerebellum but heretofore the transmitter within these cerebellar afferents has not been identified[104]. The ubiquity of serotonin containing neurons in the raphe nuclei has

led to the suggestion that they are the source of the serotonin innervation to the cerebellum[28,104]. However, this suggestion may have been premature, given that raphe neurons are not restricted to the use of serotonin as a neurotransmitter[116]. Recent studies combining serotonin immunocytochemistry and the retrograde transport of horseradish peroxidase have been instructive in this regard[21]. Following injections of horseradish peroxidase into cerebellar cortex, projections emanating from several raphe and reticular nuclei were confirmed[21]. Yet, serotonin immunoreactive fibers were deemed to be issuing almost exclusively from medullary and pontine reticular nuclei, namely: nucleus reticularis gigantocellularis, nucleus reticularis paragigantocellularis and nucleus pontis oralis[21].

iii. Ultrastructural characteristics of serotoninergic neurons

Most of our current knowledge concerning the fine structural features of serotoninergic neurons is derived from immunocytochemical and autoradiographic studies[1,13,16].

Serotonin containing and/or accumulating perikarya have been identified by means of autoradiography or immunocytochemistry in the brainstem and hypothalamus[12,30,39,109]. However, attempts at isolating recognizable cytological features unique to serotoninergic nerve cell bodies have been frustrated[13]. A majority of 5-HT neurons are endowed with axons that arborize profusely, projecting from their cell groups of origin to innervate the entire neuraxis[13]. Generally, the axons issued belong to a category of thin unmyelinated fibers that send forth billions of varicose terminals along their path[13]. These varicosities manifest as small axonal enlargements (05.-1nm in diameter) containing a mixed population of large granular (50-130nm) and small agranular (15-50nm) vesicular organelles[13,16]. Large granular vesicles are present in perikarya and are known to be transported to the 5-HT terminals where they are usually found in highly variable but sparse numbers[16]. The variability of their presence precludes a primary role for them in the storage of 5-HT. Albeit the high affinity for 3H-5HT featured by the large granular vesicles has led to the postulation of an involvement in the metabolism and/or transport of serotonin and its biosynthetic enzymes[16]. The small agranular vesicles are strictly featured within axon terminals and are thought to be responsible for the storage and subsequent release of endogenous serotonin[16].

A feature of central 5-HT varicosities with possible functional implications, is the rarity with which they form classical junctional complexes (pre- and post-synaptic differentiations) in comparison with other terminals in the surrounding neuropil[13,16]. A given 5-HT axon may be endowed exclusively with junctional or nonjunctional varicosities or possess some combination of these terminal genres[13]. Interestingly, serotonin is released at both junctional and nonjunctional sites[16]. The proclivity towards the predominance of extra-junctional varicosities exhibits a regional variation; in regions where typical synaptic contacts are scarce, serotonin is believed to be engaged as a neurohumoral agent[13].

In reality, most areas receive a dual 5-HT innervation, both junctional and nonjunctional[13]. However, exceptional cases have been cited: the subcommissural organ appears to receive a strictly junctional 5-HT input, while 5-HT terminals in the median eminence and cerebral ventricles are systematically devoid of classical synaptic contacts[13]. A modification in the incidence of synaptic junctions can be induced by alterations in the terminal field encountered within a given brain region[13]. Such is the case with 5-HT axons impinging on the rat cerebellar cortex after the x-ray induced lesioning of the external granular layer[14,108]. Sotelo and Beaudet (1979) described an evolution from a primary non-synaptic to a mainly synaptic innervation in the agranular rat cerebellum[108]. Thus, the frequency with which 5-HT varicosities establish synaptic contacts was postulated to be largely under the influence of epigenetic factors in the territory of innervation[13].

B. Functional Considerations

i. Serotonin biosynthesis

The synaptic demands for 5-HT are met by regulatory responses in the somata of serotoninergic neurons[6]. As required, synthetic enzymes and storage vesicles are conveyed via axonal transport to distal sites of synthesis in 5-HT terminals[6]. Apparently, storage vesicles for serotonin travel at a fast rate (125mm/day) while synthetic enzymes move at a slow rate (5-7mm/day)[6].

Whereas the synthesis and subsequent axonal transport of these materials is energy-dependent, the capacity of the system is greatly influenced by the ATP levels existing within the perikarya and dendrites of serotoninergic neurons[6]. A feature of serotonin biosynthesis essential for satisfying acute demands for the transmitter is the

capacity for modification in its turnover rate[6]. Serotonin biosynthesis is controlled by the availability of four factors necessary for the progress of the rate limiting step[6]. These determinants are: the substrate, an enzyme, molecular oxygen and a cofactor[6].

L-tryptophan, the substrate for serotonin synthesis, is taken up by indolaminergic neurons through an active transport process[6]. The rate limiting step involves the conversion of this amino acid by the enzyme tryptophan hydroxylase into a serotonin precursor 5-hydroxytryptophan (5-HTP)[6]. Completion of this reaction occurs only in the presence of molecular oxygen and requires BH4 as a cofactor[6]. A decarboxylation step ensues through which 5-HTP is converted to serotonin in a reaction catalyzed by the enzyme L-5HTP decarboxylase[6]. Once synthesized, serotonin is stored in vesicles until it is released or degraded[6]. Subsequent to release, serotonin is inactivated by reuptake and then degraded by a monoamine oxidase into the by-product 5-hydroxyindolacetic acid[6].

ii. Multiple binding sites for serotonin

Following in vivo administration, drugs affecting serotoninergic functions illicit profound neurochemical and behavioral alterations. The broad scope of these effects reflects the ubiquity of serotonin containing and receptive neurons in the nervous system. A more comprehensive understanding of 5-HT receptors may eventually permit the targeting of specific pathways or neuronal groups for therapeutic drug treatment. The study of 5-HT receptors has been complicated by evidence suggestive of the presence of multiple binding sites for serotonin in the nervous system. By definition, identifying a binding site for serotonin as a receptor necessitates the thorough characterization of its biochemical, pharmacological, physiological, and distributional properties.

Over thirty years ago, Gaddum and Hameed formulated the concept of multiple 5-HT receptor types[40]. Based on research into the peripheral tissues of the rat and rabbit these authors distinguished two types of 5-HT sites[40]. Namely, a uterine population readily blocked by lysergic acid diethylamide (LSD) and dihydroergotamine and a separate intestinal population, relatively insensitive to these antagonists[40]. Electrophysiological investigation into the invertebrate nervous system lent support to the postulated existence of multiple 5-HT receptor types [41,42]. In the Aplysia ganglion, 5-HT was observed to induce ionic fluxes indicative of both excitatory and inhibitory responses. After monitoring the types of ionic changes that occured and

noting the effects of various antagonists on these changes, as many as six 5-HT receptor types were defined[41,42]. Similarly, iontophoretic administration of 5-HT in the rat brain produces excitation in some brain areas and inhibition in others[3]. The inhibitory action of 5-HT is most prominent in the midbrain and forebrain nuclei, while 5-HT related facilitation of neuronal transmission has been described in the medullary reticular formation and facial nucleus[3]. Moreover, differential pharmacological profiles have been ascribed to putative 5-HT receptors mediating inhibition and excitation[91]. The excitatory effects of 5-HT are apt to be blocked by various 5-HT antagonists (LSD, methysergide and cinanserin)[91]. In contrast, serotonin mediated inhibition is not susceptible to the actions of these drugs[91].

The concept of multiple 5-HT receptor types has been corroborated by evidence derived from radioreceptor binding assays. Bennett and Aghajanian (1974) pioneered the application of this technique to the study of serotonin receptors[17]. These authors discovered the high affinity (kd = 7.5nM) saturable binding of 3H-LSD to brain membrane sites[17]. A significant observation was that the highest levels of specific binding were displayed in regions endowed with a large serotoninergic innervation[17]. Subsequently, landmark experiments by Bennett and Snyder (1975) demonstrated, for the first time, the high affinity (kd = 7nM) binding of 3H-5HT to rat brain membranes[18]. Pharmacological data suggested that both 3H-5HT and 3H-LSD were labelling 5-HT receptors; however, relevant disparities were reputed[19]. Whereas the agonist 5-HT had a greater affinity for 3H-5HT than for 3H-LSD binding sites, the reverse was true for the classical serotoninergic antagonists (cyproheptadine, cinanserin)[19]. LSD with mixed agonist-antagonist properties possessed an equal affinity for the 3H-5HT and 3H-LSD sites[19]. In view of this evidence, Bennett and Snyder (1976) maintained that two mutually exclusive states of the 5-HT receptor could be defined, with 3H-5HT binding to the agonist state and 3H-LSD to both the agonist and antagonist states[19].

A major development in the study of 5-HT receptors occurred when Leysen et al (1978) reported that the tritiated form of the neuroleptic spiroperidol, previously restricted to its use as a label of dopamine receptors, could bind specifically a putative 5-HT receptor in the rat brain[61]. In striatal membranes 3H-spiroperidol and a related compound 3H-haloperidol, label dopamine receptors[62]. However, in frontal cortex where dopamine receptors are few in number, 3H-spiroperidol alone displayed

significant degrees of specific binding[62]. Importantly, of all agents tested 5-HT along with its antagonists were the most potent displacers of this binding[62]. Moreover, the pharmacological profile of cortical 3H-spiroperidol binding correlated significantly with that of cortical 3H-LSD binding[62]. This apparent serotoninergic component of 3H-spiroperidol binding was later confirmed by other investigators[29].

Thus, by the year 1979, three distinct 3H-ligands had been shown to specifically label serotonin receptors. Peroutka and Snyder (1979) were instrumental in elucidating the binding specificities of 3H-LSD, 3H-5HT and 3H-spiroperidol in rat frontal cortex[89]. These authors noted that unlabelled LSD, 5-HT and spiroperidol revealed marked differences in potencies and displacement slopes when individually placed in competition with each of the tritiated ligands[89]. This suggested that the three tritiated ligands labelled distinct populations of receptor sites. As such, Peroutka and Snyder (1979) concluded upon the existence of two classes of non-interconverting 5-HT binding sites which they designated 5-HT1 and 5-HT2[89]. While 3H-5HT and 3H-spiroperidol labelled with high affinity 5-HT1 and 5-HT2 sites respectively, 3H-LSD appeared to bind populations equally and with high affinity[89]. Thereafter Peroutka et al (1981) reported that the pharmacological properties of serotonin-induced synaptic inhibition and excitation were suggestive of mediation by 5-HT1 and 5-HT2 sites, respectively[91].

The existence of 5-HT autoreceptors that control the firing of serotoninergic neurons has been clearly established by electrophysiological studies[80]. Martin and Sanders-Bush (1983) assessed the ability of certain 5-HT agonists to act at the 5-HT autoreceptor to prevent the K^+ evoked release of 3H-5HT[71]. Given the resemblance between the pharmacological profile of the autoreceptor population under study and that of the 5-HT site, these authors surmised that the 5-HT1 binding sites and the 5-HT autoreceptor may be related sites[71].

iii. Characteristics of 5-HT binding sites

5-HT1 binding sites

The 5-HT1 class is defined by a strong proclivity towards the binding of 3H-5HT (kd = 1-4nM)[89] and is distinct from another high affinity population (kd = 13-15nM) recently described[96].

A common structural feature of compounds that interact with high affinity at 5-HT1 binding sites is the indole nucleus[64]. Indole derivatives possessing serotonin mimetic activity, such as: tryptamine, bufotenine, and N,N-dimethyl tryptamine, potently displace 3H-5HT binding[64]. In addition, most ergot derivatives (e.g. LSD), which also have an indole nucleus in their structure, bind with high affinity to the 5-HT1 class[64]. Known serotonin antagonists (cinanserin, cyproheptadine, ketanserin and mianserin) lack an indole moiety and show very low or no binding affinity for 5-HT1 binding sites[64]. Although many compounds that interact with high affinity at 5-HT1 binding sites contain an indole nucleus, this is not a strict structural requirement. The serotoninergic agonist 8-OH-DPAT (8-hydroxy-2(n-dipropylamino) tetralin) was the first non-indole shown to potently inhibit 3H-5HT binding[52].

Apparently, the 5-HT1 class represents a heterogenous population of binding sites which has been subdivided on the basis of pharmacological data into a number of subtypes. An initial indication of this heterogeneity was provided by the demonstration that spiperone competes with 3H-5HT binding in a biphasic manner[88]. Pedigo et al (1981) designated the high and low affinity sites of the spiperone competition curve, 5-HT1a and 5-HT1b, respectively[88]. The postulated heterogeneity of 5-HT1 binding sites was further substantiated when it was revealed that some serotoninergic agonists such as RU24969[34] and 8-OH-DPAT[52], and certain beta blockers also inhibit 3H-5HT binding in a biphasic manner[77]. The 5-HT1a site can be selectively labelled with 8-OH-DPAT while beta blockers RU24969 interact preferentially with the 5-HT1b subtype[86]. Recently, a third subpopulation, designated 5-HT1c, was discerned on the basis of its high affinity recognition of 3H-LSD and the serotonin antagonist 3H-mesulergine[85].

Quantitative distributional studies have been conducted by several authors using film autoradiographs prepared from brain sections incubated with the high affinity 5-HT1 label, 3H-5HT[20,67,69,86]. Brain regions shown to be enriched with 5-HT1 sites include the: choroid plexus, nucleus raphe dorsalis, olivary pretectal nucleus, substantia nigra, entorhinal cortex, hippocampus, dorsal subiculum, dentate gyrus, globus pallidus, ventral pallidum and lateral septal nucleus[86]. More specifically, an estimation of the relative contributions made by the different subtypes towards the overall distribution of 5-HT1 binding sites has exposed some important regional variations[86]. Pazos and Palacios (1985) used 8-OH-DPAT and the beta blocker 21009 to selectively compete with 3H-5HT binding to 5-HT1a and 5-HT1b sites, respectively[86]. Binding of 3H-5HT in the presence of these selective compounds was then expressed as a proportion of the

total regional density of 5-HT1 binding sites. Brain areas where the total binding was most affected by the introduction of 8-OH-DPAT included the dentate gyrus and septal nucleus, indicating an enrichment of 5-HT1a sites in these regions[86]. This confirmed data previously obtained from the direct labelling of 5-HT1a sites with 3H-8-OH-DPAT[69]. Displacement by the 5-HT1b selective agent 21009 was most prevalent in the: globus pallidus, dorsal subiculum, substantia nigra and olivary pretectal nucleus[86]. Finally, as determined by direct labelling with 3H-mesulergine[85], 5-HT1c sites were present in very high densities within the choroid plexus.[86].

Regarding their physiological significance, 5-HT1 binding sites have been implicated in the induction of the serotoninergic syndrome involving several motor activities (Straub's tail, forepaw treading, headweaving and hindlimb abduction), illicited by serotonin and its precursors[65]. This syndrome can also be produced by 8-OH-DPAT[112] and is antagonized by beta blockers[43]. 5-HT1 sites seem to be involved in additional motor effects produced by RU-24969 and/or 8-OH-DPAT including the circling behavior in striatum lesioned rats[22,54], myoclonic jerking in guinea pigs[66] and hyperlocomotion in mice and rats[44,111]. Finally, serotonin is commonly attributed a role in the regulation of nociceptive activity[7,8,9] and it appears that 5-HT1 sites are responsible for mediating this control[100,123]. Given that the 5-HT1a subtype predominates in the dorsal horn of the spinal cord, a possible role for these sites can be envisaged[86].

Controversy persists regarding the validity of these and other physiological correlates for the 5-HT1 class. Yet, there is evidence to suggest that these sites are functional. Firstly, the 5-HT1 site appears to be linked to adenylate cyclase[38] and its affinity for 3H-5HT is regulated by the presence of guanine nucleotides[90]. Moreover, an amplification of 3H-5HT binding to rat membrane preparations has been described subsequent to the selective lesioning of the serotoninergic neuronal system with the neurotoxin 5,7-dihydroxytryptamine (5,7 DHT)[94,19,23]. The basis for the increase in 3H-5HT binding observed post-lesion has been the subject of some debate in the literature. Certain authors have explained the response to 5,7-DHT treatment in terms of an increase in the affinity of 3H-5HT for its binding sites[19]. In contrast, Quik and Azmitia (1983) attributed the change in 3H-5HT binding to an increase in the absolute number of detectable 5-HT1 binding sites[94]. In any event, the evidence that 5-HT1 binding sites on indolaminoreceptive neurons are under some kind of modulatory control is by itself suggestive of their functionality.

5-HT2 binding sites

High affinity 3H-spiroperidol binding defines a type of serotonin binding site, designated 5-HT2[89]. This class can be subclassified by competition with methysergide which discriminates 5-HT2a (high affinity) and 5-HT2b (low affinity) binding sites[70,74]. The 5-HT2 site has a pharmacological profile that reflects its micromolar affinity for serotonin and its agonists (tryptamine, bufotenine, N,N-dimethyl tryptamine and 8-OH-DPAT)[64]. Antagonists of serotonin (including cyproheptadine, cinanserin, mianserin, ketanserin, methysergide and metergoline) bind with nanomolar affinities to the 5-HT2 site[64]. Of these high affinity agents 3H-ketanserin has demonstrated the greatest selectivity for the 5-HT2 sites[63]. Distributional studies with this and other labels have revealed a heterogenous distribution of 5-HT2 densities in the rat brain[87] and post-mortem human brain tissue sections[74,101]. Briefly, these sites are most prevalent in the frontal cortex, olfactory tubercle, claustrum, nucleus accumbens and mammillary bodies[64,87]. Low concentrations have been reported in the thalamus, hippocampus, brainstem, and cerebellum[64,87].

Selective lesioning of dopaminergic and noradrenergic axons in the MFB and serotoninergic nerve cell bodies in the raphe nuclei were evaluated with regards to their effects on 5-HT2 binding[64]. These experiments suggest that 5-HT2 sites are not present on terminals of these neurons, but are rather localized on postsynaptic membranes. This interpretation is consistent with the reduction in 5-HT2 binding site in cortex, observed after injection of the perikaryal cytotoxic agent, kainic acid[64].

