Autoradiographic studies of the distribution of serotonin in the rat brain

Brent D. Wagstaff

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AUTORADIOGRAPHIC STUDIES OF THE DISTRIBUTION
OF SEROTONIN IN THE RAT BRAIN

A Thesis
Presented to the
Department of Zoology
Brigham Young University
Provo, Utah

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
Brent D. Wagstaff
August 1971
This thesis, by Brent D. Wagstaff, is accepted in its present form by the Department of Zoology of Brigham Young University as satisfying the thesis requirement for the degree of Master of Science.
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I gratefully acknowledge the technical assistance of Lennae Warnes, and the instruction and council of Dr. Donal J. Reed of the department of Pharmacology of the University of Utah medical school.

I appreciate the understanding, encouragement, and support of my wife Erna in my academic pursuits.
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Serotonin (5-hydroxytryptamine, 5-HT) was first isolated in 1948 at the Cleveland Clinic by Rapport, Green, and Page (1948). Further investigation and development of a sensitive bioassay led to the determination of the relative distribution of 5-HT in mammalian tissues (Twarog and Page, 1953). Although the majority of 5-HT (90-95%) occurs in the gastrointestinal mucosa, with lesser amounts in the blood platelets and spleen, the physiologically most significant metabolism of 5-HT occurs in the brain (Sjoerdsma, 1959).

Vogt in 1954 studied the relative distribution of 5-HT in the brain and noted high concentrations of 5-HT in the hypothalamus, area postrema, grey stratum around the aquaduct, and medial thalamus. Also in 1954, Amin and associates found the distribution of 5-HT in brain to be very similar to that of norepinephrine.

These significant findings initiated numerous investigations into the role played by 5-HT in the central nervous system. The findings at times have been highly controversial, but it has become very apparent that it is one of the most important compounds in the central nervous system. As stated by Wooley and Shaw (1954) of the Rockefeller Institute, after studying a series of 5-HT antimetabolites:
... the naturally occurring mental disorders, for example schizophrenia ... which are mimicked by these (psychotomimetic) drugs, may be pictured as being the result of a cerebral serotonin deficiency arising from a metabolic failure.

Gaddum (1954) of Edinburgh also expressed the opinion: "It is possible that the serotonin in our brains plays an essential part in keeping us sane."

My work expressed in this thesis is an attempt to determine the distribution of labelled 5-HT injected into the cerebral spinal fluid, to correlate this localization with findings related to the metabolism and physiology of 5-HT, and to make particular anatomical observations related to the mode of distribution and uptake of the original injection of $^3H-5$-HT.
LITERATURE REVIEW

Metabolism. Serotonin is a minor product of tryptophan metabolism constituting an estimated one to three percent of the metabolism of dietary tryptophan (Harper, 1963; Udenfriend, Weissbach, and Sjoerdsma, 1956). Studies of 5-HT biosynthesis indicate that the hydroxylation of tryptophan occurs first followed by a decarboxylation.

The formation of 5-hydroxytryptophan (5-HTP) is believed to occur mainly in the enterochromaffin cells of the intestinal mucosa (Erspamer, 1957). Other systems for the synthesis of 5-HTP have been demonstrated in hepatic cells, kidney, and brain tissue (Gal, Poczik, and Marshall, 1963; and Cooper and Meicer, 1961). The enzyme found in the liver has been shown to be identical in action with phenylalanine hydroxylase (Renson, Weissbach, and Udenfriend, 1962). The hydroxylation of tryptophan is apparently the rate-limiting step in the formation of 5-HT since the decarboxylation reaction occurs with zero order kinetics (Udenfriend, 1959).

Five-hydroxytryptophan decarboxylase activity is found in practically all parenchymatous tissues—gastrointestinal tract, liver, lung, kidney, testicles, brain, sympathetic ganglia, etc. (Erspamer, 1961). The enzyme appears to be non-specific since 5-HTP and DOPA compete for
the same decarboxylase in vivo and inhibitors of DOPA decarboxylase also inhibit the decarboxylation of 5-HTP (Bertler and Rosengren, 1959). Lovenberg, Weissbach, and Udenfriend (1961) have postulated the presence of an aromatic L-amino acid decarboxylase capable of decarboxylating DOPA, 5-HTP, phenylalanine, tyrosine, tryptophan, and histidine. Several investigators have demonstrated an absolute requirement for pyridoxal phosphate as a cofactor for 5-HTP decarboxylation (Buzard and Nytch, 1957; Weissbach, et. al., 1957).

Schanberg (1963) has shown that 5-HTP is actively transported into brain tissue, whereas circulating 5-HT will not penetrate the blood brain barrier. This indicates that decarboxylation of 5-HTP to 5-HT follows active transport into the brain. The 5-HT is then bound and stored or metabolized further. Five-HTP has been shown to exist as a zwitterion at physiological pH which likely augments its active transport across the blood brain barrier (Udenfriend, 1959).

Tochino and Schanker (1965) have studied an active transport system in the rabbit choroid plexus which apparently removes 5-HT and norepinephrine from the cerebral spinal fluid. Studies on the turnover of 5-HT in brain suggest its rapid formation and release with an estimated half life on the order of 35 minutes (Udenfriend and Weissbach, 1958).

