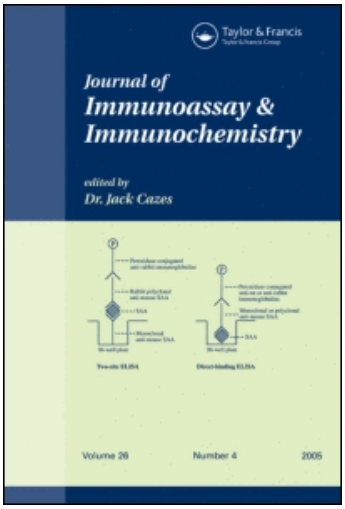

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Tissue Immunoassay for ^{19}F-Tagged 5-Hydroxytryptophan

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ABSTRACT

A new tool for magnetic resonance, L-6-heptafluorobutyryl-5-hydroxytryptophan, was synthesized and investigated using an antibody to perfluoroalkyl moieties developed previously. To be useful as an imaging agent, the compound must cross the blood brain barrier and then be concentrated in vesicles in serotonergic neurons in order to accumulate in sufficient quantity for in vivo detection to be possible. The novel imaging compound was administered in ova to domestic chicks (*Gallus domestics*) to investigate the bioavailability and uptake dynamics of the compound in this model organism. Typical immunoassay methods were ineffective, so a new technique was developed which binds amines

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and amino acids to the walls of acid-functionalized cuvettes. The first study established the presence of higher quantities of the tags in neural and liver tissue than in heart tissue. A second study investigated regional differences, with the midbrain containing more tagged compounds than the frontal lobe sample, and the frontal lobe sample containing more than the occipital or cerebellum samples. These studies demonstrate that the compound follows the pathway of endogenous serotonin. A third study investigated uptake dynamics of the novel compound. Maximum concentration of the tagged molecule in the brain was achieved three days after injecting Incubation Day 14 eggs, suggesting that it bioaccumulates in vivo. This new immunoassay technique used to detect the novel compound in tissue samples demonstrated good repeatability.

Key Words: Serotonin; MRI; Bioaccumulation; Immunoassay.

INTRODUCTION

The activity of neurotransmitters is of primary importance to the study of the brain, but reliable in vivo detection is difficult. Many of the more elegant quantitative methods for studying neural pathways sacrifice the organism to obtain tissue samples, making it impossible to study that subject again. This is especially disadvantageous in developmental studies. The neurotransmitter serotonin plays a major role during brain development and, afterwards, functions in important roles in brain functioning. The use of the Blood Oxygen Level Dependant method in magnetic resonance imaging is problematic because serotonin modulates blood vessel tone and may cause vasoconstriction. Although blood platelet serotonin is often used as a peripheral correlate of brain serotonin, this approach may be fundamentally flawed given the results of a recent positron emission tomography study.[1]

In order to investigate the role of serotonin and the development of serotonergic pathways, the precursor to serotonin, 5-hydroxytryptophan (5-HTP), has been tagged with a heptafluorobutyryl moiety for magnetic resonance spectroscopy and functional imaging. Before advancing to MRI spectroscopy, the bioavailability and bioactivity of the novel compound were assessed in order to determine if this potential imaging agent would be relatively nontoxic and follow the pathways of the endogenous precursor. Since the research reported here, synthesis of a nonafluoro-*t*-butyl tag has been accomplished. The new tag has all of the fluorines at the same chemical shift for better ^{19}F imaging.

Enzymatic modifications made to 5-hydroxytryptophan are accomplished at the opposite end of the molecule from the tag, in the region of the amino acid moiety. The tag itself is coupled to the indole ring of the molecule. The immunoassay described in this study will not differentiate between the tagged 5-HTP and its metabolites, which will respond similarly to the anti-perfluoro antibody.

Serotonin does not cross the blood brain barrier (BBB) in mammals. It is normally synthesized in the brain from tryptophan; an albumin bound amino acid that crosses the BBB by both active and passive transport. In the serotonergic neurons, tryptophan is rapidly converted to 5-hydroxytryptophan (5-HTP) by hydroxylation and is then converted to serotonin (5-HT). Most of the neurotransmitter is concentrated in vesicles until it is released to bind with receptors on post-synaptic neurons. The uptake of L-tryptophan in isolated rat brain synaptosomes had been assayed. The concentration ratio across the plasma membrane reached 9:1. Tagging the immediate precursor of serotonin allows us to bypass the rate-limiting step in the synthesis of serotonin, which is the conversion of tryptophan into 5-HTP. 5-HTP crosses the BBB, but, unlike tryptophan, it cannot be shunted into niacin or protein production.