Serotonin autoreceptors

Serotonin neurons may be recognized by their electrophysiological characteristics when recording in the nucleus raphe dorsalis[6]. As such, it has been possible to evaluate the responses of these neurons, ensuing from the iontophoretic application of 5-HT as well as several compounds possessing serotoninergic activity (e.g. LSD, bufotenine, psilocin or N,N dimethyl tryptamine)[2]. In each case, the firing of serotoninergic raphe cells was inhibited, suggestive of the presence of autoreceptors on these neurons[2]. This hypothesis was corroborated by two lines of evidence: 1) 5-HT can inhibit the K^+ induced release of 3H-5HT from loaded synaptosomal preparations[71] 2) LSD inhibits the release of 5HT from brain slices[36,46].

Serotonin autoreceptors have pharmacological properties permitting their discernment from 5-HT heteroreceptors. Cerrito and Raitairi (1979) illustrated this

contention in reporting that the 5-HT induced inhibition of 3H-5HT release from loaded synaptosomes could be blocked by methiothepin, a serotonin antagonist[27]. In contrast, other 5-HT antagonists (cyproheptadine, methysergide and mianserin), presumed by other authors to act preferentially at postsynaptic sites, were incapable of reproducing this presynaptic effect[27]. A further pharmacological distinction between pre- and post-synaptic receptors is substantiated by the fact that several hallucinogens (LSD, psilocin, N,N dimethyl tryptamine) have a more potent inhibitory action on the firing of raphe cells than on postsynaptic cells[2]. Actually, a positive correlation has been established between the hallucinogenic potencies of these drugs and their ability to discriminate between auto- and heteroreceptors[2]. This finding supports the claim that LSD and indolamine hallucinogens exert their hallucinogenic effects by interacting with 5-HT autoreceptors and not heteroreceptors.

Interestingly, the 5-HT1a selective compound 8-OH-DPAT demonstrates a capacity for blocking 5-HT autoreceptors on raphe neurons[47]. Moreover, there is evidence to suggest that the 5-HT1a subtype is localized on the 5-HT neurons in the raphe nuclei[79]. Labelling studies with 3H-5HT and 3H-OH-DPAT have revealed a posterio-anterior gradient in binding that reflects the distribution of 5-HT containing fibers and perikarya within the nucleus raphe dorsalis[79]. The labelling of nucleus raphe dorsalis with 3H-OH-DPAT is completely eradicated after 5,7 DHT treatment[79]. Although 3H-5HT binding was still evident post-lesion, the pattern of a posterio-anterior increase in labelling disappeared[79]. The remaining 3H-5HT binding sites were assumed to be of the 5-HT1b subtype present on non-serotoninergic elements[79]. From these studies, speculation emerged with regards to a possible correspondance between the 5-HT1a subtype and a 5-HT autoreceptor population commonly described on raphe neurons[79].

5-HT autoreceptors are also present on serotoninergic nerve terminals where they are involved in controlling the release of transmitter[75]. Engel et al (1983) reported that the pharmacological properties of a certain autoreceptor population in rat frontal cortex correlated with that of the 5-HT1 binding site and, in particular, with the 5-HT1b component of 3H-5HT binding[33]. The beta blocker propranolol is a stereoselective and potent displacer of binding to the 5-HT1b subtype[72]. Middlemiss (1984) successfully used the enantiomer (-)-propranolol to antagonize the inhibitory effects of 5-HT at the autoreceptor in rat frontal cortex[72]. Given that the 5-HT1a selective agonist 8-OH-DPAT is inactive at this 5-HT autoreceptor population[72], a possible relationship with the 5-HT1b site can be surmised.

In summary, there are some similarities between the pharmacological properties of two distinct 5-HT autoreceptor populations and the 5-HT1a or 5-HT1b components of 3H-5HT binding. The 5-HT1a subtype appears to be related to a 5-HT autoreceptor population in the raphe nuclei while the 5-HT1b subtype bears a pharmacological resemblance to an autoreceptor population in rat frontal cortex.

C. Receptor Autoradiography

i. Historical perspective

The topographical localization of sites mediating neurotransmitter action in the central nervous system has been facilitated by consecutive developments in the techniques of receptor autoradiography. Early studies were performed subsequent to the systemic injection of the appropriate radioligand into the intact animal. Unfixed, frozen brain sections derived from these pretreated animals were then processed for autoradiography in accordance with a procedure compatible with the detection of diffusible substances[110]. This approach permitted the initial autoradiographic identification of several transmitter receptor sites including: muscarinic cholinergic[55], opioid[92] and dopaminergic[56] binding sites.

In 1979, Young and Kuhar introduced a novel autoradiographic approach that involved the in vitro incubation of unfixed or lightly fixed brain tissue sections with the radioligand of interest[119]. Labelled sections were then processed for autoradiography by apposition to an emulsion coated coverslip. A variation of this procedure, developed later, involved the apposition of incubated sections to a radiosensitive film[95]. In vitro techniques provided several advantages over the in vivo approach in allowing for the: 1) use of radioligands that were unable to penetrate the blood brain barrier 2) labelling of the tissue in a controlled environment 3) enhancement of the signal to noise ratio of the radioligand.

These 'dry' autoradiographic techniques, however, did not provide optimal morphological preservation of the tissue. Moreover, the resolution of the autoradiographic image produced was limited. An important contribution to the field of receptor autoradiography was made when Herkenham and Pert (1980) devised a method of processing radiolabelled sections for autoradiography according to standard coating procedures[49]. A paraformaldehyde vapor fixation step was implemented which made it possible to dehydrate and subsequently dip the sections in a bath of liquid emulsion[49].

This wet autoradiographic procedure proved to be valuable for the visualization of opiate binding sites at the light microscopic level but was still incompatible with the cellular and subcellular detection of these sites[50].

More recently, Beaudet and colleagues modified this wet autoradiographic approach by introducing a glutaraldehyde fixation step[45,76]. Glutaraldehyde possesses two functional groups and is thus capable of forming stable bonds with proteins[53]. This method of fixation proved to be compatible with the light and electron microscopic detection of 125I-FK 33 824 and 125I-Neurotensin binding sites in rat brain[45,76]. Previous studies with 125I-FK 33 824 revealed that a free amino group was a prerequisite for the functioning of glutaraldehyde as a crosslinking agent[45]. As such, Hamel and Beaudet (1984) proposed that the methodology developed might be applicable to the localization of other radioligand molecules endowed with a free amino group[45].

ii. Autoradiographic visualization of 5-HT1 binding sites

Previous attempts at localizing 5-HT1 binding sites have relied on dry autoradiographic techniques, involving the apposition of sections incubated with 3H-5HT, to an emulsion coated film or coverslip[20,67,86,121]. The present project was undertaken with the intention of developing a high resolution autoradiographic approach to the visualization of these sites; based on the original method introduced by Beaudet and colleagues[45,76]. After first demonstrating the feasibility of employing this approach, it was possible to: 1) conduct a topographical and cellular analysis of the distribution of 5-HT1 binding sites 2) undertake a thorough regional quantification of the densities of 5-HT1 binding sites in the brain. The densitometric data recorded in sections processed for light microscopic autoradiography were correlated to previously documented quantitative data pertaining to the density of 5-HT varicosities as well as the frequency with which 5-HT varicosities form classical junctional complexes, in discrete regions of the rat brain.

D. Receptor Transport Studies

i. Macromolecular axonal transport

The neuronal control center for macromolecular synthesis is the cell body. Accordingly, the high degree of subcellular specialization characteristic of nerve cells must be provided for through the intraneuronal communication of metabolic materials

and membrane components over large distances from the soma[103]. To serve this purpose, an elaborate axonal transport system has evolved[103]. Anterograde transport originating from the cell body supplies the terminal field while retrograde transport operates in the reverse direction; permitting an efficient recycling of materials[103].

The bulk of materials travel anterogradely by a slow process called axonal flow[103]. In contrast, fast transport, which is an energy dependent process, distributes primarily intracytoplasmic membranes to the neuron's terminal field[103]. Microtubules and contractile proteins (neural actins and myosins) are favored candidates as the polymeric molecules that mediate this transport[103]. The polar nature of these skeletal elements affords them the potential of operating in both an anterograde and a retrograde direction[103]. Electron microscopic evidence, showing an association of vesicles and other membrane organelles with these components, provides morphological evidence for their participation in transport[103].

There is growing evidence to support the claim that neurotransmitter receptors undergo axonal transport in the brain[81]. After ligation of the vagus nerve, it was revealed that presynaptic opiate receptors could be detected autoradiographically in the nodose ganglion, while a time-dependent accumulation of receptors was seen proximal to the ligature[120]. Extensive studies have also been conducted into the axonal transport of muscarinic cholinergic receptors[59,122]. Laduron (1980) first described a bidirectional axonal transport of muscarinic cholinergic receptors in the dog splenic nerve[59]. This study was later corroborated by Zarbin et al (1982) who performed a detailed kinetic and pharmacological study of the transport of muscarinic receptors in the vagus nerve[122]. After ligation of the vagus nerve, a time-dependent accumulation of muscarinic receptors was seen both proximal and distal to the ligature, suggesting that these receptors are transported in both an anterograde and retrograde direction[122]. Moreover, the pile-up of receptors observed proximal to the ligature could be inhibited by the injection of the alkaloid colchicine into the proximal part of the nerve trunk[122]. Given that colchicine in low doses appears to selectively block fast transport by disrupting the microtubular components of the axon, it was postulated that muscarinic receptors move by a fast transport mechanism[122].

Finally, in regards to the serotoninergic neuronal system, a proximal accumulation of 5-HT2 sites was demonstrated autoradiographically after transection of the median forebrain bundle[105]. These sites could bind 3H-ketanserin and were presumed to correspond to a serotonin autoreceptor of the 5-HT2 class[105]. Autoreceptors of the 5-

HT1 class presumably undergo axoplasmic transport in a similar fashion, before being incorporated in the membrane of 5-HT axons. Indeed, 5-HT autoreceptors with biochemical and pharmacological properties, similar to those of the 5-HT1 site, have been described not only at the level of the cell bodies, but also in certain terminal fields of 5-HT neurons (see above)[75,79].

ii. Colchicine-related changes in the regional distribution of 5-HT1 binding sites: A paradigm for studying the axonal transport of 5-HT autoreceptors

In an attempt to document the axoplasmic transport of 5-HT1 binding sites in serotoninergic neurons and eventually to estimate the relative proportions of 5-HT1 autoreceptors in various 5-HT terminal fields intra-cerebral injections of the anti-tubular drug colchicine were made into the MFB, wherein lie axons en passant of these neurons. Quantitative autoradiographic studies were conducted to evaluate any changes in the distribution of 5-HT1 binding sites at the cell body level and in the terminal field of the serotoninergic neuronal system, related to the injection of colchicine into the MFB.

II. METHODS AND MATERIALS

Prerequisites for the completion of the present light microscopic autoradiographic study envisaged included verification that: 1) 3H-5HT could be used to specifically label 5-HT1 binding sites on frozen sections incubated under conditions compatible with the maintenance of tissue morphology 2) sections can be fixed with glutaraldehyde to ensure the cross-linking of 3H-5HT to its specific binding sites 3) any loss of bound 3H-5HT occuring as a result of the histological processing for light microscopic autoradiography was predictable and proportional across different brain regions 4) the regional distribution of 5-HT1 binding sites did not differ significantly in sections processed for light microscopic autoradiography as compared to film autoradiographs of adjacent sections incubated in parallel.

A. Animal Maintenance

The studies were performed on brain tissue derived from adult male Sprague-Dawley rats (175-200 g). Prior to sacrifice, these rats were maintained on a 7h-19h diurnal rhythm with free access to food and water. In total, 100 rats were used for the preliminary experiments and subsequent mapping studies described herein.

B. Histological Techniques

i. Prefixed vs fresh tissue

To ascertain whether the use of prefixed tissue was best suited for the protection of the tissue during the incubation and the prevention of its subsequent degradation, the brains of six rats were prefixed by intra-aortic perfusion with a mixture of 1% paraformaldehyde (Polysciences) and 0.25% glutaraldehyde (MECA Labs) or of 0.75% paraformaldehyde, 0.1% glutaraldehyde and 1% tannic acid (Sigma).

For this purpose, a thoracotomy was performed under sodium pentobarbital (MTC Pharmaceuticals) anaesthesia (0.12cc/kg i.p.) to expose the heart within the ribcage. After clamping the descending aorta, the heart was transected at the apex. Subsequently, a catheter connected to a varistaltic pump by a rubber hose was clamped into place within the ascending aorta. At this time, the fixative was delivered into the ascending circulation, thus reaching the brain.

ii. Tissue extraction and storage

The animals were then decapitated and the brains immediately removed from the skull, and frozen in isopentane at -52°C for twenty seconds. Preceding storage, the frozen brain was wrapped in aluminum foil and left to rest on dry ice for 15 minutes. The brain was stored in a freezer at -80°C until sectioning. Fresh brains were similarly dissected, frozen and stored.

iii. Preparation of slides

Microscope slides were cleaned in a solution of sulfochromic acid, rinsed in distilled water, and left to dry in a dustfree environment. Clean slides were immersed in a 2% gelatin (Sigma) solution containing 0.05% chromium potassium sulfate and again left to dry. Once dry, slides were then stored until use in a refrigerator at 4°C in the presence of a desiccant (Drierite; W E Hammond Co).

iv. Preparation of sections

In preparation for sectioning, the brain was removed from storage and placed within the refrigeration unit of a Reichert-Jung cryostat. The temperature of the cryostat was maintained between -16°C and -20°C and the brain was given 45 minutes for its temperature to equilibrate with that of the cryostat. Thereafter, the brain was mounted onto a microtome chuck using Tissue-Tek (Miles Scientific) embedding medium.

In all cases, twenty micron sections were cut from the brains in the coronal plane, by proceeding in the caudo-rostral direction. From those brains used in preliminary tests, sections were taken within the rat mesencephalon. When a complete mapping was required, two parallel series of adjacent sections were collected every two hundred microns, between the level of the spinomedullary junction and the caudal extent of the olfactory bulb. Sections were collected by thaw-mounting onto gelatinized slides (two sections per slide). The slides were placed in a microscope slide box kept within the cryostat refrigeration unit. At this stage, the sections were susceptible to the accumulation of frost. This was curtailed by positioning a jar containing desiccant within the cryostat. Sections that showed signs of frost buildup, despite this treatment, would be discarded.

When a box was filled with its complement of slides, it was quickly transferred to a freezer at -20°C for storage until use.

C. In Vitro Labelling of 5-HT1 Sites

i. Basic protocol

The tissue sections were removed from storage at -20°C and then allowed to warm to room temperature for a period of five minutes prior to incubation. All sections were incubated at room temperature in 50 mM tris buffer (pH = 7.6) with 5 nM 3H-5HT (specific activity 20-30 Ci/mmol; New England Nuclear). The incubation medium always contained 0.001% ascorbic acid (antioxidant; BDH Chemicals) and 10 micromolar pargyline (MAO inhibitor; Sigma) to prevent the degradation of 3H-5HT, as well as 10 micromolar fluoxetine (5-HT uptake site inhibitor; Sigma), to prevent its interaction with the 5-HT uptake site. Designated sections were incubated concurrently in the presence of unlabelled 5-HT for the determination of non-specific binding. After the incubation had progressed for 60 minutes, excess unbound ligand was removed by carrying the sections through three successive rinses of two minutes each in 50 mM tris buffer (pH = 7.6) at 4°C. Sections destined for counting or film autoradiography were then rapidly rinsed in deionized water at 4°C and allowed to air dry at room temperature. Sections destined for light microscopic autoradiographic studies were postfixed immediately after the rinses in buffer.

When a comprehensive mapping of the 5-HT1 site distribution was conducted, two series of adjacent unfixed sections were incubated in parallel in the presence of 3H-5HT. For the determination of non-specific binding, every 15th slide from each series was incubated with 2 micromolar unlabelled 5-HT. One series was treated for film autoradiography and the other processed for light microscopic analyses.

ii. Establishment of optimal binding conditions

In determining the conditions appropriate for maximizing the ratio of specific over nonspecific 3H-5HT binding, the factors taken into consideration included the: a) ligand concentration b) kinetics of binding c) value of preincubation.

a) Ligand concentration: The 5-HT1 site has long been recognized as a high affinity site for 3H-5HT. Saturation on membrane preparations and intact sections have rigorously characterized the binding parameters of this radioligand[37,67,98]. By referring to saturation curves documented in the literature, a concentration of 5nM was chosen for the 3H-5HT ligand[67].

b) Binding kinetics: A timecourse for the binding of 3H-5HT to its 5-HT1 binding sites was derived for the purpose of determining the time to equilibrium of the reaction. Adjacent unfixed sections were incubated with the 3H-5HT label for different periods of time ranging from 0 to 60 minutes. At each timepoint, total and nonspecific binding was measured and a curve plotted depicting the variation of these periods as a function of time.

c) Role of preincubation: Previous investigators have implicated the preincubation of tissue sections with evoking the release of endogenous 5-HT stores and thus, enhancing the specific labelling of 5-HT1 sites with 3H-5HT[67,78]. Preincubation preceded exposure to the tritiated probe and involved placing frozen sections for 30 minutes at room temperature in 50mM tris buffer (pH = 7.6). The release of endogenous 5-HT was promoted by including 4mM CaC12 in this medium. Adjacent unfixed sections, preincubated or not, were then incubated with the 3H-5HT label.

iii. Optimizing morphological preservation

The introduction of fresh tissue into a buffer solution can result in the separation of the sections from the gelatinized slides and/or osmotic lysis of neuronal and/or glial elements. To try to prevent these disruptive phenomena, various osmotic conditions and buffer supplements were tested in regards to their value in maintaining the integrity of the tissue.

An osmotic environment conducive to the retention of tissue integrity was established by evaluating the following incubation media: 50mM tris buffer, 50mM tris buffer with 0.25 M sucrose and 0.17 M tris buffer. The former was hypoosmolar and the latter two were isoosmolar with plasma.