Five-HT is converted by monoamine oxidase (MAO) to 5-hydroxyindole aldehyde which is further oxidized to 5-hydroxyindole acetic acid (5-HIAA), the major excretory metabolite, by a DPN-catalyzed aldehyde dehydrogenase (White,
Handler, and Smith, 1964). The rate-limiting step is the formation of the aldehyde. Both the aldehyde and the acid are physiologically inert (Sjoerdsma, 1959). MAO is believed to be strictly a mitochondrial enzyme (DeRobertis, 1964), and is non-specific for 5-HT, acting on several substrates, notably tyramine, tryptamine, adrenalin, and noradrenalin (Weissbach, Redfield, and Udenfriend, 1958).

**Physiology.** Low doses of 5-HTP, when injected i.v. (5-20 mg/Kgm), produce a decrease in spontaneous activity in dogs and cats, and induces tranquilization in rabbits (Bogdanski, Weissbach, and Udenfriend, 1958; Costa, et. al., 1959). Controversy exists as to the behavioral and neurological effects of the i.v. injection of larger doses of 5-HTP (30-100 mg/Kgm). Several investigators have noted excitement and disorientation in dogs, cats, and rabbits, accompanied by depressant effects on reflexes, motor control and sensory functions (Udenfriend, Weissbach, and Bogdanski, 1957a; Bogdanski, Weissbach, and Udenfriend, 1958; Costa, et. al., 1959). Lewis (1958) was unable to produce such symptoms in cats. He observed no signs of central excitation, rather the usual effect of 5-HTP was found to be slight depression.

Injections of 5-HT into the lateral ventricles of cats produces apathy (Feldberg and Sherwood, 1954).

Giorman (1956) has noted that an excess of 5-HTP in nervous tissue blocks its own enzymatic decarboxylation thereby inhibiting 5-HT formation from the exogenous 5-HTP. Brodie, et. al., (1966) have shown that 5-HTP, in doses that
elicit excitation, also causes the release of brain norepinephrine. Such findings make it difficult to draw definite unambiguous conclusions as to the function of endogenous brain 5-HT especially with the use of exogenously administered 5-HTP.

**Neurophysiology.** Considerable evidence exists suggesting that 5-HT acts as a transynaptic mediator in certain areas of the nervous system. This contention is supported by the high 5-HT concentration within certain neurons together with the enzymes for its synthesis and inactivation (Bogdanski, Weissbach, and Udenfriend, 1957; Fuxe, 1965; Garattini and Valzelli, 1965), its storage within synaptic vesicles (Maynert and Kuriyama, 1964), and its high rate of turnover in nervous tissue (Udenfriend and Weissbach, 1958). Furthermore, biochemical and fluorescent histochemical studies have shown that 5-HT is accumulated in nerve terminals making close contacts with other nerve cells and that it is released on nerve stimulation (Anden, et. al., 1964; Dahlstrom, et. al., 1965; Aghajanian and Bloom, 1967). DeRobertis (1964) has shown that 5-HT is contained in nerve endings which sediment at the same density as those containing acetylcholine and norepinephrine. Studies of nerve endings and synaptic vesicles indicate that about 4% of 5-HT is unbound with approximately 50% bound in the crude mitochondrial fraction and 40% in the microsomes (DeRobertis, 1964).

Further evidence comes from Brodie and Shore (1957) studies using reserpine, a tranquilizer which apparently
acts by impairing the storage mechanism of 5-HT and reducing the overall cellular content of 5-HT. These findings led to the speculation that the sedative action of reserpine is mediated through free 5-HT which persistently occupies neuronal receptor sites. Brodie and Shore (1957) further proposed 5-HT to be a chemical transmitter in the central parasympathetic system, based on the high concentration of 5-HT in the brain stem, where the major part of autonomic integration occurs, and the parasympathomimetic activity induced by reserpine. More recent histochemical techniques have, however, shown 5-HT nerve terminals to be found in both parasympathetic and sympathetic nuclei (Fuxe, Hokfelt, and Ungerstedt, 1968).

Five-HT concentrations increase within the reticular activating system particularly during light sleep and dream states (Jouvet, 1967). Marazzi and Hart (1955) consider 5-HT to be an inhibitory neurohumor since it is 20 to 25 times as potent as adrenalin in causing cerebral synaptic inhibition.

Five-HT may well be necessary for the proper formation and development of the nervous system. Pregnant women excrete as much as ten times more xanthurenic acid (a major metabolite of tryptophan) than do nonpregnant women following a dose of tryptophan (White, Handler, and Smith, 1964) indicating an enhanced tryptophan metabolism. Kaarki, Kuntzman, and Brodie (1947) have shown that the concentration of 5-HT in the rat brain parallels the muscular coordination and
neurological maturation of the animal, whereas guinea-pigs, which are physiologically more mature at birth have brain levels of 5-HT at parturition similar to those found in the mature adult. Phenylketonuria (PKU), a hereditary metabolic disease in which the enzyme necessary for the hydroxylation of phenylalanine to form tyrosine is missing, is characterized as resulting from inhibition of 5-HTP decarboxylation due to the direct action of increased phenylalanine metabolites such as phenylpyruvic acid, phenyl-lactic acid, and phenylacetic acid (Davison and Sandler, 1958). Idiocy early in infancy occurs with PKU. The mental defect of experimentally induced PKU in mice is prevented if melatonin or 5-HTP is administered continuously from birth to maturity (Woolley and van der Hoeven, 1964).