To explore the uptake and storage of this novel imaging compound in vivo, we selected in ova chicks as a model organism for the following reasons:

(1) their synthetic pathway for serotonin is similar to that of mammals,
(2) their BBB is permeable to serotonin early in development,
(3) the distribution and development of their serotonergic system have been studied,
(4) they develop in only 21 days,
(5) their brains are more accessible to imaging during development than in utero mammals, and
(6) they are not considered vertebrate animals under government regulations before hatching.[2–4]

Taken together, these factors recommend the use of this species for rudimentary in vivo toxicity and bioavailability screening which accords with Congressional intent to ''replace, reduce, refine'' the use of animals in research.

Serotonin in the developing chick brain increases in a linear manner from Incubation Day 6 until it reaches a maximum at one week posthatching.[5,6] Serotonergic neurons are first observed in this species

on Incubation Day 4, and by Incubation Day 8 virtually all the raphe nuclei serotonergic neurons found in the adult animal have appeared. These neurons appear to be fully developed by Incubation Day 16. Terminal arborization of the fibers starts about Incubation Day 16, and is maximized within one week of hatching. The pattern of serotonin neuron development, including migration and cell differentiation, is similar for chickens and mammals.

Four goals were set for the research reported here. First, the novel immunoassay developed for this project must be shown to be able to differentiate between tagged and untagged 5-HTP. Second, the distribution of the tags in various organs was assessed. Third, the distribution of the tags between regions of the brain was investigated. Serotonin varies by brain region and it was important to determine whether the novel compound is localized in regions of the brain with high levels of it.[7] Fourth, the time course of the bioaccumulation of the imaging compound was measured, as this will be useful in planning MR studies.

EXPERIMENTAL

Materials

Unless otherwise specified, all chemicals are available from Sigma-Aldrich. Most reagents do not need to be of special purity. ACS-grade chemicals are recommended.

The anti-perfluoroalkyl antibodies were developed under an Air Force STTR by the predecessor of Inovatia Laboratories, LLC, Fayette Environmental Services, Inc.[8,9] The cell lines are in liquid nitrogen storage at the Cell & Immunology Core Facility of the University of Missouri–Columbia. Vials of sera from the last bulk-up operation in 1999 are in cold storage at Inovatia Laboratories, LLC. These IgM antibodies must be pre-treated according to the procedure given in the Methods section.

The L-6-heptafluorobutyryl-5-hydroxytryptophan (PF-5-HTP) was synthesized by Inovatia Laboratories, LLC, for this and related research. Related compounds (L-6-(2-(nonafluoro-*t*-butoxy)acetyl-5-hydroxytryptophan, L-5-(2-(nonafluoro-*t*-butoxy)acetyl-DOPA, and 5-(2-(perfluoro-*t*-butoxy)acetyl-dopamine) have been synthesized recently to provide better MR signals, but antibodies do not yet exist for these tags. Each compound is about 80% pure, with the remainder being an isomer of the major project. For the 5-HTP, the 7-isomer is the minor product. For the DOPA and dopamine, the 6-isomers are the minor products.

Methods

Administration of Tagged Compounds to Eggs

The bioavailability of tagged 5-HTP (PF-5-HTP) was investigated by injecting eggs containing live *Gallus domesticus* with 5 μg PF-5-HTP or 5 μg 5-HTP in a vehicle of 100 μL of dilute phosphate buffered saline (PBS). Control eggs were injected with just PBS. The buffer was diluted by a factor of 10 from the customary phosphate buffered saline solution (0.016 mol/L phosphate, 0.9% sodium chloride) because the high salt content appeared to cause damage to developing chicks in a prior study. Various tissues were then assayed for the presence of the novel compound using antibodies to the perfluoroalkyl moiety in a custom immunoassay. Compounds were administered by drilling a 1 mm diameter opening in the egg shell over the air space using a Dremel drill. Injections were administered with sterile, 0.5 cc, disposable insulin syringes. In studies other than that investigating the time course of the compound, two to five days after injections, on Incubation Day 19, chicks were quickly removed from their eggs, observed briefly for gross abnormalities and immediately sacrificed with a lethal injection of diazepam. Tissues used for analyses were promptly removed and placed on a drop of ice-cold perchloric acid solution for processing.