Additional tests were conducted to determine whether the inclusion of a protein and/or protease inhibitor could curtail the damage incurred by the tissue during incubation. The beneficial influence of these agents has been previously documented[50,76]. The supplementation of incubation media with protein has been shown to enhance the resistance of neuronal elements to osmotic shock[50,76]. With regards to the protective effect of protease inhibitors, this may derive from their ability to restrict the degradation of tissue proteins[50]. To assess the potential benefit of supplementation, unfixed sections were incubated in the media listed above with or without either 0.2% Bovine serum albumin (BSA; Sigma) + 0.03% bacitracin (Sigma) or

0.1% gelatin. Following incubation, these sections were stained with cresyl violet (cf; Neuroanatomical staining) and then viewed under the light microscope. The effects of buffer supplementation on the binding properties of 3H-5HT were assessed by incubating some sections in the presence of the radioligand.

D. Measurement of Total Bound Radioactivity

Measurements of the bound radioactivity in incubated tissue sections were necessary: 1) to ensure the specificity of 3H-5HT binding at each phase of this study 2) in establishing optimal binding conditions for 3H-5HT 3) to evaluate the effects of tissue prefixation, buffer supplementation and histological processing (see below) on 3H-5HT binding.

To measure bound radioactive content, sections incubated in the presence of 3H-5HT were scraped off their slides with a razor blade and then individually placed in a scintillation vial. Dissolution of the tissue was achieved by adding 0.5 ml of soluene (United Technologies Packard) to the vials. After allowing a digestion time of 1-2 hours at 60°C, 9.5 ml of toluene cocktail was added as a scintillant. The vials were left to sit overnight at room temperature prior to counting. The radioactive content was measured by a Beckman Beta rack counter and expressed in terms of counts per minute (CPM). Counts from sections incubated in the presence of labelled 3H-5HT alone were averaged, yielding a mean of total bound radioactivity. Sections incubated with 3H-5HT in the presence of cold 5-HT were used to produce a mean value for nonspecific binding. By subtraction, an estimate of specific 3H-5HT binding was derived and expressed as a percentage of total bound radioactivity.

E. Crosslinking of 3H-5HT Molecules

Following incubation, sections destined for light microscopic autoradiography were immersed for 30 minutes at 4°C in a 4% glutaraldehyde solution prepared in 0.4M phosphate buffer. Glutaraldehyde possesses fixative properties deriving from its ability to crosslink the amino groups on proteins[53]. This fixation step ensures the preservation of tissue morphology and is designed to prevent the dissociation of bound ligand during the subsequent processing of the sections for light microscopic autoradiography[45,76].

Following fixation, the sections were dehydrated by passage through a sequence of ethanol or acetone baths of increasing concentration (70%(2*5min), 95%(2*5min), 100%(2*5min). They were then defatted in xylene (2*5min) before being rehydrated

through an inverse sequence of ethanol or acetone baths (100%-70%) as described above. Finally, they were rinsed in three successive baths of distilled water (5min each). This defatting procedure has been shown to be essential for: 1) reducing the quenching of the emitted radiation from the ligand 2) improving the staining properties of the tissue 3) removing any unfixed, diffusible radioligand and 4) ensuring an even coating of the sections with the emulsion[50].

During the course of postfixation, a certain percentage of bound 3H-5HT molecules can be expected to dissociate from their 5-HT1 binding sites. Presumably, any free 3H-5HT molecules would be available for fixation by glutaraldehyde. To rule out any extensive participation of artifactually crosslinked dissociated ligand molecules to the autoradiographic signal, incubated sections were placed in 0.4M phosphate buffer with or without glutaraldehyde and the loss bound radioactivity compared in the two conditions. Measurements of tissue-bound radioactivity were also made on postfixed sections, delipidated in either acetone or ethanol so as to evaluate the effects of these conditions of histological processing on the retention of bound 3H-5HT molecules.

To further assess the effects of the postfixation/delipidation procedures on the retention of bound 3H-5HT tests were conducted in which two series of adjacent sections were incubated in parallel. The first series served as a control and was immediately rinsed and air dried after incubation, while the second series was subsequently fixed in 4% glutaraldehyde and delipidated in acetone. Sections from both series were apposed to an autoradiographic film. A quantitative distributional analysis was conducted on the film autoradiographs produced from the control and postfixed/delipidated sections and the results then compared.

F. Autoradiographic Processing

i. Film autoradiography

a) Exposure: Following incubation, slide-mounted sections destined for film autoradiographic processing were allowed to dry overnight at room temperature. Exposure was performed in a darkroom maintained at 18°C, by fixing the slides within an x-ray film cassette and then apposing a tritium sensitive film (LKB Ultrofilm) to the tissue sections. Films were exposed in the presence of a desiccant for a period of 6-11 weeks. During this time, they were stored in the dark within a refrigerator.

b) Film development: The film was removed from the cassette and immersed for five minutes in a bath containing developer (Kodak GBX) at 13°C. Development was

terminated by introducing the film into a bath of distilled water for 20 seconds. Subsequently, the film was fixed (Kodak GBX) for a period of 15 minutes and then rinsed under running water at 18°C for 45 minutes. Persuant to development images of the sections appear on the film, reflecting the regional variations in 3H-5HT labelling. These regional variations were visible as degrees of grey on the film autoradiographs.

ii. Light microscopic autoradiography

a) Dipping procedure: A warm bath (40°C) of NTB-2 nuclear track emulsion (Kodak diluted 1:1) was prepared in the dark, one hour prior to dipping. During this intermittent period the emulsion was carefully stirred at twenty minute intervals. After the final stirring, the homogeneity of the emulsion was verified and any air bubbles removed by dipping 5-10 clean test slides into the emulsion. When this was done, incubated and processed slide mounted sections were dipped into the emulsion and withdrawn in one continuous motion. Once dipped, the sections were left to dry upright for 4-6 hours on a slide rack. The slides were then placed in a light impermeable microscope slide box in the presence of a bag containing desiccant. The slide box was sealed and stored in a refrigerator at 4°C during the period of exposure lasting 6-8 weeks.

b) Development: Autoradiographs were developed (Dektol 1:2; Kodak) for a period of 1.5 minutes at 17°C. After development the slides were rinsed for 20 seconds in a bath of deionized water at 4°C and then fixed (Ektaflo diluted 1:3) at 4°C for a period of 10 minutes. Subsequently, the slides were rinsed under running tap water for a period of 45 minutes and then rinsed again in two successive baths of deionized water (5 minutes each) at room temperature.

In light microscopic autoradiographic preparations, silver grains are distributed over the sections themselves. Regional variations in labelling densities were visible as grades of luminescence when viewed in dark field with a light microscope.

iii. Neuroanatomical staining

Neuroanatomical analyses were performed at the light microscopic level on sections stained with cresyl violet. The staining procedure involved the immersion of the sections in 0.5% cresyl violet for five minutes followed by a rapid rinse in distilled water. The stain was differentiated by carrying the sections through a series of alcohol baths of increasing concentration (70%-100%). The amount of time spent in each of

the alcohol baths was determined by visual judgement of the staining. Following differentiation, the sections were placed in xylene for 15 minutes. Glass coverslips were then mounted on the sections with Permount glue (Fisher Scientific).

iv. Wet vs film autoradiography

In mapping the distribution of 5-HT1 binding sites in the rat brain, two series of adjacent sections were incubated in parallel. One series was processed for film and the other for wet autoradiography. To validate the distribution of 5-HT1 binding sites revealed in sections processed for autoradiography by standard dipping techniques, specific labelling densities recorded in several key brain areas on film and wet autoradiographs were compared (see below).

G. Densitometric Analysis

A computer-assisted quantitative distributional analysis of autoradiographically labelled 5-HT1 binding sites was conducted using a Bioquant microdensitometric software package (Apple). Autoradiographs were viewed under the light microscope and the magnified image projected to the video screen of an Apple IIe computer. For each brain analyzed, the system was calibrated to accomodate the range of tissue radioactivity values, corresponding to the differing degrees of ligand binding. This amounted to the establishment of a 'sensitivity window' admitting for measurement all labelling densities intrinsic to the tissue. To ensure a linearity between the recorded grain density and the radioactive content of the tissue, within these set limits, radioactive standards were prepared for both film and light microscopic autoradiography. Densitometric measurements on these standards were used to plot a standard curve.

i. Preparation of standards and plotting of standard curves

Standards were manually prepared from rat brain homogenates. The procedure followed has been routinely used in similar quantitative distributional analyses on other receptor systems[115]. Accordingly, the brains from three rats were removed and the white matter from them excised where possible. The grey matter was homogenized and aliquoted into Beem capsules (Sarstedt). To each aliquot, differing known amounts of 3H-leucine (Specific activity 5 Ci/mmol; New England Nuclear) were added and stirred into the homogenate. After allowing 60 minutes for diffusion and binding of the ligand,

the capsules were frozen in isopentane at -52°C for 20 seconds. The blocks formed contained differing concentrations of radioactivity homogeneously distributed throughout the tissue aliquot. Two series of adjacent 20 micron sections were cut from each block and thaw-mounted onto gelatinized slides. One series was apposed to film and the other postfixed in 4% glutaraldehyde and delipidated in acetone at 4°C. The latter series was dipped in liquid emulsion for use in light microscopic autoradiographic analyses. All autoradiographs were developed after six weeks of exposure as described above.

Densitometric measurements were made on these autoradiographs using the Bioquant system. The mean density recorded from sections of a given block was then plotted as a function of radioactive content. Figure 1 shows a typical standard curve obtained from a series of standards processed for autoradiography by standard dipping techniques. Evident from this curve is the linear increase of densitometric measurements within the range of tissue radioactivity levels commonly observed in intact sections labelled with 3H-5HT. The standard curves derived from homogenate sections processed for either film or wet autoradiography, were verified using commercially prepared standards (Amersham).

ii. Distributional analyses

Autoradiographs were viewed under high power using a light microscope (American Optics) and the image projected onto the computer video screen. Regions of interest were encircled using a Bioquant Hipad Digitizer (Apple) and a density within the arbitrary scale (0-53) established during calibration of the Bioquant system. For the determination of nonspecific binding, average values were calculated for each of the divisions of the brain (hindbrain, midbrain, forebrain). Depending on the location of the region under study, the appropriate value for nonspecific binding was subtracted from the total measurement. The final result of this calculation was an index of the amount of specific 3H-5HT binding in that particular region.

A fine topographical analysis of the distribution of 3H-5HT binding sites was conducted at the light microscopic level, on sections processed by wet autoradiographic methods and stained with cresyl violet. The objective of these studies was to describe the regional and cellular distribution of silver grains within all brain areas subject to quantification. This entailed characterizing any discrete distributional patterns within

given brain regions or describing the relationships between the grains and any recognizable neuronal elements.

H. Receptor Transport Studies

i. Animal subjects and treatment

In total, 30 male Sprague-Dawley rats (175-185g) were used for these experiments. Ten animals received a periaxonal injection of colchicine (20 mgm; Sigma), delivered with a saline vehicle, into the MFB. Six animals served as sham controls and were injected with an identical volume of a normal saline solution in the absence of the drug. A total of nine rats, six from the colchicine treated group and three shams, were sacrificed 24 hours after injection of the drug. The remaining seven animals were sacrificed 48 hours postoperatively.

ii. Identification of the injection

The stereotaxic co-ordinates of the injection site were chosen with reference to the intra-aural line according to the Paxinos and Watson atlas of the rat brain (A.P. = +5.0; H = -1.5; V = +1.0)[84]. Prior to beginning the actual drug studies, test injections were made with a dye (fast green), so as to verify the location of the injection site. From each animal receiving an injection of colchicine or saline, a series of brain sections was stained with cresyl violet so that the path taken by the micropipette in reaching the injection site could be mapped.

iii. Injection protocol

The rat was anaesthetized with sodium pentobarbital (0.12 cc/kg) and then placed within a stereotaxic apparatus. Having previously established the location of the injection site, with reference to the intra-aural line, surgery was performed to expose the brain above this site. A glass micropipette attached to a Hamilton syringe was lowered into the brain tissue until the injection site was reached. The drug which was dissolved in one microliter of vehicle (normal saline) was delivered into the MFB over a period of five minutes. After allowing 10 minutes for diffusion, the micropipette was withdrawn from the brain tissue. The animals were allowed to survive for periods of 24 or 48 hours postoperatively, whereupon, they were sacrificed by decapitation.

iv. Tissue processing

Following sacrifice of the animal, the brain was removed, frozen and sectioned as previously described. Serial sections were collected from each brain between the level of the inferior colliculus and the caudal extent of the olfactory bulb. Slide-mounted sections from drug treated animals and sham operated controls were incubated in parallel, with 5nM 3H-5HT and processed for film autoradiography (see above).

v. Quantitative distributional analysis

A quantitative distributional analysis of 3H-5HT binding within a number of brain regions was conducted using the Bioquant system, on film autoradiographs prepared from brain sections of colchicine treated animals. At each timepoint tested, the regional densitometric data obtained from the colchicine treated group was compared with results compiled from the appropriate sham control group.

III. RESULTS

A. Methodology

i. Prefixed vs fresh tissue

The binding of 3H-5HT to prefixed and fresh tissue is compared in Table 1. Perfusion prefixation with a mixture of 1.0% paraformaldehyde and 0.25% glutaraldehyde resulted in a 62% decrease in specific binding concomitant with a significant (33%) increase in nonspecific binding of the ligand. On the other hand, when a fixative mixture containing 0.75% paraformaldehyde, 0.1% glutaraldehyde and 1% tannic acid was used, a 36% loss in specific 3H-5HT binding was observed; again accompanied by a significant increase in nonspecific binding.

ii. Timecourse of 3H-5HT binding

Figure 2 depicts the timecourse of 3H-5HT binding in unfixed sections. The reaction reaches equilibrium after 60 minutes of incubation, at which time the specific over total binding ratio of the ligand ranges between 91%-93%. An incubation period of 60 minutes was utilized for all subsequent experiments.

iii. Tissue preincubation

The specific and nonspecific components of 3H-5HT binding were not altered significantly by a 30 minute preincubation in tris buffer. As shown in Table 2, 77% of 3H-5HT molecules bound to nonpreincubated sections were specifically associated with the tissue. Similarly, the specific labelling of preincubated sections presented 79% of total 3H-5HT binding.

iv. Buffer osmolarity and supplementation

The adherence of slide-mounted sections to gelatinized slides was dependent on the osmolar environment provided by the incubation medium tested. Within an isoosmolar milieu the adherence of the tissue to gelatinized slides was enhanced. The incubation medium containing the combination of 50mM tris + 0.25M sucrose was more effective in this regard than the medium containing 0.17 M Tris buffer. The utilization of an isoosmolar milieu by itself, was not sufficient to provide consistent protection against cell lysis and damage. However, the addition of either 0.1% gelatin or 0.2% BSA + 0.03% Bacitracin to the medium containing 50mM tris + 0.25M sucrose resulted in consistently uniform staining and improved morphological preservation.

The addition of 0.1% gelatin to this isoosmolar incubation medium reduced total 3H-5HT binding by 52% as compared to control sections incubated in 50mM tris (Table 3). Moreover, the specific to nonspecific binding ratio of the ligand was reduced by 6% in the presence of gelatin (Table 3). Inclusion of the BSA/Bacitracin supplement reduced total 3H-5HT binding (by 34%) but significantly improved (by 11%) the specific to nonspecific binding ratio of the ligand (Table 3).

v. Effects of histological processing on bound 3H-5HT

The effects of histological processing on the retention of 3H-5HT are depicted in Table 4. After glutaraldehyde postfixation, about 84% of bound radioactivity is retained in the tissue. Identical amounts of radioactivity were measured in sections placed in a bath of phosphate buffer in the absence of glutaraldehyde.

After defatting in acetone, over 70% of bound 3H-5HT molecules were still detected in sections postfixed with glutaraldehyde. In contrast, the radioactivity was totally washed out from sections immersed in phosphate buffer alone. Approximately 60% of bound radioligand molecules were retained in sections processed in ethanol instead of acetone (Table 4). Irrespective of whether the sections were defatted in acetone or ethanol, comparable proportions of specifically and nonspecifically bound molecules remained in the tissue after histological processing (Table 4).

Densitometric data compiled within eight midbrain regions from film autoradiographs of adjacent control and processed sections are compared in Table 5. Across the brain areas examined, similar proportions of specifically bound 3H-5HT molecules were retained after postfixation/delipidation. The regionally proportionate retention of bound 3H-5HT in the tissue is reflected in the correspondence between the topographical distribution of bound molecules on film autoradiographs prepared from control and histologically processed sections (Figure 3).

vi. Effects of light microscopic processing on the distribution of 3H-5HT binding

The topographical distribution of 3H-5HT was similar on sections prepared for autoradiography either by apposition to an emulsion coated film or by standard dipping techniques (Figure 4). Moreover, quantitative distributional analyses revealed a highly significant correlation between normalized densitometric data obtained from film and wet autoradiographs in 15 selected regions of the rat brain.

B. Distributional analysis of 5-HT1 binding sites

i. Regional distribution

Regional densities of specific 3H-5HT binding sites as quantified in the rat brain from light microscopic autoradiographs are shown in Table 7. Specific 3H-5HT binding sites are distributed throughout the grey matter of the central nervous system, giving rise to a heterogeneous labelling pattern (Figure 4 a-h). The white matter (corpus callosum, anterior commissure, internal capsule, fornix) was selectively devoid of specific 3H-5HT binding (Figure 4 a-h).

Metencephalon

The medulla contained a number of areas in which close to background levels of 3H-5HT binding were observed, including the: medullary reticular formation, dorsal accessory olive, nucleus ambiguous and dorsal motor nucleus of the vagus. Low densities of 5-HT1 binding sites were recorded in the raphe nuclei magnus and obscurus and in the hypoglossal, solitary and spinal trigeminal nuclei.

In the pons, silver grains were evenly distributed throughout the periventricular grey and over the parabrachial and lateral dorsal tegmental nuclei, all of which showed low to moderate labelling densities. Labelling densities, only slightly above background, were recorded in the locus coeruleus, motor Vth and mesencephalic Vth. The reticular formation of the pons was markedly spared of 3H-5HT binding.

The cerebellar cortex showed only minimal labelling densities in all three layers; 3H-5HT binding was slightly more pronounced in the deep cerebellar nuclei.