Mental Illness. Wooley and Shaw (1954) were perhaps the first to note the apparent relationship between mental illness and a metabolic failure of 5-HT. They studied a series of 5-HT antimetabolites and found them to be psychomimetic drugs inducing schizophrenic-like behavior. These drugs included the ergot alkaloid derivative lysergic acid diethylamide (LSD), harmaline, yohimbine, and a synthetic antimetabolite called medmain—each structurally related to 5-HT. Bufotenine (N, N-dimethylserotonin) is an active component of the psychotropic snuff called cahoba used by the Mura Indians of Rio Negro during annual assemblies (McIsaac, 1961). A similar drug psilocybin (the phosphoric ester of N, N-dimetyly-4-hydroxytryptamine) was isolated by
Hofmann, et. al. (1958) from a sacred mushroom eaten during ancient religious rites by the Mazatec Indians of Mexico to induce exhilaration, incoherence, and colorful visual fantasies. The O-methyl derivative of 5-HT caused trained rats to make as many mistakes as bufotenine. The most potent compound tested was the O-methyl derivative of bufotenine (McIsaac, 1961). O-methylation and N-acetylation of 5-HT occurs in the formation of melatonin in the pineal gland (Lerner, Case, and Takahashi, 1960). Evidence for the presence of 5-methoxytryptamine in the pineal gland has also been found by McIsaac, Kreder, and Page (1960). Under physiological conditions of pH and temperature, amines like 5-methoxytryptamine will condense with aldehydes and keto acids to give a harmalan similar to 10-methoxyharmalan (derived from the removal of H₂O from melatonin), which, when given to a trained rat, causes trembling and reduces its capability in performing simple conditioned behavior (McIsaac, 1961). McIsaac (1961) has also found reason to believe the removal of H₂O from melatonin with cyclization to form the harmala alkaloid, 10-methoxyharmalan, may occur. The harmala alkaloids, besides causing hallucinations, are potent monoamine oxidase inhibitors (Udenfriend, et. al., 1958) and could therefore, once formed, prevent the normal breakdown of 5-HT and shunt it down pathways leading to the production of more 5-methoxytryptamine, melatonin, and 10-methoxytryptamine, thus initiating a pathological self-perpetuating cycle (McIsaac, 1961).
Giorman and Freedman (1960), in a limited study of the pineal glands of deceased mental patients vs. those of deceased patients with somatic failure (cancer, heart attack, etc.), found that the mental patients (7) showed a great variability in pineal weight (.115 - 1.89 gms) and 5-HT concentration (.36 - 22.82 μg/gm) as compared to the three patients with somatic failure (pineal weight: .095 - .130 gms, and 5-HT concentration: 2.18 - 5.77 μg/gm).

Administration of exogenous 5-HTP plus a monoamine oxidase inhibitor as a means for raising the brain 5-HT level has proven beneficial in the relief of severe depression (Kline and Sacks, 1963). Such relief from melancholia is notably increased if methyl phenidate (Ritalin) is given following the administration of the 5-HTP. Ritalin is believed to facilitate or catalyze the synthesis of 5-HT from 5-HTP (Robie and Flora, 1965). Administration of 5-HTP to schizophrenic patients has been of no value and in some instances has intensified the disease (Woolley, 1962).

It is also interesting that no 5-HT has been detected in the cerebrospinal fluid of phenylketonurics and mongoloids, even after monoamine oxidase blockade. (Perry, Shaw, and Walker, 1961).

**Distribution of Serotonin in the Brain.** Twarog and Page in 1953, using a bioassay, demonstrated the presence of 5-HT in acetone extracts of dog, rat, and rabbit brain, the values varying from 0.1 - 0.36 μg/gm/gm of tissue. A year later Amin and coworkers (1954) found the distribution of 5-HT in
brain to be very similar to that of nor-epinephrine, which is notably high in hypothalamus and medial thalamus. Bogdanski, Weissbach, and Udenfriend (1957) have noted the greatest 5-HT concentration in the brain of dog and cat in the brain stem, rhinencephalon, and neostriatum. A similar study by Pscheidt and Hibovich (1963), using rhesus monkey brain, concluded that 5-HT concentrations were highest in midbrain (hypothalamus), thalamus, pons-medulla, caudate, hippocampus (amygdala), lenticular thalamic mass, temporal pole, and various cortical structures. Studies of the 5-HT concentration in the pineal gland show that this structure has the highest 5-HT concentration in the brain (Giarman and Freedman, 1960). Numerous other studies to determine the regional distribution of 5-HT in larger vertebrates have been conducted. Table 1 gives but a few of the comparative values obtained.

Early autoradiographic studies by Lewis (1958) of brain tissue obtained after injection of radioactive 5-HP, indicated 5-HT to be distributed fairly evenly throughout the brain with no relation to nervous elements. A more recent autoradiographic study using tritiated (labelled) 5-HT injected into a lateral ventricle of rat brain found $^3$-5-HT activity predominantly localized in nerve endings and unmyelinated axons in the paraventricular regions examined (Aghajanian and Bloom, 1967).

Falck, Hillarp, and co-workers (1962) have developed a histochemical fluorescence method which demonstrates morphologically the existence of central 5-HT neurons containing
Table 1. Distribution of Brain Serotonin.