Mesencephalon

Dorsally, the inferior colliculus was notably deprived of 3H-5HT binding. However, a distinct laminar pattern of labelling was observed in the superior colliculus (Figure 4 a,b,c). The zonal and superficial layers showed intermediate labelling densities while the intermediate and deep layers were labelled at low levels. Around the aqueduct of Sylvius, intermediate labelling densities were seen in the central and periaqueductal grey (Figure 4 a-b). More caudally, the nucleus raphe dorsalis was intensely labelled (Figure 7 a). In contrast, the nucleus raphe medianus (Figure 7 a) and oculomotor nuclei showed only low densities of 5-HT1 binding sites. The distribution of 5-HT1 binding sites in the tegmentum of the midbrain was patterned in a distinct manner. In the substantia nigra, the pars compacta displayed intermediate labelling densities, while the pars reticulata was very intensely labelled (Figure 4 a,b and 7 c).

Within the interpeduncular nucleus complex, the innerposterior nucleus, paramedian and central subnuclei revealed a low, intermediate and high density of 5-HT1 binding sites, respectively (Figure 4 a). Finally, the ventral tegmental area and red nucleus were devoid of labelling (Figure 4 a,b).

Diencephalon

Specific 3H-5HT binding was minimal in the majority of thalamic nuclei including the: medial geniculate (Figure 4 a,b), parafascicular nucleus, dorsolateral geniculate and ventrobasal complex (Figure 4 c,d). Intermediate densities of autoradiographic grains were recorded in the: olivary pretectal nucleus (Figure 4d and 5b), magnocellular part of the ventral geniculate nucleus (Figure 5 a) and central medial nucleus.

Of all the hypothalamic regions, labelling was heaviest in the superior and medial mammillary nuclei. Intermediate values were recorded in the preoptic area (Figure 4 e,f), ventromedial nucleus and in the lateral hypothalamus within and around the MFB. Regions characterized by low levels of 3H-5HT binding included the: arcuate nucleus (Figure 4 c,d), dorsomedial nucleus, suprachiasmatic nucleus and median eminence (Figure 4 c,d).

In the subcommissural organ, lying in the roof of the third ventricle, low levels of 5-HT1 sites were observed.

Basal ganglia and forebrain

3H-5HT binding in the telencephalon revealed an important distribution of 5-HT1 binding sites. High labelling densities were observed in the lateral septum (Figure 4 g,h and 5 d), while the medial septum (Figure 4 g,h) and fimbrioseptal nucleus (Figure 4 f) contained only intermediate silver grain densities. Within the basal ganglia, high and intermediate densities of 5-HT1 binding sites were observed in the globus pallidus and neostriatum, respectively (Figure 4 e-h). The selective sparing of myelinated fiber bundles passing through these areas produced a reticular labelling pattern. A more homogeneous distribution of silver grains was seen in the nucleus accumbens, with density values falling in the intermediate range (Figure 4 g,h). Intermediate labelling densities were also recorded in the entopeduncular nucleus (Figure 5 c). In the basal forebrain, intense labelling was seen over the ventral pallidum, while intermediate densities were recorded in the bed nucleus of the stria terminalis (Figure 4 e,f) and olfactory tubercle (Figure 4 e-h). Both the horizontal and diagonal bands of Broca showed low levels of 3H-5HT binding (Figure 4 g,h).

Amygdala

Labelling within the various subnuclei of the amygdala was generally low. An exception was the basolateral subnucleus where slightly higher values were recorded, falling in the intermediate range (Figure 4 a-d).

Hippocampal formation

3H-5HT binding was particularly enriched in the hippocampal formation where a laminar pattern of labelling was observed (Figures 4 a-d and 6 a). High densities of 5-HT1 binding sites were recorded in the subiculum and in the inner and outer molecular layers of the dentate gyrus. Additionally, within the dentate gyrus, the outer and inner granule layers revealed intermediate and low labelling densities, respectively. In the CA II region of the hippocampus, labelling was intense in the stratum radiatum and stratum oriens while density values in the stratum pyramidale were in the intermediate range. All the aforementioned layers displayed intermediate levels of 3H-5HT binding in the CA I region. Labelling within the CA III region varied along the rostro-caudal extent of the hippocampus. A distribution pattern emerged in which the medial and caudal aspects of CA III were seen to contain substantially higher grain densities than the dorsal aspect (Figure 4 a-d). In the CA III region, densitometric measurements were taken where labelling densities were most intense. High levels of 3H-5HT binding were recorded in the molecular and granule layers, while intermediate labelling densities were recorded in the pyramidal layer.

Cerebral cortex

The labelling of the cerebral cortex varied across the different regions considered. High grain density readings were only recorded within the principalis external layer of entorhinal cortex. The principalis internal and internal layers of this cortical region contained intermediate and low levels of 5-HT1 binding sites, respectively. In the posterior cingulate, retrospinal and piriform cortical areas, labelling densities were low, with autoradiographic grains distributed evenly over all layers (Figure 4 a-h). Regarding the striate, sensorimotor, anterior cingulate and frontal cortical areas; grain densities were generally in the low or intermediate range (Figure 4 a-h). Moreover, labelling within these areas adhered to the boundaries of the histologically distinct layers characteristic of cerebral cortex. The typical interlaminar labelling pattern was such that grains were preferentially distributed in layers I and V

with relative sparing of layers II, III and VI (Figure 4 a-h). Grain densities within layer IV were quite variable from one region of cortex to the next.

ii. Light microscopic localization of 5-HT1 binding sites

A fine topographical analysis of the distribution of 5-HT1 binding sites was conducted on sections processed for light microscopic autoradiography. In all brain areas analyzed, silver grains appeared to be homogeneously distributed over cell bodies and the neuropil. Figure 7 shows the homogeneity of the cellular distribution of 5-HT1 binding sites as seen in the nucleus raphe dorsalis and substantia nigra.

iii. Correlation of 5-HT1 site densities with the 5-HT innervation of discrete areas in rat brain

The application of light microscopic autoradiography to the visualization of 5-HT1 binding sites provided a degree of resolution sufficient to permit the recording of labelling densities at high magnification within discrete areas of the rat brain. The areas selected were those for which data on 5-HT innervation densities (number of 5-HT terminals/mm^3) had been documented in the literature. As shown in Table 8, there is no apparent correlation between density of 5-HT1 binding sites and that of 5-HT varicosities in the regions examined. The locus coeruleus, dorsal accessory olive and suprachiasmatic nucleus all contain very low densities of 5-HT1 binding sites, yet reportedly receive among the heaviest 5-HT innervations in the rat brain. In contrast, layer V of frontal cortex, which was labelled to a much greater extent than these aforementioned structures, contains only low densities of 5-HT terminals. In the cerebellar cortex, in which there is reportedly a sparse 5-HT innervation, background levels of 3H-5HT binding were recorded.

The densities of 5-HT1 binding sites recorded from light microscopic autoradiographs were also correlated to the quantitative data available concerning the extent to which 5-HT varicosities form morphologically defined synapses in certain discrete areas of rat brain. As seen in Table 9, there is no apparent correlation between the density of 5-HT1 binding sites and the density of synaptic contacts established by 5-HT varicosities in the brain areas examined. In the locus coeruleus, a highly synaptic 5-HT innervation had been previously described, yet this structure is shown to contain a low density of 5-HT1 binding sites. Frontal cortex, which is labelled

to a much greater extent, had been previously ascribed a low incidence of synaptic 5-HT varicosities. In the motor Vth and cerebellar cortex, low levels of 3H-5HT binding are associated with a paucity of 5-HT synapses.

C. Effects of Colchicine Injection on the Distribution of 3H-5HT Binding

Table 10 compares the regional 3H-5HT labelling densities recorded from film autoradiographs of brain sections obtained 24 and 48 hours after the periaxonal injection of saline or colchicine into the MFB. Twenty-four hours following the intracerebral injection of colchicine, a significant decrease in 3H-5HT binding was recorded in several brain areas (Table 10). The magnitude of this effect was subject to some degree of regional variation. Among the brain areas tested, the reduction in 3H-5HT binding was most pronounced in the: lateral hypothalamus, frontal cortex, dorsal raphe and globus pallidus.

The specific binding of 3H-5HT in sections from colchicine treated animals was not statistically different from that of sham operated rats 48 hours after injection (Table 10). The regional distribution of 5-HT1 sites observed in sections from sham operated animals was similar 24 and 48 hours post-injection. In contrast, regional densities recorded in sections from the colchicine treated group were significantly different at the 24 and 48 hour timepoints (Table 10).

IV. DISCUSSION

A. Methodology

i. Tissue prefixation

The prefixatives used adversely influenced the binding properties of 3H-5HT to an extent that precluded their implementation in preparing rat brain tissue for high resolution light microscopic autoradiography. Moreover, in regards to the preservation of tissue morphology in frozen sections from uncryoprotected brains, perfusion prefixation can be counterproductive. The high water content of the perfused brain lends itself to the formation of ice crystals. This leaves the tissue susceptible to damage during incubation. Accordingly, a protocol was devised for the in vitro labelling of 5-HT1 binding sites in fresh tissue sections.

ii. Specificity of 3H-5HT binding

The distribution of 5-HT1 binding sites in the rat brain was mapped in frozen sections incubated with 5nM 3H-5HT. Previous authors have thoroughly characterized the biochemical parameters of 3H-5HT binding in brain sections, under incubation conditions identical to those documented in this thesis[67,98]. Rostene et al (1983) reported that the dissociation constant of this ligand ranges between 1-4nM, while the maximal capacity of binding can vary up to 600 fmol/mg protein, depending on the brain area examined[98]. Figure 8 is a saturation curve obtained from Magre (1983) depicting the binding of 3H-5HT to intact sections from rat midbrain[67]. The specific binding of the ligand reaches saturation at a concentration of 8nM. A concentration of 5nM 3H-5HT lies above the dissociation constant of the ligand, near the plateau of the specific binding curve. At this chosen ligand concentration, approximately 80% of the total binding is specific. The specific over total binding ratios (over 80%) recorded in the present study after incubation of tissue sections with 5nM 3H-5HT are in accordance with these previous findings[67,98].

At nanomolar concentrations, such as that which was used in the current study, 3H-5HT is known to label a single class of binding sites first designated as 5-HT1 by Peroutka and Snyder (1979)[89]. The 5-HT1 class is characterized pharmacologically by its high affinity for serotonin and those 5-HT agonist compounds which feature an indole nucleus (see above)[64]. The various subtypes of the 5-HT1 class, however, could not be differentiated within the experimental paradigm described herein.

The 5-HT2 class of binding sites, with only a micromolar affinity for 3H-5HT, could not be labelled to any significant extent with the ligand concentration chosen herein[64]. Indeed, the topographic distribution of labelled sites observed in the current study is distinct from previously reported findings on the distribution of 5-HT2 binding sites in the rat brain (see below)[64,87]. We, therefore consider the 3H-5HT probe to be labelling with an elevated degree of specificity, a single class (presumably comprised of several subtypes) of high affinity binding sites for serotonin.

iii. Kinetics of 3H-5HT binding

Specific 3H-5HT binding to intact frozen sections is saturable and reaches a state of equilibrium after 60 minutes of incubation. This timecourse is in accordance with documented findings on the binding kinetics of 3H-5HT[67].

These results support the choice of a 60 minute incubation period, subsequently used for optimizing the specific binding of 3H-5HT to frozen brain sections.

iv. Tissue preincubation

Tissue preincubation reportedly promoted the release of endogenous transmitter stores and has been recommended as a method for increasing the specific binding of a number of radioligands[50,67]. Test experiments described herein, reveal that preincubation does not significantly affect the specific binding of 3H-5HT to frozen brain sections. This suggests that the presence of endogenous 5-HT does not significantly influence the binding of 3H-5HT. Earlier data showed that preincubation can increase the specific binding of 3H-5HT by as much as 400%[67]. Such a discrepancy might be explained as follows. Tissue preincubation involved the introduction of frozen brain sections into a hypoosmolar medium. The results of histological studies documented in this thesis show that placing frozen sections in such an environment can result in osmotic damage to the cellular membranes of the tissue. Presumably, the disruption of tissue integrity leads to an increase in the number of specific binding sites which are available to the label during incubation. This assumption is consistent with our finding that the binding of 3H-5HT to intact tissue sections is related to the quality of the morphological preservation achieved during incubation (see below). Another possibility is that the damage sustained by tissue membranes in a hypoosmolar milieu itself results in a release of endogenous 5-HT stores. The release of the endogenous ligand in this manner during incubation might prevent its interference with the binding

of 3H-5HT. As such, the discrepancy between our findings and those of previous authors in regards to the value of preincubation, may be accounted for by postulating that the reputed beneficial effects of this procedure on the specific binding of 3H-5HT stem from its impact on tissue integrity. Whether an enhancement of specific 3H-5HT binding to preincubated sections is observed, or not, may depend on the state of preservation of the cellular membranes when the tissue is exposed to the label.

Given that the preincubation step constituted another threat to the integrity of the tissue and that a satisfactory labelling specificity could be achieved even upon its exclusion, this preambulatory step was omitted in devising a protocol for the in vitro labelling of 5-HT1 binding sites.

v. Incubation media

The preservation of tissue integrity was fundamental to the completion of the high resolution autoradiographic study undertaken. The use of an isoosmolar milieu was sufficient to ensure the adherence of slide-mounted tissue sections, yet did not consistently prevent tissue disruption and/or cell lysis in the course of the incubation procedure. To improve morphological preservation of the tissue, it appeared necessary to carry out the incubation in an isoosmolar supplemented medium. After evaluating several possible incubation milieu, it appeared that a medium containing 50 mM tris buffer, 0.2M sucrose, 0.2% BSA and 0.03% bacitracin, offered the best conditions for preserving the morphological integrity of the tissue. This medium has also previously been shown to provide an optimal milieu for the incubation of frozen sections with 125I Neurotensin[76]. However, the use of this supplemented incubation medium did result in a substantial decrease in specific 3H-5HT binding. The discrepancy observed between the binding of 3H-5HT onto sections incubated in a supplemented and hypoosmolar milieu may be related to differences in the quality of morphological preservation achieved under these conditions (see above).

A major disadvantage of the reduction in 3H-5HT binding, associated with the incubation of tissue sections in a supplemented milieu, results from the mandatory increase in exposure time required to assure a strong autoradiographic signal. With high specific activity iodinated compounds, the decrease in specific binding concomitant with the enhanced preservation of tissue morphology does not pose a problem in this regard[76]. However, 5-HT can only be tritiated to a low specific activity (20-40 Ci/mmol). This explains why it was necessary to carry out some of the incubations for

the present study in a hypoosmolar, unsupplemented medium so that we could deal with reasonable durations of exposure.

vi. The effects of histological processing on the retention of bound 3H-5HT

During the 30 minute postfixation procedure in the glutaraldehyde bath, only 15% of bound 3H-5HT molecules dissociated from the tissue. Comparable amounts of radioactivity were lost from tissue sections left in a phosphate buffer solution without glutaraldehyde. These observations are consistent with earlier data pertaining to the binding kinetics of 3H-5HT which reveals that the rate of dissociation of this ligand from intact sections is slow[67]. The fact that most of the ligand had not dissociated after 30 minutes in phosphate buffer suggests that the major fraction of 3H-5HT molecules were crosslinked onto their binding sites when glutaraldehyde was present. The similarity between the distribution of bound 3H-5HT molecules, as visualized in film autoradiographs before and after fixation, is consistent with this interpretation. Moreover, our findings indicate that dissociated radioligand molecules were not artifactually crosslinked to the tissue. Indeed, had this been the case, a higher proportion of 3H-5HT molecules would have been retained in the glutaraldehyde than in the phosphate buffer bath.

Assessments made of the radioactive content of whole sections revealed that 70% of specifically bound 3H-5HT molecules had been retained in the tissue when postfixation with 4% glutaraldehyde was followed by: dehydration in acetone and defatting in xylenes. In contrast, when sections were placed in phosphate buffer in the absence of glutaraldehyde, bound radioactivity was almost completely washed away during the defatting procedure. This observation illustrates the importance of effectively crosslinking 3H-5HT molecules to their specific binding sites during the postfixation step, to ensure the retention of the label through the course of the histological processing which ensues. During the defatting procedure, the amount of radioactivity associated with the tissue was reduced by an additional 15%, from what had been retained in the tissue after postfixation in glutaraldehyde. This further loss of bound radioactivity is probably due to non-dissociated 3H-5HT molecules which had not been crosslinked to the tissue. An interpretation which is supported by documented evidence indicating that glutaraldehyde fixation cannot be expected to ensure the crosslinking of all available molecules[53]. Therefore, it is likely that the loss of bound 3H-5HT is a non-selective chance event. In this regard, the observation that similar

proportions of specifically and nonspecifically bound 3H-5HT molecules remained in the tissue after histological processing strongly suggests that the minor loss of the ligand was a random event. The percentage of 3H-5HT remaining in tissue after histological processing is consistent with previous data concerning the retention of 125I-FK 33 824 and 125I-Neurotensin[45,76].

Densitometric measurements from film autoradiographs revealed that a fairly uniform proportion of bound 3H-5HT molecules was retained in all brain areas examined after fixation and defatting. These findings further alleviate the concern that an artifactual redistribution of the ligand may have occurred due to a preferential regional retention of bound 3H-5HT. However, a significant difference was noted betwen the magnitude of the retention data compiled by densitometry and from counts of tissue radioactivity. This apparent discrepancy can be explained in light of evidence demonstrating that the presence of lipids within tissue sections can produce film autoradiographic quenching, when tritiated probes are used[58]. The densitometric retention data reported herein were obtained by comparing measurements recorded from defatted sections, in which this type of film autoradiographic quenching was no longer a problem, to those recorded from control sections where the presence of tissue lipids had presumably reduced the autoradiographic signal. As such, the differences in regional labelling densities observed between control and histologically processed sections may not reflect, in absolute terms, the extent to which 3H-5HT molecules had been retained from one brain region to another. Accordingly, the 70% retention value obtained from measurements of the total radioactive content of whole sections is probably a more accurate index of the efficiency of glutaraldehyde postfixation in crosslinking specifically bound 3H-5HT molecules to their receptor sites.

B. Distributional Analyses of 3H-5HT Binding

The similarities in both the topographic and quantitative distributions of 3H-5HT binding on film (in fixed as well as unfixed sections) and wet autoradiographs (sections fixed and defatted) indicate that specifically bound radioligand molecules were retained in the tissue through the dipping procedure. This demonstrates the feasibility of using conventional dipping techniques for the detection and quantification of specific 3H-5HT binding sites in the central nervous system.

Densitometric data on the distribution of 5-HT1 binding sites presented in this thesis were generally in agreement with the results of quantitative dry autoradiographic

studies previously reported in the literature[20,67,86]. Confirmed herein were the high labelling densities reported in the: ventral pallidum, globus pallidus, lateral septum, substantia nigra, hippocampal formation and nucleus raphe dorsalis. As previously described in the literature, regions containing intermediate levels of 3H-5HT binding included the: nucleus accumbens, striatum, bed nucleus of the stria terminalis, entopeduncular nucleus, medial mammillary nucleus, central grey and superior colliculus[20,67,86]. Also, in accordance with preceding studies was the detection of low labelling densities in the: cerebellar cortex and in most regions of the thalamus and hindbrain[20,67,86]. An important area of labelling, not previously noted, was found in the magnocellular part of the ventral lateral geniculate nucleus.

One advantage of the wet autoradiographic method established herein was that it permitted the obtention of densitometric data at high magnification in discrete brain areas, wherefrom precise measurements were previously difficult to obtain due to the limited resolution afforded by available dry autoradiographic methods. Moreover, the resolution of the wet autoradiographic method allowed us to assess the intra-regional and cellular distribution of 5-HT1 binding sites at the light microscopic level. Specific 3H-5HT binding sites were thus shown to be confined to the grey matter, to the exclusion of the white matter bundles of the brain. Within the grey matter, silver grains were homogeneously distributed over both perikarya and neuropil. This ubiquitous distribution made it difficult to distinguish somatodendritic from axonal labelling. In a similar light microscopic autoradiographic study of the fine topographic distribution of 125I Neurotensin binding sites, Moyse et al (1986) reported a fairly uniform labelling of neuropil and cell bodies through most of the grey matter[76]. However, these authors were also able to discern selective accumulations of silver grains over cell bodies and their proximal dendrites in specific regions of the rat brain. As discussed above, it is unlikely that the homogeneity observed in the distribution of 3H-5HT binding sites stems from an artifactual redistribution of specifically bound molecules. Rather, it would appear from our observations that 5-HT1 binding sites may be ubiquitously distributed over different compartments. Data derived from pharmacological and lesion studies, reveal the presence of membrane associated 5-HT1 binding sites on the dendritic trees and axon terminals of serotonin-containing neurons[27,37,46,67,71,79]. A post-synaptic localization of specific 3H-5HT binding sites

has also been described; presumably these correspond to heteroreceptors on indolaminoceptive neurons[19,23,94]. Finally, 3H-5HT may label auto- and heteroreceptors in the course of synthesis and/or transport from one brain region to another (see below).

When a tritiated probe is used, the self-absorptive properties of the tissue influence the efficiency of the autoradiographic signal emitted from a radiolabelled source which is more than one micrometer below the surface of a fixed/defatted section[97]. As such, Beta particles emitted from sources below one micrometer of section will be largely absorbed, prior to reaching the emulsion[97]. Therefore, in autoradiographs from twenty micron thick sections, such as those used for the current study, the majority of Beta particles reaching the emulsion could be from the neuropil overlying cell bodies, rather than from the cell bodies themselves. A more complete study of the cellular localization of 5-HT1 binding sites will obviously necessitate the visualization of these sites on semi-thin sections at the light microscopic level and the eventual extention of the analysis to the electron microscopic level. The wet autoradiogaphic procedure described herein, forms the basis for the establishment of a method to permit the visualization of 5-HT1 binding sites in both semi-thin and thin sections. A similar wet autoradiographic approach has indeed proven to be compatible with the electron microscopic visualization of 125I-FK 33 824 and 125I-Neurotensin binding sites in the central nervous system[45,76]. However, at present, the low specific activity of the available 3H-5HT ligands is an obstacle to this further step in increasing the resolution of the distributional analysis of 5-HT1 binding sites.

By allowing the visualization of 5-HT1 binding sites in discrete and precisely delineated brain areas, the present approach made it possible to quantify 5-HT1 binding sites in regions or even subregions for which estimates of the number of 5-HT terminals per cubic millimeter of tissue were available in the literature[10,24,60,99,106,107,118]. These estimates were derived from counts of varicosities labelled after specific uptake of 3H-5HT, per square units of light microscopic autoradiograph, by taking into consideration the thickness of section and the mean diameter of varicosities as determined in electron microscopic autoradiographs. Earlier studies have compared and found no close relationship between the distribution of 5-HT1 binding sites and endogenous 5-HT in the brain[20,51]. The present study represents the first attempt at

comparing the densities of 5-HT1 binding sites with the actual density of 5-HT axon terminals, i.e. to correlate receptor densities with putative endogenous release sites rather than with 5-HT content.

The high densities of 5-HT1 binding sites recorded in the substantia nigra and globus pallidus are consistent with the dense 5-HT innervation previously demonstrated within these structures[109]. Similarly, the low densities of 5-HT1 binding sites observed in the cerebellar cortex conformed with the reputedly sparse 5-HT innervation therein[14]. In brain regions where there was an apparent 'mismatch' between the 5-HT terminal and receptor, two types of discrepancies were possible. Firstly, in the locus coeruleus, dorsal accessory olive and suprachiasmatic low receptor densities were associated with the purportedly heavy 5-HT innervation received by these structures[24,60,118]. On the other hand, in layer V of the frontal cortex, the discrepancy was in the opposite direction, with relatively high densities of 5-HT1 binding sites being associated with the sparse 5-HT innervation previously described therein[10]. Statistical analysis revealed no apparent correlation between the densities of 5-HT terminals and 5-HT1 binding sites across the different brain areas sampled. This finding suggests that a unique relationship does not exist betwen the 5-HT transmitter and high affinity receptor maps in the brain.

Although the 5-HT1 class has not been ascribed a clear physiological role in the brain, there is documented evidence which suggests that these high affinity binding sites are functional[19,23,38,91,94]. As such, the results of the correlation study documented herein do not preclude a functional role for these sites. Rather, several proposals can be offered to account for the apparent lack of correlation between the 5-HT innervation of the brain regions sampled and the densities of 5-HT1 binding sites recorded within these areas.

Non-neuronal and non-membrane bound receptors

The presence of high affinity binding sites for 3H-5HT on non-neuronal elements such as glial cells has been previously described and might account, in part, for the apparent lack of a relationship between the distribution of 5-HT terminals and 5-HT1 binding sites[117]. High affinity binding to sites of receptor synthesis may be a factor at the level of the cell bodies of serotoninergic neurons (raphe nuclei, dorsomedial nucleus of hypothalamus). This possibility could be of particular relevance in regards to the receptor map within the nucleus raphe dorsalis where high labelling densities were

recorded. Meanwhile, if 3H-5HT labels 5-HT1 auto- and heteroreceptors in transit, any significant regional variations in the size of this component of 3H-5HT binding, may, in part, account for the apparent lack of correlation between the 5-HT innervation and 5-HT1 binding site densities in the brain.

Transmitter co-localization

Serotonin co-exists with substance P or enkephalin in a number of medullary raphe neurons[25]. In certain brain regions, some substance P or enkephalin-immunoreactive terminals demonstrate the ability of accumulating 3H-5HT[25]. Morevoer, 3H-GABA was found to be taken up within 5-HT-immunoreactive terminals and dendritic processes in rat supraependymal plexuses and nucleus raphe dorsalis, respectively[25]. A neuron which is able to synthesize more than one transmitter might selectively release a given transmitter in different regions of its terminal field. As such, a plausible explanation for an apparent dense 5-HT innervation in regions devoid of 5-HT1 binding sites is that serotonin might be co-stored with another transmitter at terminals where it is not released. Conversely, 5-HT receptors might be coupled to peptide receptors in areas with low 5-HT innervation and high 5-HT receptor densities. This interpretation was invoked by Schultzberg and Hokfelt (1986) to account for the apparent 'mismatch' in the adrenergic/noradrenergic transmitter systems[102]. The neuropeptide NPY is known to be present in the vast majority of adrenergic cells and in a proportion of noradrenergic cells[102]. These authors showed that the distributional pattern of alpha-2 and NPY receptors in the medulla oblongata were very similar (high labelling densities were recorded in the superficial laminae of the spinal trigeminal nucleus and dorsal vagal complex)[102]. At the same time, immunocytochemical analysis revealed overlapping distributions of adrenalin/noradrenalin and NPY in the dorsal vagal complex[102]. However, the superficial laminae of the spinal trigeminal nucleus received a dense NPY innervation, but only a sparse network of noradrenalin fibers[102]. In view of the apparent 'mismatch' in this latter brain region, these authors proposed that alpha-2 and NPY receptors might be coupled in areas where the presynaptic neurons contain only one of the ligands[102].

Multiple receptors for serotonin

The multiplicity of 5-HT receptors in the brain has long been recognized (see above)[80,89]. The presence of multiple receptors for serotonin might account for the

type of 'mismatch' observed in the locus coeruleus, dorsal accessory olive and suprachiasmatic nucleus[24]. In regions such as these which receive a dense 5-HT innervation, but are devoid of 5-HT1 binding sites, the existence of 5-HT2 binding sites, or another yet unidentified low affinity receptor, might compensate for the paucity of high affinity binding sites[24,60,118].

Correlation between 5-HT1 sites and synaptic contacts

It has been well-documented that the incidence of morphologically defined synapses formed by 5-HT varicosities can vary across different regions of the central nervous system[10,14,24,60,99,106,107,118]. Therefore, an exclusive association of sites with synaptic contacts could perhaps be invoked to explain the lack of correlation between the densities of 5-HT axon terminals and that of 5-HT binding sites in the brain. To test this hypothesis, estimates of the frequencies with which 5-HT varicosities form classical junctional contacts within a number of discrete brain areas were obtained from the literature and correlated with densities of 5-HT1 binding sites recorded in these same brain areas[10,14,24,60,99,106,107,118]. Estimates of synaptic frequencies were calculated by extrapolation from data obtained on the frequencies of labelled synaptic 5-HT varicosities observed in electron microscopic autoradiographs, using a stereological formula developed by Beaudet and Sotelo (1981)[14].

As was the case when the densities of 5-HT1 binding sites were correlated to the densities of axon terminals; a unique relationship could not be defined between the distribution of 5-HT1 binding sites and 5-HT synaptic contacts in the brain. In the cerebellar cortex[14] and motor Vth[99], both low densities of 5-HT1 binding sites and synaptic contacts were noted. Similarly, there was no apparent 'mismatch' in the striatum where relatively high densities of 5-HT1 binding sites and 5-HT synaptic contacts were present[106]. A discrepancy was apparent in the locus coeruleus and suprachiasmatic nucleus where low densities of 5-HT1 binding sites were associated with high densities of synaptic contacts[24,60]. The opposite type of 'mismatch' was observed in frontal cortex where relatively high densities of 5-HT1 binding sites were concomitant with low densities of synaptic contacts[10]. The conclusion, derived from this correlation study, is that 5-HT1 binding sites are not exclusively associated with synaptic contacts. This conclusion may have important implications in regards to possible modes of action of serotonin in different regions of the central nervous system.

Action at a distance

Considerable evidence has been accumulated indicating that neurochemical transmission can occur at loci where specialized junctional contacts are absent[11,31,32]. In view of this evidence, the classical concepts of synaptic transmission have been extended to accomodate the neurohumoral model of transmitter function. Within this paradigm, the transmitter is thought to be released into the extracellular space before diffusing to neighboring (but not contiguous) sites of action. A neurohumoral role for serotonin was originally proposed in the cerebral cortex[11] and later in other brain areas[108,118]. In brain areas, such as the substantia nigra or globus pallidus, where high 5-HT innervation densities[109] are associated with high densities of 5-HT1 binding sites, serotonin may be acting as a classical transmitter. On the other hand, in certain cases where there was an apparent 'mismatch' between the 5-HT transmitter and high affinity receptor maps, serotonin may be acting as a neurohumoral agent; particularly in regions with high densities of 5-HT1 binding sites and low innervation densities. Action at a distance has already been invoked as a possible explanation for several 'mismatches' between different transmitters and their receptors[51]. A putative role for serotonin as a 'neurohumoral' agent might similarly explain the apparent 'mismatch' observed in areas such as layer V of frontal cortex which received a sparse 5-HT innervation[10] but contains an important concentration of 5-HT1 binding sites. In areas where a very dense 5-HT innervation is associated with low densities of 5-HT1 binding sites such as the: locus coeruleus, dorsal accessory olive and suprachiasmatic nucleus, other explanations, such as those described above (non-neuronal non-membrane bound receptors, low affinity receptors, transmitter co-localization) would seem more likely.

Previous authors have suggested that there could be a link between the density of 5-HT varicosities and the frequencies with which these varicosities form synaptic contacts, in given brain areas[24]. It is possible that in areas which receive a dense 5-HT innervation, high affinity binding sites might not be necessary. In these areas which would presumably receive a highly synaptic innervation, the actions of serotonin might be mediated by low affinity receptors with more classical functions. On the other hand, in areas which receive a sparse and a mainly nonsynaptic innervation, high affinity binding sites might be necessary so that serotonin could act on distant targets after diffusion in the tissue.

C. Colchicine-Related Changes in the Distribution of 5-HT1 Binding Sites

The results of our preliminary experiments show reversible changes in 3H-5HT binding at the cell body level and in the terminal field of the serotoninergic neuronal system, twenty-four hours after the injection of colchicine into the MFB. When the results of a quantitative distributional analysis of 3H-imipramine binding sites (a label of the 5-HT uptake site) conducted by Palkovits et al (1981)[82] were compared to the observed regional decreases in 3H-5HT binding, a high degree of positive correlation was noted between the distribution of this putative presynaptic marker and the scope of the colchicine-associated changes (Table 10). Although several fiber contingents, including those from catecholaminergic systems, also pass through the median forebrain bundle (MFB)[30,114], this observation suggests that the decreases in 3H-5HT binding recorded may be due to the actions of colchicine on serotoninergic neurons which transmit their fibers through the MFB. Colchicine-sensitive axonal transport has been previously demonstrated within fibers of serotoninergic neurons passing through the MFB. Evidence for this is provided by the findings of Areneda et al (1980)[4]. These authors described a preferential accumulation of 3H-5HT in neurons of the nucleus raphe dorsalis, twenty-four hours after injection of the label into the olfactory bulb[4]. When the anti-tubular drug colchicine was administered by periaxonal injection into the MFB, twenty-four hours prior to the introduction of the label into the olfactory bulb, the retrograde accumulation of 3H-5HT was reduced by 80%[4].

In view of the reversible nature of the observed changes in 3H-5HT binding, it is unlikely that they represent a response to a cytotoxic effect of the drug. Considering the anti-tubular actions of colchicine, it is tempting to postulate that the reduction in 3H-5HT binding recorded in the various terminal fields of serotoninergic neurons may be due to an interruption in the transport of serotonin autoreceptors of the 5-HT1 class. This is supported by the evidence of large decrease noted in the region of the MFB (lateral hypothalamic area) after colchicine injection.

However, the apparent lack of a pile-up of binding sites in the nucleus raphe dorsalis or in the MFB suggests that the periaxonal injection of colchicine may have also indirectly affected receptor synthesis within the serotoninergic neuronal system. This interpretation is supported by the discovery of a decrease in 3H-5HT binding in the terminal field of 5-HT neurons not only distal but also proximal to the injection site, and namely at the level of 5-HT nerve cell bodies within the nucleus raphe dorsalis. This putative inhibition of receptor synthesis may have occurred as a retrograde

response to the disruption of the cellular transport mechanism or might represent a redirection of the cell's metabolic machinery towards repairing colchicine induced cellular damage.

The regional variability in the scope of the colchicine-associated changes observed suggests that the size of the autoreceptor component of 3H-5HT binding differs from one brain region to another. Large decreases in 3H-5HT binding were observed in the nucleus raphe dorsalis and frontal cortex. Pharmacological studies have previously demonstrated the presence of 5-HT autoreceptors within these brain areas[33,72,75,79].

If 3H-imipramine binding is assumed to provide an accurate index of the densities of 5-HT terminals in the areas studied, the apparent relationship between the scope of the colchicine-related changes and the binding of this presynaptic marker might imply that 5-HT autoreceptors are distributed in a fairly homogeneous manner on the terminals of serotoninergic neurons. Such an interpretation is compatible with the apparent lack of a relationship between the 5-HT transmitter and high affinity receptor maps in the brain.

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TABLE 1

Effects of Prefixation on 3H-5HT Binding[1]

Fixation Solution	Specific 3H-5HT Binding Control	Prefixed	Decrease (%)
1% paraformaldehyde + 0.25% glutaraldehyde	1574 ± 204	585 ± 89*	62
0.75% paraformaldehyde + 0.1% glutaraldehyde + 1% tannic acid	1567 ± 220	1006 ± 269*	36

1 Data expressed in cpm/section. Each value corresponds to the mean (± S.D.) of more than 12 different sections from two different animals.

* Significantly different from control with $p \leq 0.05$.

TABLE 2

Effects of Preincubation on 3H-5HT Binding[1]

Condition	3H-5HT Binding		
	Total	Nonspecific	Specific
With Preincubation	1426 ± 260	302 ± 81	1124 ± 222
Without Preincubation	1799 ± 109	412 ± 62	1387 ± 156

1 Data expressed as cpm/section. Each value corresponds to the mean (± S.D.) of more than eighteen different sections from three animals.

TABLE 3

Effects of Buffer Osmolarity and Supplementation on 3H-5HT Binding[1]

Incubation Medium	3H-5HT Binding*		
	Total	Nonspecific	Specific (% total)
Control (50 mM Tris)	1619 ± 20	413 ± 52	74
50 mM Tris + 0.25M Sucrose + 0.1% Gelatin	770 ± 58*	247 ± 52*	68
50 mM Tris + 0.25M Sucrose + 0.2% BSA + 0.03% Bacitracin	1073 ± 152*	158 ± 44*	85

1 Data expressed in cpm/section. Each value corresponds to the mean (± S.D.) of more than twelve different sections from three animals.