<table>
<thead>
<tr>
<th>Area</th>
<th>Rat</th>
<th>Cat</th>
<th>Dog</th>
<th>Human</th>
</tr>
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<tbody>
<tr>
<td>Amygdala</td>
<td>0.49</td>
<td>1.6</td>
<td>2.1</td>
<td>0.32</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>0.50</td>
<td>2.5</td>
<td>1.65</td>
<td>0.81</td>
</tr>
<tr>
<td>Caudate nucleus</td>
<td>1.6</td>
<td>0.76</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>Pons</td>
<td>0.67</td>
<td>0.33</td>
<td>0.38</td>
<td>0.7</td>
</tr>
<tr>
<td>Septum</td>
<td>2.0</td>
<td>1.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.45</td>
<td>0.66</td>
<td>0.57</td>
<td>0.62</td>
</tr>
<tr>
<td>Tuberculus olfactorius</td>
<td>2.0</td>
<td>0.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area postrema</td>
<td></td>
<td>0.22</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.32</td>
<td>0.64</td>
<td>0.09</td>
<td></td>
</tr>
</tbody>
</table>

(values in μg/gm)

(Costa and Aprison, 1958; Kuntzma, et. al., 1961; Udenfriend, 1957; Udenfriend, et. al., 1957b; Anden, et. al., 1966; Fuxe, et. al., 1968; Anden, et. al., 1967.)

Low concentrations of 5-HT in the cell bodies and high concentrations in the terminal parts of the cell. The 5-HT nerve cell bodies are localized mainly in the raphe nuclei of the lower brainstem. Some are also found in the medioventral part of the caudal tegmentum and surrounding the pyramidal tract. No 5-HT cell bodies are found in the diencephalon, the telencephalon, or the spinal cord (Dahlstrom and Fuxe, 1966; Fuxe, 1965). Five-HT nerve terminals are found with high to medium density in the hypothalamus; with low to medium density in the thalamus, amygdala, and septal area;
and with low density in the hippocampus, cerebral cortex, tuberculum olfactorium, and reticular formation (Fuxe, 1965). The axosomatic contacts of the 5-HT nerve terminals probably occur in the lumbosacral part of the spinal cord and the motor nucleus of the trigeminal nerve; whereas axodendritic contacts probably occur in various parts of the hypothalamus and the limbic lobe (Fuxe, 1968). Studies using selectively placed lesions, after which anterograde and retrograde degeneration are observed using the fluorescence histochemical method, have shown ascending 5-HT axons, from cell bodies situated in the raphe nuclei of the mesencephalon, running uncrossed mainly in the medial forebrain bundle innervating the limbic forebrain structures and the hypothalamus (Anden, Fuxe, and Larssen, 1966). Five-HT axons are observed in the striae terminales, the dorsal fornix, and the fimbriae of the hippocampus (innervating the amygdala and hippocampus). The 5-HT axons innervating the cortex probably ascend in the medial forebrain bundle bypassing the septal area to enter the cingulum (Anden, Fuxe, and Understedt, 1967). Lesions destroying the medial forebrain bundle which interconnects the hypothalamus with the basal telencephalon and midbrain have a pronounced effect (36% decrease) on the rat brain 5-HT level, indicating that the integrity of this neuronal system is necessary to maintain normal 5-HT levels in brain (Heller and Moore, 1968).
MATERIALS AND METHODS

In this study four male albino rats weighing between 250 and 325 grams were used. The two experimental rats were first injected intraperitoneally with 200 mg of iproniazid per Kgm body weight (Marsalid Phosphate, Hoffman LaRoche, Inc.). Two hours later, under ether anesthesia, the experimental rats were injected with .05 ml of a saline solution containing 20 microcuries 5-hydroxytryptamine$^3$-H (Nuclear Chicago Corp.). The injection into the brain was made through a small burr hole in the rat's skull located 5 mm posterior from the bregma and 3 mm lateral from the sagittal suture. The syringe was tilted 10° from the vertical plane corresponding to the sagittal suture; thus the needle tip projected laterally. A size 22 needle was used which had been sealed at its end, and a small hole for injection was made in the side of the barrel about 1 mm from the tip. The .05 ml injection was given slowly over a 30 second interval at a depth of 3 mm below the dura mater. Following the operation the experimental rats recovered and appeared fully awake. One hour after injection of the labelled 5-HT, the experimental rats were decapitated and the brain removed and placed in a 10% formalin solution. The brain was then dehydrated in graded series of ethanol solutions and embedded with paraffin wax. One brain was cut in serial cross...
sections, and the other longitudinally, at five microns thickness. The sections were then fixed to precleaned slides using Haupt's gelatin fixative.