* Significantly different from controls with $p \leq 0.05$.

TABLE 4

Effects of Fixation and Defatting
on Bound 3H-5HT[1]

Postincubation Treatment	Total Binding (% Control)	Specific Binding (% Total)
Control (air dried)	100	79.6 ± 2.12
0.4M Phosphate Buffer	80.5 ± 2.66	79.0 ± 1.41
4% Glutaraldehyde	83.7 ± 3.51	77.3 ± 4.32
4% Glutaraldehyde + Acetone	71.5 ± 5.86	84.2 ± 4.61
4% Glutaraldehyde + Ethanol	59.8 ± 5.90	76.5 ± 4.01

1 Data measured in cpm/section. Each value corresponds to the mean (± S.D.) of at least twenty-four different sections from three animals.

TABLE 5

Regional Retention of Specifically Bound
3H-5HT After Postfixation and Delipidation

Region	Specific 3H-5HT Binding*		% Retention
	Control	Postfixed and Delipidated	
Substantia Nigra	49.8 ± 2.60	45.6 ± 0.50	91.6 ± 3.46
Central Grey	23.6 ± 2.35	20.9 ± 3.10	88.5 ± 2.65
Superior Colliculus	20.1 ± 2.19	17.5 ± 2.78	87.1 ± 3.06
Striate Cortex	22.6 ± 2.32	19.0 ± 2.82	84.1 ± 4.00
Subiculum	46.9 ± 1.13	40.4 ± 1.07	86.1 ± 2.31
Hippocampus	47.4 ± 1.77	41.7 ± 3.74	87.9 ± 6.03
Medial Geniculate	10.2 ± 1.48	8.56 ± 2.34	84.0 ± 3.65
Interpeduncular Nu	44.2 ± 3.10	39.3 ± 3.25	88.9 ± 3.06

* Data expressed in optical density units (Arbitrary Scale: 0-53). Densitometric measurements from film autoradiographs of sections histologically processed or not were made at the level of the midbrain. Each value corresponds to the mean (± S.D.) of three different experiments.

TABLE 6

Comparison of Densitometric Data Obtained
from Film and Wet Autoradiographs

Region	Specific Binding (% maximum)[1]	
	Control (Film)	Postfixed/Delipidated* (Dipped)
Periventricular Grey	19.7 ± 3.68	16.6 ± 2.22
Raphe Dorsalis	72.6 ± 4.40	67.5 ± 3.25
Interpeduncular Nu	77.7 ± 1.28	76.7 ± 5.40
Substantia Nigra (Reticulata)	85.6 ± 1.91	90.6 ± 5.54
Central Grey	43.6 ± 4.49	44.2 ± 1.63
Superior Colliculus	40.0 ± 3.19	37.0 ± 4.85
Subiculum	74.2 ± 2.24	75.6 ± 1.31
Medial Mammillary Nu	63.9 ± 4.23	66.6 ± 2.78
Zona Incerta	25.7 ± 2.12	22.5 ± 0.50
Ventral Pallidum	82.6 ± 1.66	85.2 ± 4.56
Globus Pallidus	76.9 ± 0.30	72.9 ± 4.42
Accumbens Nu	58.9 ± 4.76	53.6 ± 3.26
Striatum	33.3 ± 5.64	37.5 ± 3.98
Bed Nu Stria Terminalis	42.6 ± 3.24	44.8 ± 4.87
Lateral Septum	81.6 ± 4.00	82.3 ± 4.87

1 Data expressed in optical density units (Arbitrary Scale: 0-100). Each value corresponds to the mean (± S.D.) of forty-eight measurements from three animals.

* Significantly correlated wtih control (r = 0.99).

TABLE 7

Quantitative Distributional Analysis of
5-HT1 Binding Sites in Rat Brain

Region	Specific 3H-5HT Binding[1]
Hindbrain:	
Locus Coeruleus	4.29 ± 1.18
Mesencephalic Vth	1.66 ± 0.31
Periventricular Grey	8.74 ± 2.04
Parabrachial Nu	11.20 ± 2.01
Motor Vth	1.76 ± 0.06
Dorsal Accessory Olive	4.21 ± 2.41
Raphe Obscurus	6.94 ± 1.89
Raphe Magnus	4.90 ± 1.38
Cerebellum:	
Granule Cell Layer	0.26 ± 0.08
Molecular Cell Layer	0.89 ± 0.01
Purkinje Cell Layer	0.85 ± 0.04
Deep Nuclei	9.71 ± 1.28
Midbrain:	
Substantia Nigra	
Pars Reticulata	47.70 ± 2.99
Pars Compacta	26.80 ± 3.15
Superior Colliculus (superficial)	19.60 ± 2.62
Inferior Colliculus	3.26 ± 1.04
Raphe Dorsalis Nu	35.80 ± 3.25
Raphe Medianus Nu	7.94 ± 2.65
Oculomotor Nu	7.71 ± 1.91
Central Grey	23.10 ± 0.88
Diencephalon:	
Hypothalamus	
Arcuate Nu	6.92 ± 0.04
Dorsomedial Nu	9.68 ± 1.45
Ventromedial Nu	17.20 ± 0.86
Median Eminence	7.58 ± 1.96
MFB (Lateral Hypothalamus)	19.90 ± 3.00
Superior Mammillary Nu	38.70 ± 3.86
Medial Mammillary Nu	35.30 ± 1.23

Table 7 (cont'd)

Region	Specific 3H-5HT Binding[1]
Diencephalon (cont'd):	
Hypothalamus (cont'd)	
Preoptic Area	13.30 ± 1.76
Suprachiasmatic Nu	5.03 ± 0.86
Thalamus	
Olivary Pretectal Nu	27.40 ± 2.24
Dorsolateral Geniculate	0.49 ± 0.02
Ventrolateral Geniculate (Magnocellular)	30.10 ± 1.22
Medial Geniculate	8.24 ± 1.36
Medial Habenula	2.40 ± 0.48
Centromedian Nu	15.00 ± 1.93
Reuniens Nu	9.95 ± 2.27
Lateral Dorsal Nu	12.10 ± 1.47
Zona Incerta	11.90 ± 0.27
Parafascicular Nu	2.53 ± 0.73
Subcommissural Organ	3.65 ± 1.51
Telencephalon:	
Basal Forebrain	
Lateral Septum	43.20 ± 2.63
Striatum	19.80 ± 3.98
Globus Pallidus	40.80 ± 2.39
Ventral Pallidum	45.20 ± 2.46
Nucleus Accumbens	28.40 ± 1.76
Bed Nu Stria Terminalis	23.70 ± 3.79
Diagonal Band of Broca	14.70 ± 0.35
Entopeduncular Nu	31.20 ± 2.89
Olfactory Tubercle	18.00 ± 2.23
Amygdala	
Medial Nu	13.80 ± 1.27
Posterior Medial Nu	18.30 ± 2.25
Posterior Lateral Nu	15.50 ± 1.69

Table 7 (cont'd)

Region	Specific 3H-5HT Binding[1]
Telencephalon (cont'd):	
Amygdala (cont'd)	
Lateral Nu	17.60 ± 0.96
Basolateral Nu	22.00 ± 2.20
Central Nu	13.70 ± 1.36
Hippocampal Formation:	
Subiculum	39.20 ± 0.71
CA I	
Oriens	37.10 ± 1.83
Pyramidale	24.10 ± 3.42
Radiatum	37.10 ± 2.81
CA II	
Oriens	41.20 ± 1.05
Pyramidale	29.60 ± 3.26
Radiatum	42.90 ± 3.34
CA III	
Molecular	51.20 ± 1.25
Pyramidale	38.20 ± 3.97
Granule	43.50 ± 4.13
CA IV	
Dentate Gyrus	34.40 ± 1.62
Internal Molecular	50.00 ± 1.63
Internal Granular	23.40 ± 0.90
Outer Granular	12.10 ± 2.24
Outer Molecular	44.30 ± 1.89
Cerebral Cortex:	
Entorhinal	
Principalis External	50.50 ± 2.25
Principalis Internal	38.90 ± 1.72
Internal	14.90 ± 0.72
Piriform	17.20 ± 2.88

Table 7 (cont'd)

Region	Specific 3H-5HT Binding[1]
Cerebral Cortex (cont'd):	
Frontal	
I	23.00 ± 1.50
II	20.00 ± 2.04
III	16.00 ± 1.99
IV	17.80 ± 3.19
V	23.40 ± 2.04
VI	7.22 ± 2.44
Striate	
I	25.00 ± 1.32
II	19.40 ± 2.17
III	21.10 ± 0.97
IV	23.80 ± 2.73
V	26.90 ± 1.25
VI	21.10 ± 1.50

1 Data expressed in optical density units (Arbitrary Scale: 0-53). Densitometric measurements were obtained from wet autoradiographs. Each value corresponds to the mean (± S.D.) of more than twenty-four readings from three animals.

TABLE 8

Correlation of 5-HT1 Binding Site Densities
with 5-HT Innervation Densities**

Region	Innervation Density* (million varicosities/mm^3)		Specific 3H-5HT Binding[1]
Locus Coeruleus	10	(60)	4.30 ± 1.1
Dorsal Accessory Olive	4.5	(118)	4.20 ± 2.4
Motor V	1.8	(99)	1.80 ± 0.06
Cerebellar Cortex	0.24	(14)	0.67 ± 0.08
Oculomotor Nu	1.3	(107)	7.80 ± 1.9
Suprachiasmatic Nu	3.5	(24)	5.00 ± 0.86
Striatum	1.2	(106)	20.0 ± 3.9
Frontal Cortex:			
Layer I	1.8	(10)	23.0 ± 1.5
II	1.5	(10)	20.0 ± 2.0
III	1.1	(10)	16.0 ± 2.0
IV	1.1	(10)	18.0 ± 3.2
V	0.57	(10)	23.0 ± 2.0

1 Data expressed in optical density units (Arbitrary Scale: 0-53).

* Data obtained from literature, calculated by extrapolation from counts of silver grain clusters in light microscopic autoradiographs. References given in parentheses.

** $r = -0.403$

TABLE 9

Correlation of 5-HT1 Binding Site Densities with Densities of 5-HT Synaptic Contacts**

Region	Frequency of Synaptic Contacts*		Density of Synaptic Contacts (million varicosities/mm^3)	Specific 3H-5HT Binding[1]
Locus Coeruleus	42	(60)	4.2	4.30 ± 1.1
Dorsal Accessory Olive	14	(118)	0.63	4.20 ± 2.4
Motor V	14	(99)	0.25	1.80 ± 0.06
Cerebellar Cortex	8.8	(14)	0.02	0.67 ± 0.08
Oculomotor Nu	43	(107)	0.56	7.80 ± 1.9
Suprachiasmatic Nu	40	(24)	1.4	5.00 ± 0.86
Striatum	29	(106)	0.35	20.0 ± 3.9
Frontal Cortex (I, II, III)	17	(10)	0.24	20.0 ± 2.5

1 Data expressed in optical density units (Arbitrary Scale: 0-53).

* Data on frequency of synaptic boutons taken from the literature. References given in parentheses. Absolute frequencies were derived from proportion of 5-HT terminals showing a synaptic differentiation in single thin sections by the formula:

$$\rho = \frac{1}{\pi} \int_0^{\pi} \frac{d \sin\alpha + w}{D} = \frac{d}{D} \cdot \frac{2}{\pi} + \frac{w}{D}$$

where:

- D = varicosity diameter
- d = active zone
- w = section thickness
- α = angle at which the plane of section intersects the active zone

** $r = -0.224$

TABLE 10

Colchicine Related Changes in the Regional Distribution of 5-HT1 Binding Sites

Region	Specific 3H-5HT Binding[1] Sham	Colchicine	Decrease (%)*
24 hrs Postlesion			
Raphe Nucleus Dorsalis	43.7 ± 3.90	28.5 ± 2.68	-34.7*
Substantia Nigra (Reticulata)	53.3 ± 2.25	37.9 ± 4.01	-28.8*
CA III (Molecular)	47.5 ± 1.75	35.4 ± 3.25	-25.5*
Lateral Hypothalamus	24.5 ± 7.25	9.4 ± 4.31	-61.8*
Globus Pallidus	40.8 ± 6.15	26.5 ± 3.65	-34.9*
Frontal Cortex (I, II, III)	20.0 ± 2.25	12.2 ± 1.75	-39.1*
Lateral Septum	42.2 ± 2.83	33.2 ± 2.26	-21.4*
48 hrs Postlesion			
Raphe Nucleus Dorsalis	41.7 ± 7.42	34.1 ± 3.88	-18.2
Substantia Nigra (Reticulata)	48.9 ± 5.08	48.9 ± 4.69	0
CA III (Molecular)	45.2 ± 6.50	47.4 ± 5.14	+ 4.87
Lateral Hypothalamus	17.1 ± 5.65	12.8 ± 4.15	-25.2
Globus Pallidus	36.1 ± 7.57	38.3 ± 6.50	+ 6.09
Frontal Cortex (I, II, III)	16.1 ± 4.95	14.0 ± 1.68	-13.0
Lateral Septum	38.8 ± 6.20	42.6 ± 8.05	+10.8

1 Densitometric measurements expressed in optical density units (Arbitrary Scale: 0-53). Each value corresponds to the mean (± S.D.) of more than twenty-four measurements from film autoradiographed sections of four colchicine and three saline treated animals at the 24-hour timepoint and three colchicine and two saline treated aminals at the 48-hour timepoint.

* Significant decrease after colchicine treatment with $p \leq 0.05$.

TABLE 11

Correlation Between Regional Changes after Colchicine and 3H-Imipramine Binding*

Region	3H-5HT Binding Decrease after Colchicine (%)[1]	3H-Imipramine Binding[2] (fmol/mg protein)
Raphe Nu Dorsalis	34.7	93.3
Substantia Nigra (Reticulata)	28.8	121.4
CA III (Molecular)	25.5	75.2
Lateral Hypothalamus	61.8	203.7
Septum	21.4	57.4
Globus Pallidus	34.9	148.7
Frontal Cortex	39.1	47.9

1 Data represents the percentage decrease in 3H-5HT binding relative to saline-treated control animals 24 hours after the periaxonal injection of colchicine.

2 Data taken from Palkovits et al (1981). Specific 3H-imipramine binding in each region was determined using at least six membrane preparations.

* Correlation significant with $p \leq 0.10$.
$r = 0.734$

Figure 1

Standard curve for tritium labelled sections.

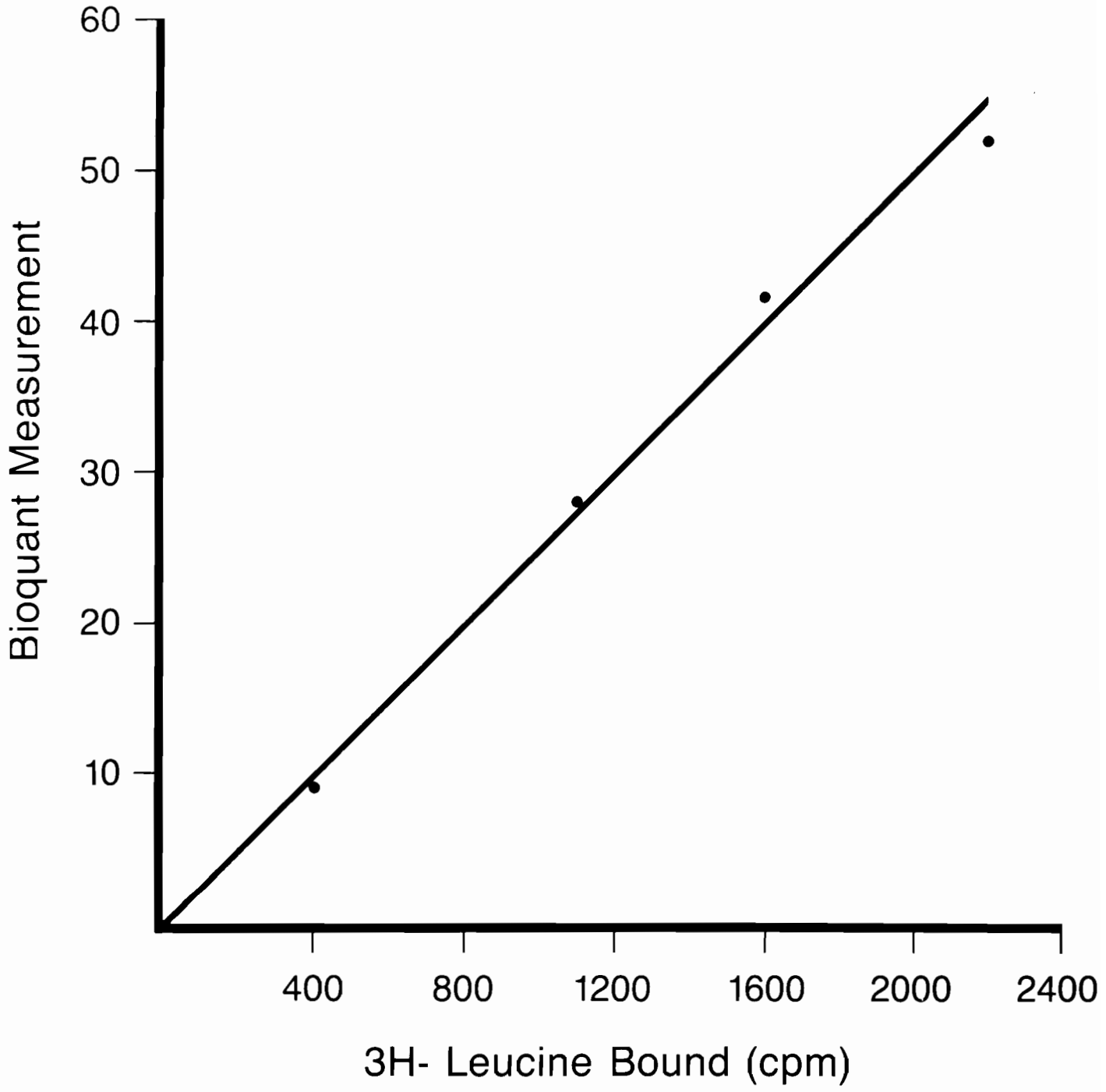


Fig.1 Standard curve derived from radiolabelled sections of rat brain homogenates processed for wet autoradiography. Densitometric measurements in optical density units (Arbitrary scale 5-58). Each corresponds to the mean of more than 12 determinations.