The two control animals were decapitated without treatment, and their brains removed and fixed for cross and longitudinal sections for autoradiography in the same manner as with the experimental animals. The procedure used for radioautography was that outlined by Jofte (1963). Autoradiographs were made by first removing the paraffin with zylene, rehydrating the tissue, and then dipping the slides in NTB2 nuclear emulsion from Eastman Kodak. The nuclear emulsion was placed in a suitable container for dipping and immersed in a water bath in the darkroom and warmed to 40° C. The slides were then taken from the water in which they were rehydrated. Each slide was immersed in the emulsion with the long axis vertical so that the tissue went about half an inch below the surface. It was immediately withdrawn and allowed to drain for a few seconds onto a gauze pad held in the other hand for about ten seconds. After the slide had drained, the gauze pad was used to remove all emulsion from the bottom of the slide. The slide was then placed horizontally in a tray for the emulsion to gel. The slides in open trays were then placed in an air-tight exposure chamber charged with CO₂ (dry ice) and drierite, to reduce oxygen and moisture, and were stored for four weeks at 50° C for exposure.
Following the exposure period the slides were developed using Kodak D19 developer. About 15 slides were placed in a metal staining rack for immersion into the photographic processing solutions. The process schedule for immersion was 5 minutes in Kodak D19 developer, 15 seconds in Kodak SB5a stop bath, 2 minutes in Kodak fixer, and 10 minutes wash period in running tap water. All solutions were kept at 18°C. The stop bath reduces swelling and the probability of silver grain shifting, and the acid fixer hardens the emulsion layer which helps prevent damage from subsequent handling. The tissue sections were then stained with Delafield's hematoxylin, and eosin stains.

Rat brain stereotaxic atlases by Pellegrino and Cushman (1967) and Konig and Klippel (1963) were used to help determine regional anatomy. Zeman and Innes's (1963) text on neuroanatomy was used to help determine vascular distribution in the rat brain.
RESULTS

Originally, upon surveying our autoradiographs, we were impressed with unexpected results. Consistently radioactivity was seen to be localized near blood vessels, especially peripheral arteries, rather than having a more uniform distribution as we had anticipated (Figures 6, 13, 14, 18, 20, 21, 22, 25). Furthermore radioactivity was gradually attenuated in the brain tissue proportional to the distance proceeding away from the perivascular spaces surrounding blood vessels (Figures 6, 8, 12). Therefore areas deep in the parenchyma of the brain as well as peripheral areas lacking immediate association with perivascular spaces were apparently inaccessible to the injected H³⁻⁵-HT. We further discovered that the injections of labelled 5-HT were made by mistake into a space between the thalamus-midbrain and the hippocampus (Figures 22-24), instead of into the lateral ventricle. This was apparently due to inaccurate coordinates; i.e. the injections appear to have been given about 2 mm too far caudally. Because of this situation, we felt it impractical to make any conclusive quantitative regional comparisons. However, since it has previously been shown that interventricularly injected H³⁻⁵-HT is believed to be taken up by true serotonergic fibers (Aghajanian and Bloom, 1967), we did make a regional
survey of autoradiographic localization. About 45% of the total brain isotope was retained by tissue after processing for autoradiography, which presumably represents firmly bound labelled 5-HT (Aghajanian, et. al., 1966).

The predominant sites of intense autoradiographic activity in the telencephalon (Figure 1) were localized in the olfactory bulb, tract (Figures 5, 6), and tubercle. The greatest activity was found in the lamina molecularis and glomerulosa of the olfactory bulb, in the medial olfactory tract, and ventral tuberculum olfactorium. Moderate activity was noted in the rostral hippocampus (pars anterior).

In the diencephalon (Figure 2) major autoradiographic activity was observed in the medial forebrain bundle, the lateral nucleus of the thalamus, the central and marginal nuclei of the medial geniculate body, ventral hippocampus, the lateral optic tract, and the lateral geniculate body. Moderate accumulation of grains occurs in the ventral hypothalamus, the medial nucleus of the amygdala, the medial habenular nucleus, and the optic chiasm. Activity in this area of the brain is noted to be unilaterally high in the area of the lateral thalamic nuclei and the fimbria of the hippocampus (Figures 7-9). Grain accumulation is markedly decreased on the opposing side (Figures 10, 11). This distribution is interpreted to indicate the injection side and subsequent local dispersion and accumulation of the labelled 5-HT.
In the midbrain (Figure 4) area heavy autoradiographic accumulations are observed in the substantia nigra, the interpeduncular nucleus, the cerebral peduncle, the subiculum and dentate gyrus of the hippocampus, and the medial and lateral mammillary nuclei. Moderate activity occurs in the superficial stratum of the superior colliculus and the brachium of the inferior colliculus. Activity in this area is also unilaterally higher in the area of posterior thalamus and hippocampus thereby marking the side of injection (Figures 22-24).

Caudally in the hindbrain moderate to heavy activity is localized ventrally in the pyramidal tracts, ventral pons, and dorsally in the dorsal raphe area.

Because the injection entered a comparatively vascular region, we were able to study the previously mentioned "perivascular space" distribution of the labelled 5-HT. Even though the space between the thalamus-midbrain and hippocampus in the living condition is a narrow cleft containing a thin layer of connective tissue, it is potentially large enough to easily contain the amount of fluid injected (.05 ml). This is especially so caudally near the midbrain, whereas rostrally there are enough blood vessels for the perivascular space in the surrounding connective tissue to accommodate the injection. In fact it appears that the majority of the radioactive 5-HT moved from the site of injection forward and ventrally along a perivascular space of blood vessels and connective tissue in the cleft between the thalamus and
hippocampus. In our cross sections the cleft appears wider near the midbrain (Figure 22) than further forward (Figure 7). However larger blood vessels, mainly choroidal arteries, pass along the cleft laterally to the rostral part of the thalamus.