Figure 2

Timecourse of 3H-5HT binding to intact frozen sections of rat midbrain.

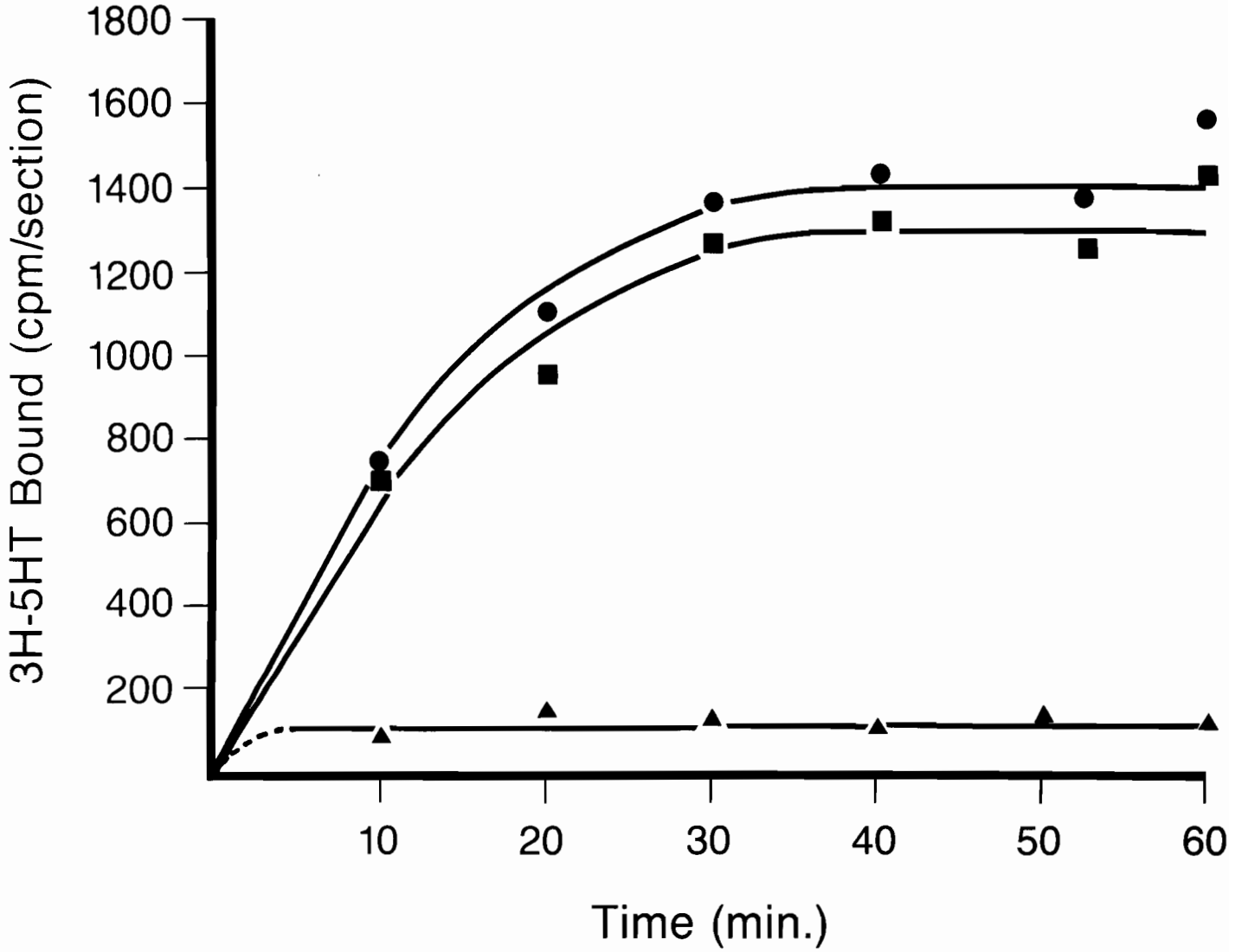


Fig.2 Binding of 3H-5HT in sections of rat midbrain with time. The sections were incubated with 5mM 5-HT with (nonspecific binding) or without (total binding) unlabelled 5-HT. Each point corresponds to the mean of three different determinations.

Figure 3

Effects of fixation and dehydration on the topographical distribution of 3H-5HT binding sites within the midbrain. Film autoradiographs were processed without fixation (a), after fixation alone (b) and after fixation and dehydration (c). No modification in the distribution of specifically bound 3H-5HT is observed after histological processing. Note the apparent enhancement of labelling intensities subsequent to dehydration (c).

Exposure time: 6 weeks

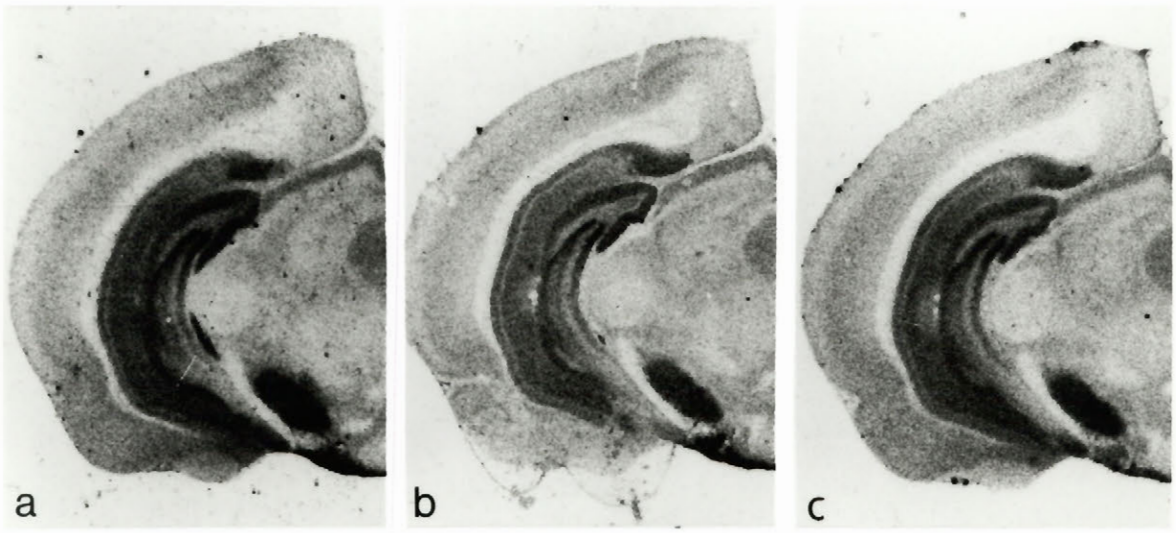
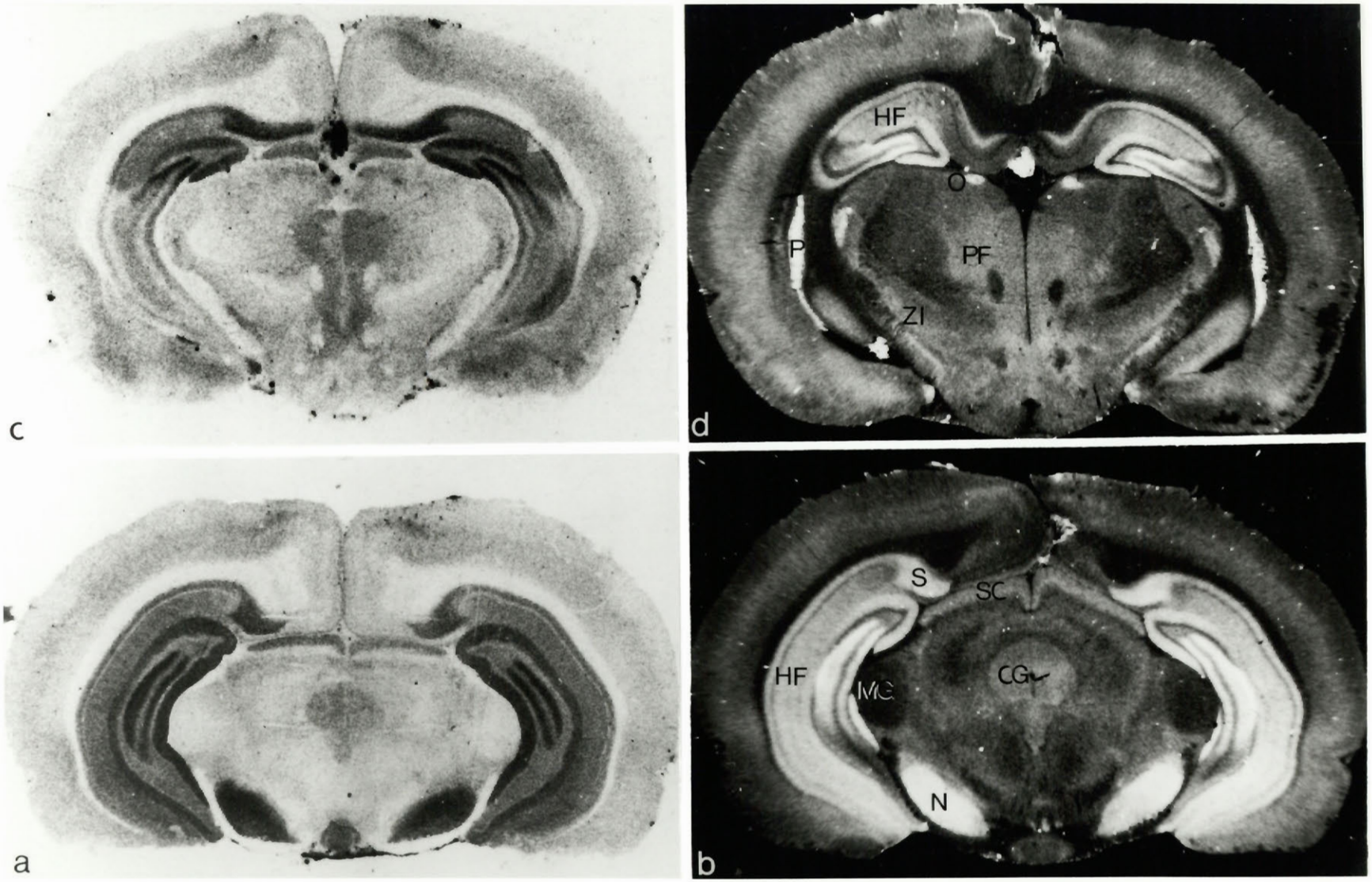

a
b
c

Figure 4

Topographical distribution of 3H-5HT labelled binding sites at four successive levels of the rat brain as seen on autoradiographs prepared by apposition to tritium sensitive film (a,c,e,g; unfixed sections) or by classical dipping techniques (b,d,f,h; fixed and dehydrated sections). Note the similarities between the distribution of 5-HT1 binding sites in sections processed by either wet or dry autoradiography.
Exposure time: 6 weeks

Abbreviations:

ac = anterior commissure
cc = corpus callosum
BST = bed nucleus stria terminalis
CG = central grey
GP = globus pallidus
HF = hippocampal formation
LS = lateral septum
MG = medial geniculate
N = substantia nigra
O = olivary pretectal nucleus
OT = olfactory tubercle
P = choroid plexus
PF = parafascicular nucleus
S = subiculum
SC = superior colliculus
STR = striatum
VP = ventral pallidum
ZI = zona incerta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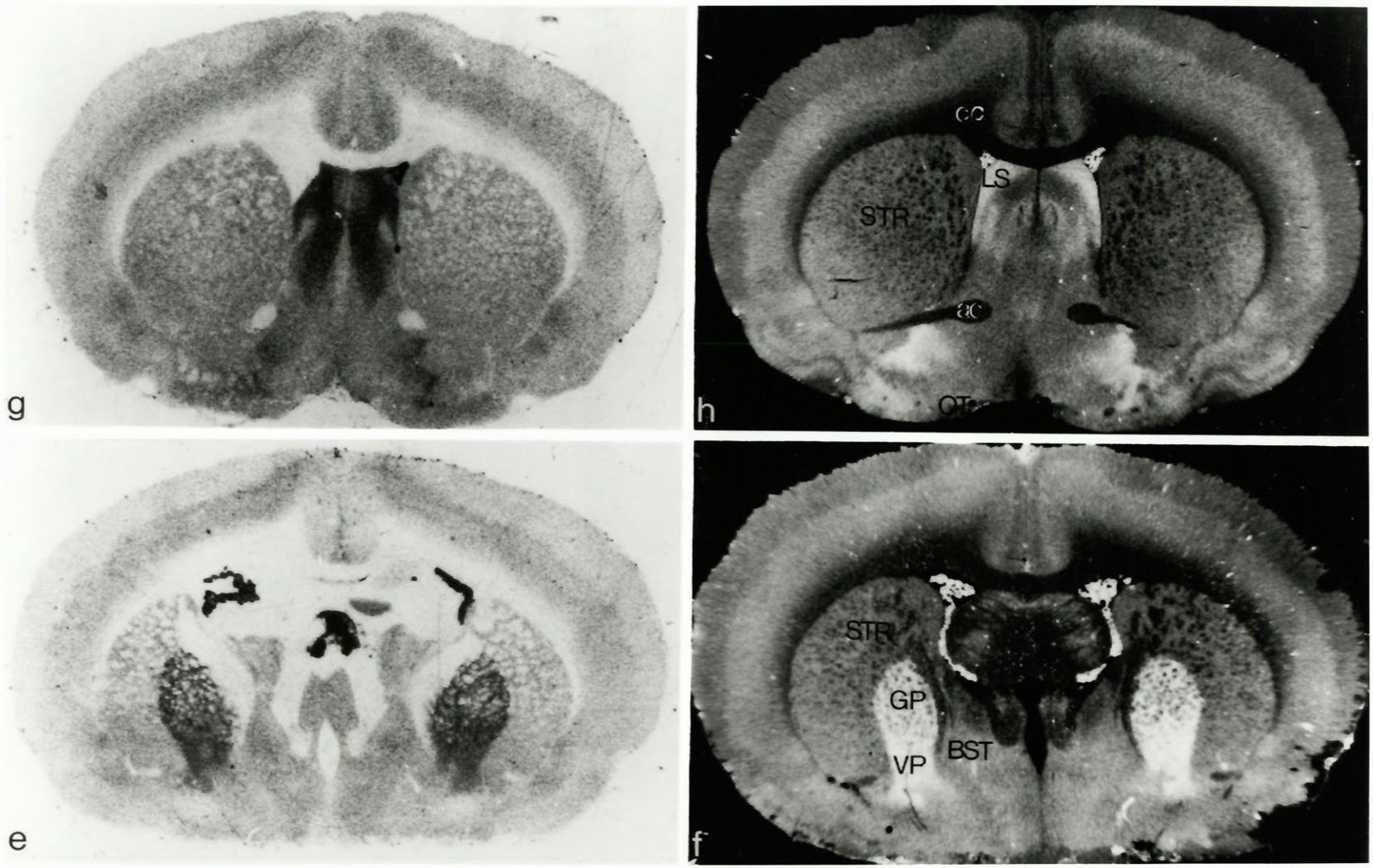
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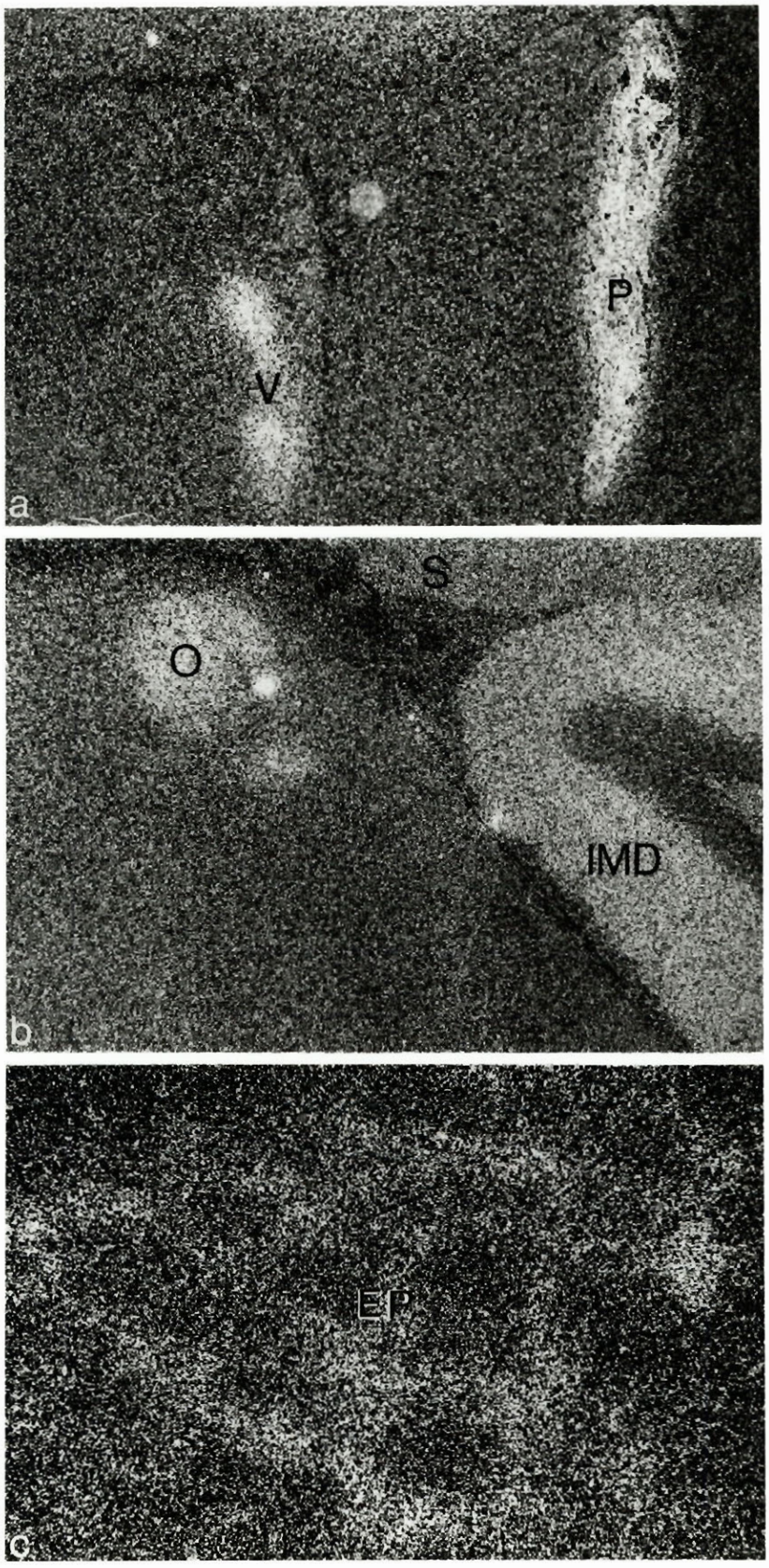
GP
VP
BST