Heavy radioactivity is seen ventrally in the diencephalon especially on the side of injection (Figures 12-16) along the perivascular spaces and the surrounding brain tissue. Evidently the injection was distributed along the arteries of the circle of Willis, up into the cleft on the non injection side, and along ventral arteries both rostrally and caudally. A moderate amount of activity is found along the anterior cerebral arteries to the olfactory bulb, where it is mainly concentrated between them (Figures 5, 6). A fairly heavy amount is also seen along the ventral midbrain, pons, and medulla oblongata, where it evidently followed the perivascular space associated with arteries of the circle of Willis, basilar artery, and vertebral arteries.

The only dorsal or lateral cerebral area with evidence of significant amounts of activity is observed in the longitudinal fissure between the hemispheres (Figure 25) caudal to the splenium of the corpus callosum. The injection evidently spread to the opposite cleft along blood vessels and other cleft spaces near the pineal gland (Figures 19-21). It then moved dorsally from the dorso-lateral cleft area with veins draining blood to the superior sagittal sinus. There appears to be very little, if any, falx cerebri in that region.
DISCUSSION

The "perivascular space" distribution of the original injection was very evident and deserves further comment. Considerable disagreement has existed concerning the spatial relationship of the leptomeninges (arachnoid and pia mater) to the underlying nervous tissue and the blood vessels contained therein (Wollam and Millen, 1954). This is due mainly to artifacts encountered with tissue shrinkage during histological preparation.

Concerning the nature of the reticular perivascular sheath of the larger vessels of the nervous system, Hughson (1925) regarded the cells of the pia mater as turning inwards with blood vessels to form the outer wall, and the cells of the arachnoid covering the parent vessel as it traversed the subarachnoid space, as contributing to the formation of the inner wall. Schaltenbrand and Bailey (1928) considered the perivascular sheath to be simply a sheet of reticular connective tissue equivalent to the pia-arachnoid. Wollam and Millen (1954) believe the larger vessels in the central nervous system to be surrounded by a reticular perivascular sheath representing a prolongation of the leptomeninges, and that it is easy to define an outer pial and an inner arachnoid layer in the sheath surrounding the largest vessels entering the cord and brain. They further suggest that the
perivascular sheath be regarded as a loose envelope of connective tissue surrounding the blood vessels of the central nervous system, consisting of two layers blending imperceptibly with each other. The perivascular space surrounding penetrating blood vessels appears to be continuous with the subarachnoid space for cerebral spinal fluid circulation, and disappears as smaller vessels are reached (Woolam and Millen, 1955; Nelson, Blinzinger, and Hager, 1961).

Generally it is concluded that the superficial portion of the rat brain is composed of astrocytic cells and their processes covered by a basement membrane that is shared by the pia mater (Pease and Schultz, 1957; Peter, Palay, and Webster, 1970). The pial membrane seems to be composed of single layered cells containing delicate cytoplasmic extensions which slightly overlap, and occasionally, but not always, extend over a penetrating vessel (Nelson, Blinzinger, and Hermann, 1961). Some investigators have found evidence that the pia membrane does not always closely adhere to the underlying basement membrane and may not form a uniformly continuous sheath (Waggener and Beggs, 1967; Ramsey, 1965). This, along with the observation that macromolecules placed in the subarachnoid space accumulate rapidly beneath the pial cells along the astrocytic plasmalemmata (Brightman, 1965) suggest a direct pathway from the glial surface to the cerebrospinal fluid.
Our observations suggest that there exists a perivascular space associated with cerebral blood vessels, particularly peripheral arteries, in which fluid flow is highly uninhibited. It appears that this space is discontinuous with the subarachnoid space; however, such a conclusion cannot be definite from our present study (Figure 12). One would expect more general surface distribution of the labelled 5-HT if it had entered the subarachnoid space in appreciable amounts. Since darkening of the emulsion only occurs in the vicinity of major blood vessels in the cleft and along the surface of the brain, the connections between the perivascular spaces and the subarachnoid are absent or minimal in the rat. An alternate possibility is that there is communication between the perivascular spaces and subarachnoid, yet limited distribution of the subarachnoid space. Such a contention is supported by the electron microscopy studies on rat brain meninges by Pease and Schultz (1958) who observe that in some areas there is no separation of arachnoid and pia, there being but a single sheet of overlapping cells without extracellular spaces; and therefore cerebrospinal fluid does not bathe all parts of the cerebral mantle.

We also were unable to observe that the perivascular space surrounding penetrating vessels enhanced access of the labelled 5-HT into the nervous tissue.

Consideration of the perivascular spaces and subsequent enhanced route of fluid flow should be of significance
in analyzing data from injections into the cerebral spinal fluid; i.e. such as studies involving the spread and localization of pathogens injected into the cerebral spinal fluid (Nelson, Blinzinger, and Hermann, 1961) as well as studies involving injections of the catecholamines, 5-HT, and other substances impermeable to the blood brain barrier, and their subsequent uptake and localization (Fuxe and Ungerstedt, 1967; Fuxe and Ungerstedt, 1968; Glowinski, Axelrod, and Iverson, 1966; Aghajanian, et. al., 1966; Aghajanian and Bloom, 1967). Several investigators have observed that interventricularly injected catecholamines and 5-HT penetrate the parenchyma of the brain only in a zone (200-300 µ thick) close to the ventricles and the ventral part of the subarachnoid space (Fuxe, et. al., 1968).

Our results, though incomplete, show 5-HT to be highly associated with the limbic system, which plays a key part in controlling the emotions and the basic drives of fear, hunger, pleasure, and sex. From this standpoint it is interesting and speculative to make comparisons of the anatomical localization of 5-HT with its metabolism and physiology.

It has been observed that when an electrode is placed in the medial forebrain bundle in the brain of a rat, the area considered necessary for maintenance of normal 5-HT brain levels (Heller and Moore, 1968), the animal will ignore all other pursuits and stimulate itself up to 8000 times per hour until exhaustion occurs (Olds, 1962). Olds suggests
that this indicates that the medial forebrain bundle and its connections play a central role in the mediation of reward. Depression on a psychological level is assumed to be a disorder of positive reinforcement or reward function (Stein, 1962). Raising the brain 5-HT level by administering a mono-amine oxidase inhibitor followed by 5-HTP has been used therapeutically to relieve severe depression (Kline and Sacks, 1963). Robie and Flore (1965) postulated that the easily depressed patient has a defective 5-hydroxytryptophan decarboxylase system and that methyl phenidate (Ritalin) facilitates or catalyzes the synthesis of 5-HT from 5-HTP. Bowers (1970) has treated depressed patients with L-tryptophan and vitamin B₆ and found the 5-HIAA level of the cerebrospinal fluid to be unaffected in 8 of 10 patients, indicating these patients were unable to utilize L-tryptophan optimally to form 5-HT.

Since the conversion of 5-HTP to 5-HT occurs with zero order kinetics (Udenfriend, 1959), and the decarboxylase enzyme is nonspecific, acting on DOPA as well as 5-HTP (Bertler and Rosengren, 1959), yet requires pyridoxal phosphate (vitamin B₆) for 5-HTP decarboxylation but not for DOPA decarboxylation (Weissbach, et. al., 1957), I find it interesting and significant that norepinephrine, a product of DOPA, is capable of inhibiting 5-HT formation by forming a schiff-base structure with pyridoxal phosphate (Buzard and Nytch, 1959). Furthermore, the periventricular nucleus of the hypothalamus, rich in norepinephrine fibers (Aghajanian
and Bloom, 1967), is part of the paraventricular system noted in self stimulation studies to be a self-punishment area which opposes the medial forebrain bundle reward system (Olds, 1962).

If the 5-hydroxytryptophan decarboxylase enzyme is defective in the depression syndrome, then DOPA decarboxylase and the subsequent formation of epinephrine and norepinephrine would be effected. This, however, appears not to be the case. Funkenstein (1955) has shown that depressed patients are well capable of elevating their blood epinephrine level under anger-evoking situations. Perhaps more attention should be focused on the availability and role of pyridoxal phosphate in the 5-HTP decarboxylase system.

Smith (1960) has observed that 5-HTP decarboxylation is inhibited in vitro by DOPA, dopamine, and epinephrine, as well as norepinephrine; and that conversely, DOPA decarboxylation is inhibited by 5-HTP but not by 5-HT. The mutual inhibition of 5-HTP and DOPA decarboxylation by mixtures of the two substrates, and the inhibition of the 5-HTP decarboxylation by the catecholamines, if operative in vivo, may produce a mechanism of regulation whereby the ratio of 5-HT and catecholamines is regulated (Erspamer, 1961). This mechanism, if defective or unbalanced, could result in the inducement of mental aberrations, such as depression.

Another interesting point is the observation that 5-HT induces ACTH secretion from the pituitary (Fiore-Donate, Pollice, and Chi eco-Bianchi, 1959). This may play a key
role in the correlation noted between illness and depression. In a psychiatric study done at the University of Rochester School of Medicine on hospital patients suffering from a wide variety of ailments it was found that 97% of the patients, shortly before the onset of their illness, had had a depressing emotional experience such as marital separation, financial setback, parental disownment, loss of a close friend, etc., that left them with a feeling of helplessness or hopelessness (Gibson, 1967). Seventy four percent of the patients developed the initial stage of illness within one week of the emotionally depressing experience, and in 38% the onset occurred within 24 hours. Such a relationship was also observed by Dr. Jack B. Trunnell, M. D., former director of cellular research at the Brigham Young University, in patients who had developed prostatic cancer. He also noted a reciprocal relationship in that viral infections may lead to severe depression. (Personal interview)

Selye (1956) has demonstrated the necessary role of the adrenal cortical hormones in resistance to disease. He has shown that differences in the ratio of pro-inflammatory (mineralcorticoids) to anti-inflammatory (glucocorticoids) can induce pathological states--increased desoxycorticosterone (pro-inflammatory) induces renal disease, hypertension, and arthritis; increased cortisol (anti-inflammatory) may induce tuberculosis. Perhaps the depressed patient renders himself less resistant to disease via inhibition of the pituitary adrenal axis with decreased hypothalamic 5-HT
or decreased stimulation of serotonergic fibers therein. Further studies in this area may prove to be of considerable therapeutic importance.
CONCLUSIONS AND SUMMARY

Attempts were made to inject tritium labelled serotonin (5-hydroxytryptamine, 5-HT) into the cerebrospinal fluid of the lateral ventricle, thereby bypassing the blood brain barrier; and to determine the distribution, uptake, and discrete anatomical localization of injected $^3$H-5-HT in the rat brain.

The injections were made by mistake into a vascular cleft region between caudal thalamus and hippocampus, instead of into the lateral ventricle. The distribution of the injection from the injection site was in association with perivascular spaces, which appear to be discontinuous with the subarachnoid space.