Figure 5

High resolution autoradiographic distribution of 5-HT1 binding sites within selected brain regions (darkfield). Labelled structures include the magnocellular part of the ventrolateral geniculate body (V), choroid plexus (P), olivary pretectal nucleus (O), entopeduncular nucleus (EP) and the lateral septum (LS)

Exposure time: 6 weeks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IMD
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EP
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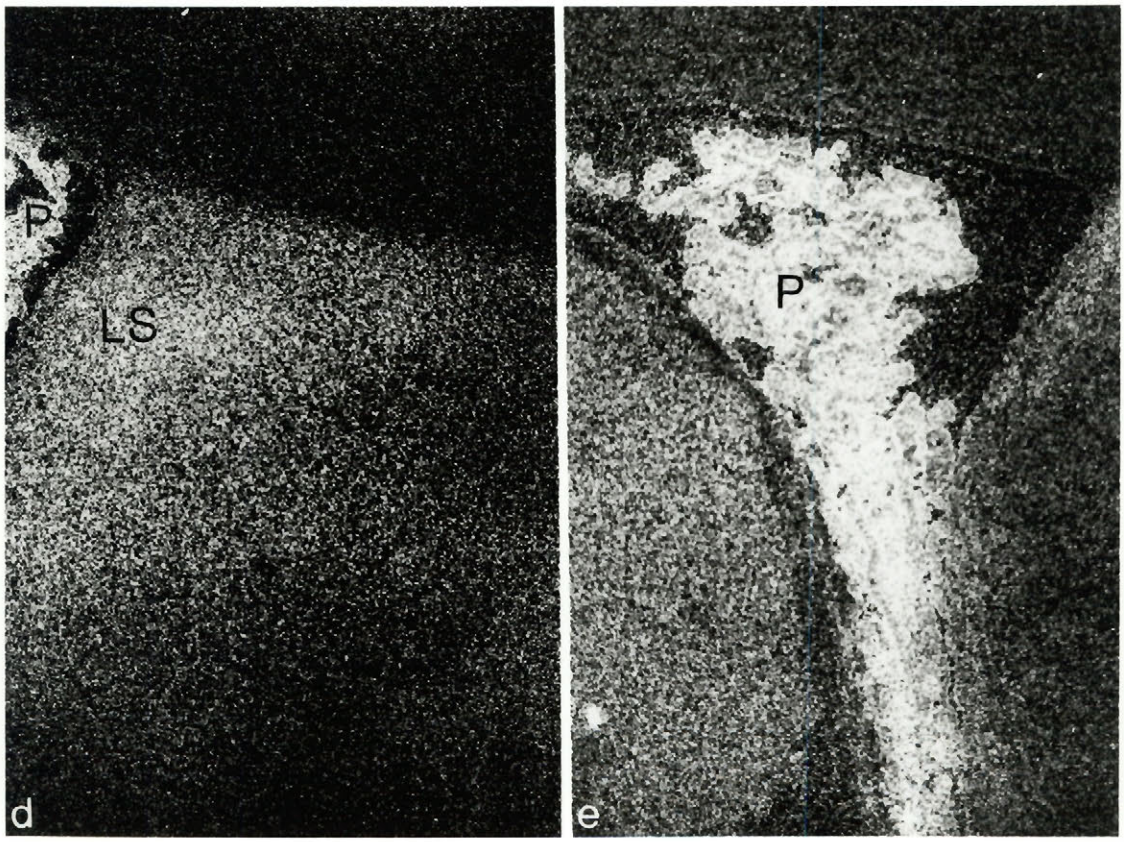

P
LS
d
P
e

Figure 6

(a) Darkfield micrograph showing the distinct laminar labelling pattern characteristic of 3H-5HT binding within the hippocampal formation. (b) The histologically distinct layers of the hippocampal formation can be recognized when observed in brightfield. Note the intense labelling within the molecular layers of the dentate gyrus and the CA III region, with relative sparing of the pyramidal and granule cell layers.

Exposure time: 6 weeks

Abbreviations:

H = hilus
IGD = internal granular layer of dentate gyrus
IMD = internal molecular layer of dentate gyrus
M = CA III (molecular layer)
OGD = outer granule layer of dentate gyrus
OMD = outer molecular layer of dentate gyrus
SP = CA III (stratum pyramidale)

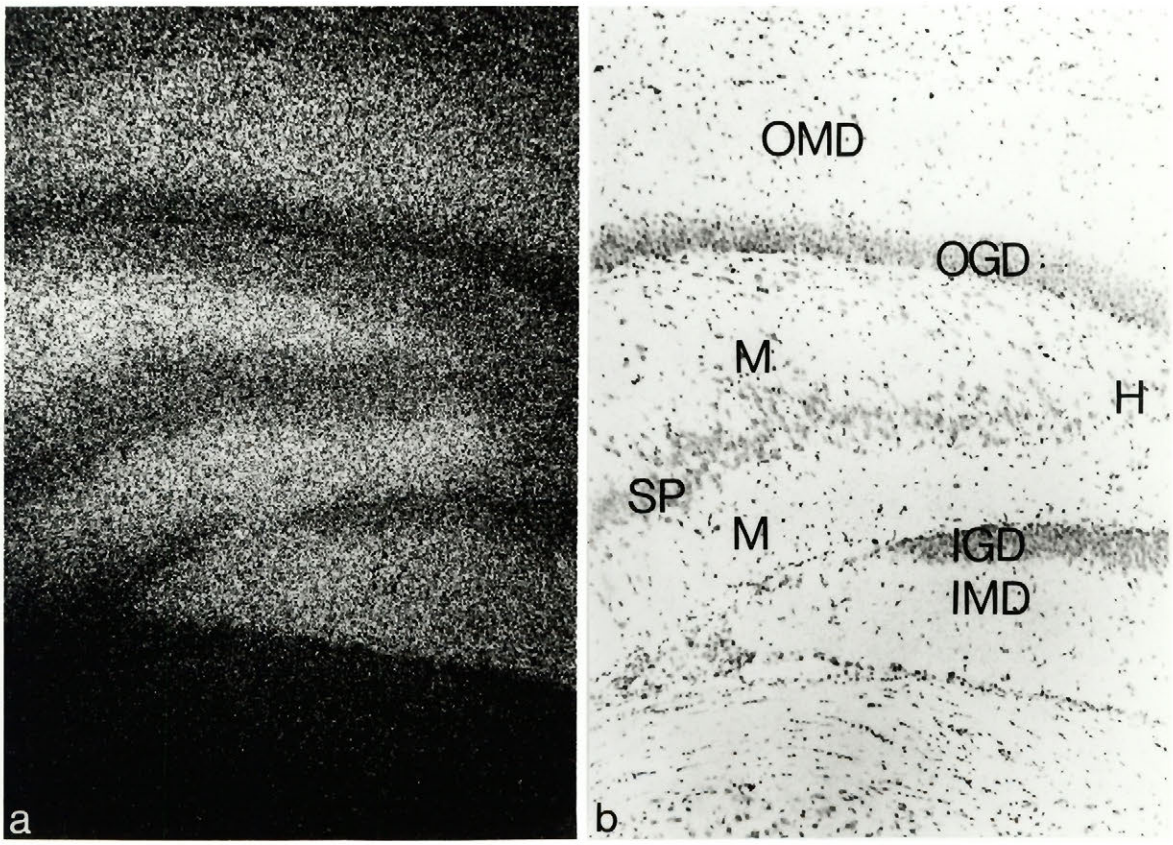

a
OMD
OGD
M
H
SP
M
IGD
IMD
b

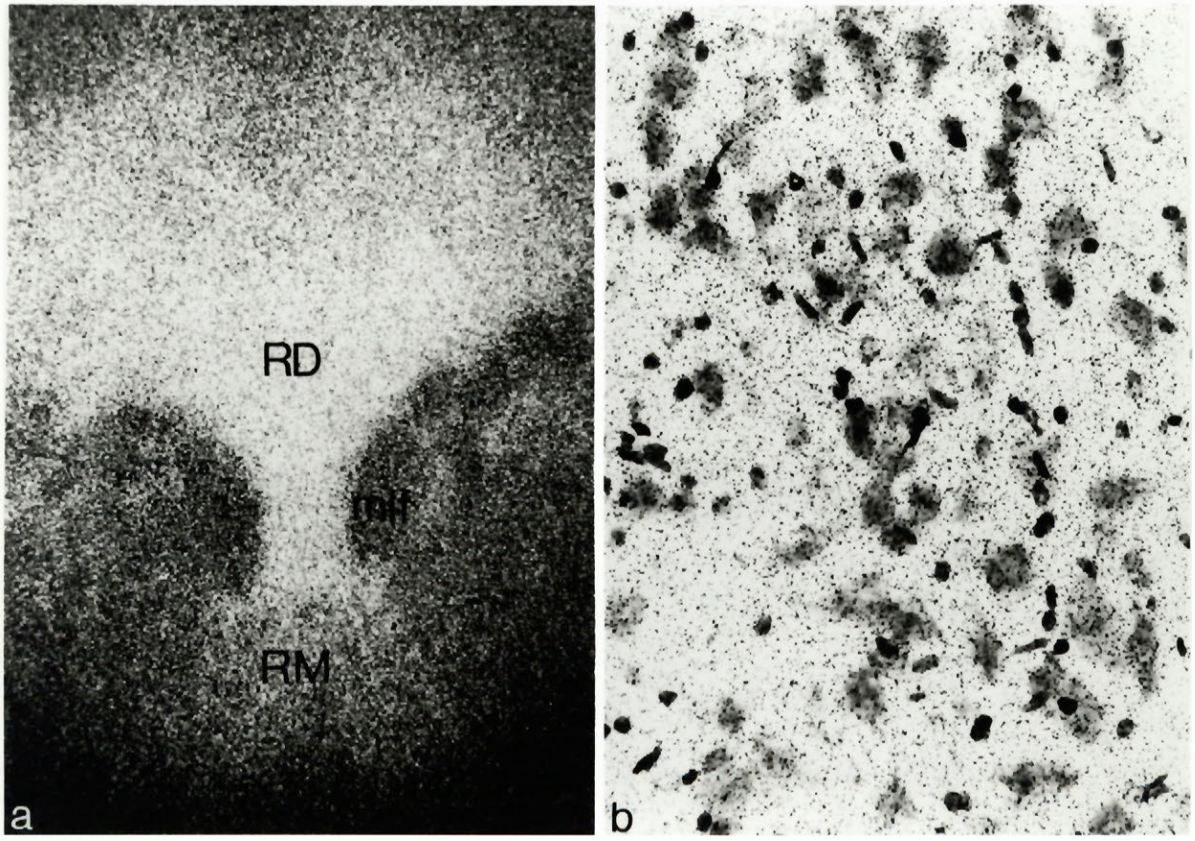
Figure 7

(a,c) Darkfield micrographs revealing the regional distribution of 3H-5HT labelled binding sites in the raphe nuclei dorsalis and medianus and in the substantia nigra. At high magnification in bright field silver grains are seen to be homogeneously dispersed over the neuropil and perikarya in the raphe nucleus dorsalis (b) and in the substantia nigra reticulata and compacta (d).

Exposure time: 6 weeks

Abbreviations:

mlf = medial longitudinal fasciculus
NC = substantia nigra (compacta)
NR = substantia nigra (reticulata)
RD = raphe nucleus dorsalis
RM = raphe nucleus medianus


RD
rdf
RM
a
b

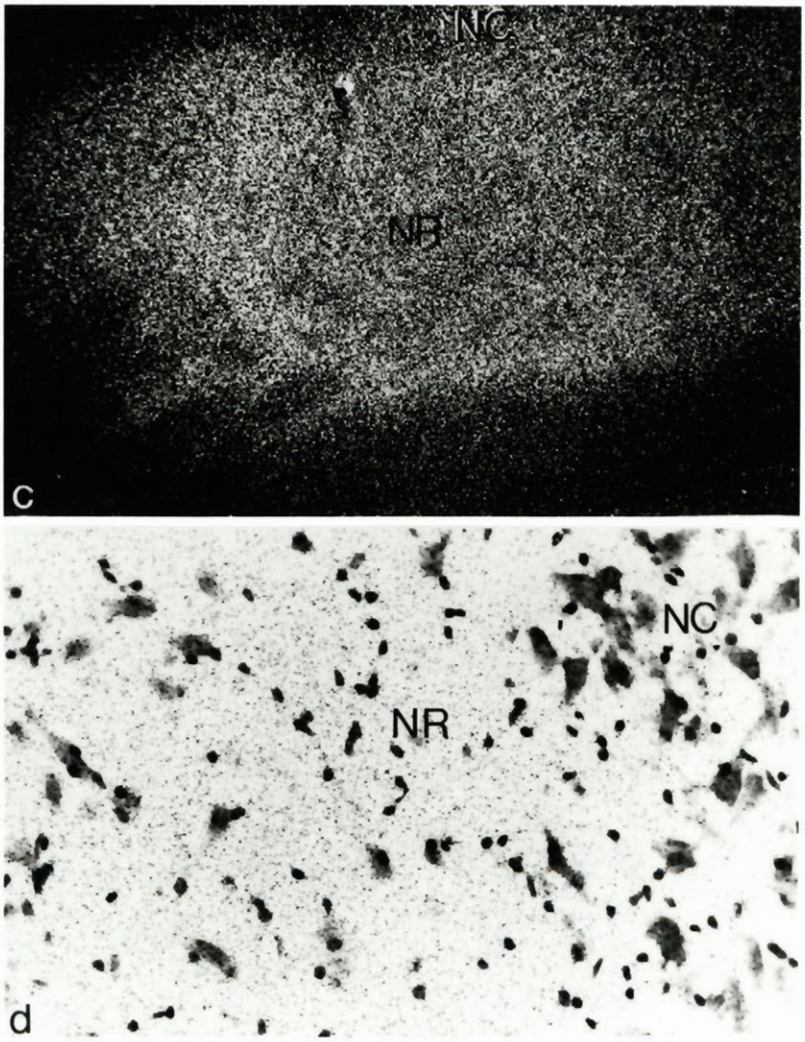

NC
NB
c
NC
NR
d

Figure 8

Saturation curve for 3H-5HT binding.

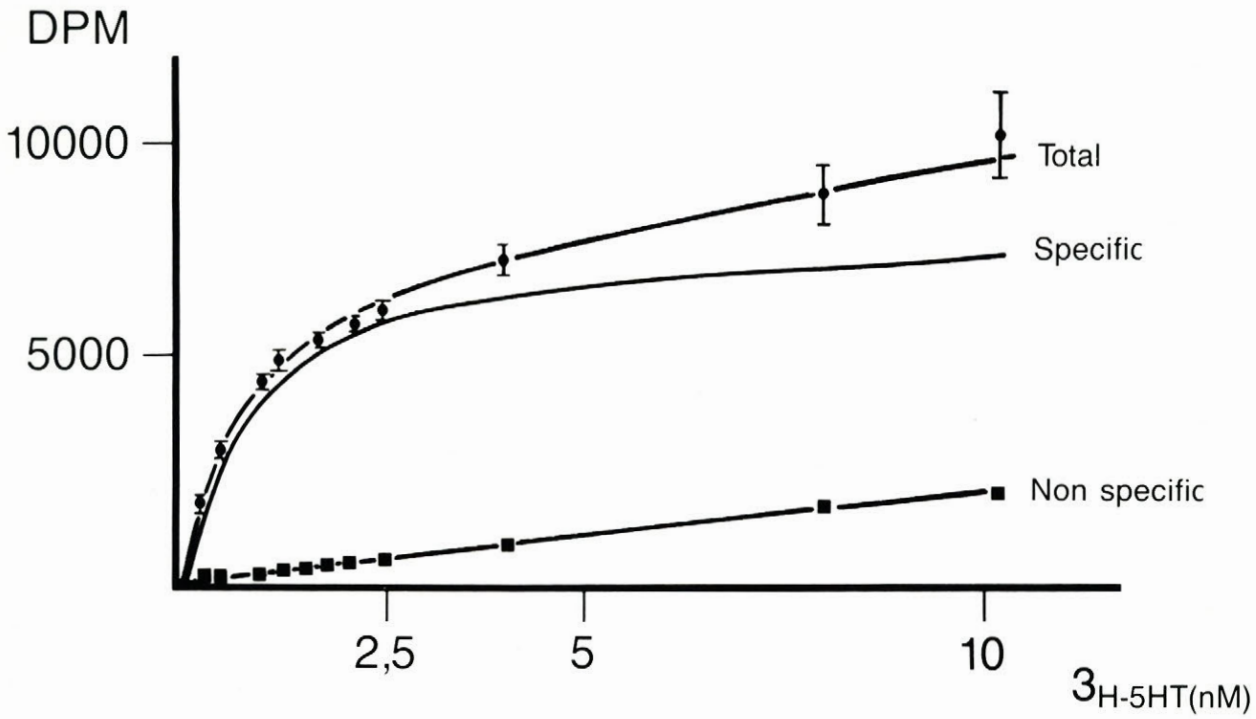


Fig. 8 3H-5HT Saturation Curve
Adapted from Magre (1983).
Tissue sections were incubated with known concentrations of 3H-5HT with (total binding (•)) or without (nonspecific binding (∘)) unlabelled 5-HT. Each value corresponds to the mean ± SEM of three determinations.