We observed that (1) there exists a cerebral perivascular space, associated especially with larger arteries peripherally located and those in the connective tissue between the subdivisions of the brain, which allows comparatively uninhibited fluid flow as compared to subarachnoid space, (2) $^3$H-5HT penetration of brain tissue parenchyma is restricted, (3) radioactivity is markedly attenuated going away from the surface of access associated with the perivascular spaces of distribution, (4) the injected $^3$H-5-HT is taken up in areas considered to contain true serotonergic fibers, and (5) 5-HT is highly localized in the paleocortex.
We have suggested that the perivascular space observed to have relatively uninhibited fluid flow is an important anatomical feature of the brain of the rat.
LITERATURE CITED


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Fig. 1. Telencephalon, gross view. Frontal lobes overlie the olfactory bulb and tract. X2.

Fig. 2. Diencephalon, gross view. Note hippocampus above the thalamus (see arrows). X2.
Fig. 3. Midbrain-diencephalon, gross view. Note area near the pineal gland (see arrow). X2.

Fig. 4. Midbrain-diencephalon, gross view. Note the injection site (see arrow). X2.
Fig. 5. Telencephalon, frontal lobe overlies olfactory bulb and tract (see arrow). X25.

Fig. 6. High power view of a branch of the anterior cerebral artery in the medial cleft between frontal lobe and olfactory bulb. The radioactivity decreases going away from the artery. X160.
Fig. 7. Diencephalon. The cleft on the injection side between the hippocampus and thalamus (lower arrow). Note the lateral ventricle (upper arrow). X25.

Fig. 8. Membranes within the cleft. Radioactivity can be seen as a shadow beneath the membranes, in lateral thalamus (see arrow). Radioactivity is attenuated going away from the cleft. X63.

Fig. 9. High power view along the cleft. Note the intense radioactivity, higher in thalamus below the cleft membranes, than in hippocampus above. X160.
Fig. 10. Diencephalon. Cleft region on non injection side (lower arrow). Above the fimbria of the hippocampus is the lateral ventricle (upper arrow). X25.

Fig. 11. High power view along the cleft between hippocampus and thalamus on non injection side. Note the reduced radioactivity as compared to the opposing side (Figure 9). X160.
Fig. 12. Ventral diencephalon on injection side. Note the perivascular area around two arteries ventrolaterally, almost completely defined by membranes (see arrow). X25.

Fig. 13. High power view of artery in perivascular space between cerebral cortex with medial amygdala (left arrow), and medial forebrain bundle area (right arrow). Note intense activity especially in the medial forebrain bundle. X160.
Fig. 14. High power view of the larger artery medial to the artery previously shown (Figure 13). Note intense activity in lateral thalamus above and the membranes loosely surrounding the artery (see arrow), X160.

Fig. 15. High power view medial to Figure 10. Note how the membrane defining the perivascular space becomes part of the thinner layer associated with cerebral tissue (see arrow). X160.

Fig. 16. High power view medial to Figure 11. Note how radioactivity decreases in the hypothalamus going away from the perivascular space. X160.
Fig. 17. Ventral diencephalon of non injection side, X25.

Fig. 18. High power view of perivascular space associated with medial forebrain bundle (left arrow), optic tract (middle arrow), and medial amygdala within surrounding cortex (right arrow). Note that activity is decreased over that of the opposing side (Figure 13.). X160.
Fig. 19. Midbrain, ventricle area near the pineal body. Note the cleft membranes laterally, between hippocampus and superior colliculus (see arrows). X25.

Fig. 20. High power view of cleft near ventricle on injection side. X160.

Fig. 21. High power view of cleft near ventricle on non-injection side. Note decreased activity as compared to the opposing side (Figure 20). X160.
Fig. 22. Midbrain, injection site. The middle arrow indicates the needle puncture wound. Note the vascular nature of the widened cleft space between hippocampus above, and superior colliculus (upper arrow) and medial geniculate (lower arrow) below. X25.

Fig. 23. High power view of the puncture wound (see arrow). Note the intense activity in the surrounding tissue. X160.

Fig. 24. High power view of area across cleft space, located below superior colliculus, also showing intense activity. X160.
Fig. 25. Cerebral cortex and superior sagittal sinus (see arrow). Very light radioactivity is observed. X160.
AUTORADIOGRAPHIC STUDIES OF THE DISTRIBUTION OF SEROTONIN IN THE RAT BRAIN

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ABSTRACT

Serotonin (5-hydroxytryptamine, 5-HT), considered to be a synaptic transmitter within the central nervous system, has been shown to be localized mainly in the paleocortex of the brain. Others have shown disturbances of normal 5-HT metabolism to be associated with mental illness and disease.

Attempts to inject tritium labelled 5-HT into cerebrospinal fluid of the rat brain for anatomical localization studies were made. The following observations were noted, (1) there exists a perivascular space in which fluid flows and distribution is relatively uninhibited as compared to subarachnoid space, (2) the labelled 5-HT penetration of brain tissue parenchyma is restricted and radioactivity is attenuated going away from the surface of access, and (3) the injected labelled 5-HT is absorbed into areas believed to contain true serotonergic fibers, especially throughout the paleocortex.

Suggestions concerning the anatomical importance of the perivascular space observed are made. Also comparisons of 5-HT brain localization with metabolism and physiology of 5-HT are made in relation to mental illness.