Preparing Homogenate

Tissues samples were placed into test tubes containing about 2.0 mL perchloric acid solution (0.1 mol/L with 1.0 g/L ascorbic acid and 0.15 g/L cysteine hydrochloride as antioxidants). An Omni PCR Tissue Homogenizer with disposable tips was used at 30,000 rpm to reduce the samples to particles of 2 to 5 μm. The homogenate was briefly stored in an ice bath prior to centrifugation (1380 g, 8 min). After centrifugation the supernatant was removed with a sterile disposable pipette into another test tube and centrifuged for an additional 12 min. The final samples had a volume of about 1.5 mL of fluid. The pellets were discarded. Samples for the first three studies were shipped on dry ice to Inovatia Laboratories, LLC, where the immunoassays were performed. The time course study was conducted at Marist College using antibodies provided by Inovatia.

Immunoassay for Tagged 5-HTP and Metabolites

The immunoassays were performed in modified 4.5 mL methylmethacrylate spectrometer cuvettes using a modified anti-perfluoroalkyl IgM antibody. Inovatia Laboratories, LLC, developed both the method and the antibody. The modification methods for the cuvette and IgM are given below in addition to the immunoassay method.

Anti-perfluoroalkyl IgM Treatment

The contents of one vial of IgM concentrate (FES PFC412) is added to 15 mL Tris buffer (727 mg *tris*(hydroxymethyl)aminomethane in 15 mL distilled water, adjusted to pH 8 with 1 N HCl) with stirring. To this, is added 1 mL of fresh 2-mercaptoethylamine solution in distilled water (2-MEA, 1.2 mg/mL). This is diluted to 20 mL with distilled water and stirred, covered, for 1 hour at room temperature. This causes the IgM to break into IgG-like fragments by the action of the 2-MEA on the disulfide bonds of the IgM.

After the 1 hour reaction time, the solution is transferred to 14,000 MWCO dialysis tubing (6.4 mL/cm) and dialyzed in a covered 250 mL beaker in 200 mL PBS/Tween (phosphate buffered saline solution, pH 7.2, with 0.5 mL Tween 20 per liter). This is stirred at room temperature 4 h with two changes of PBS/Tween. The entire beaker and contents are then stored in a refrigerator ($\sim 4^{\circ}$C) overnight. During this time, the small molecules, including unreacted 2-MEA and various wastes from the cell culturing process, are removed from the antibody concentrate.

The following day, the contents of the 14,000 MWCO tubing are transferred to 300,000 MWCO tubing (0.32 mL/cm). This tube, appropriately folded, is placed into a 100 mL graduated cylinder covered with parafilm containing approximately 75 mL PBS/Tween. This also is placed in a refrigerator overnight. The larger MWCO tubing allows the fragments to diffuse into the cylinder, while the unreacted IgM and the large core ring of reacted IgM (which has no antibody functionality) remain in the tubing.

On the following day, the liquid is squeezed slowly from the dialysis tubing into the graduated cylinder, forcing the remainder of the IgG-like fragments into the graduated cylinder. When the tubing contains less than 2 mL, it may be discarded. The solution remaining in the graduated cylinder is diluted to 100 mL with PBS/Tween and transferred to a screwtop polypropylene bottle. The concentration of IgG-like antibodies, assuming 75% recovery, is about 30 ng/mL.

Modification of Methylmethacrylate Cuvettes

Methylmethacrylate spectrometer cuvettes (4.5 mL) were submerged in sodium hydroxide solution (2.5 mol/L) in a large beaker with a large stir bar. The solution was heated to 70°C overnight. Then, the cuvettes were rinsed thoroughly with distilled water. This process hydrolyzes the methyl esters on the surfaces of the cuvettes to produce acid groups. The heat is controlled and stirring is maintained in order to prevent deformation of the cuvettes.

Immunoassay for Perfluoroalkyl Tags

Each set of samples should include a negative control and a positive control. The negative control is a cuvette in which the sample consists of phosphate buffer saline at pH 4.5. The positive control is made by adding 2 μg of a perfluoroalkyl-tagged 5-HTP to phosphate buffered saline at pH 4.5. A good practice is to use 2 or 3 replicates of the positive and negative controls.

Up to 1.4 mL of digested and centrifuged sample is added to a cuvette. If the sample volume were more than 1.4 mL, the remainder is retained for later treatment in the same cuvette. The pH of samples and controls is adjusted to a value between 5.5 and 6.0 using 0.25 mol/L sodium hydroxide solution. The final volume is adjusted to 2.0 mL with morpholino ethane sulfonic acid buffer (MES, 0.05 mol/L, pH 5.5, prepared from 9.76 g MES in 1 L of water, adjusted to pH 5.5 with 1.0 mol/L HCl). The volume may be slightly higher if good stirring can be maintained. Since the cuvettes are too light and top-heavy to stand alone while being stirred (7 mm × 2 mm magnetic stir bars), the cuvettes are packed into a beaker of a convenient size. The stir bar must cause good stirring to the top of the liquid. The height of the liquid in the cuvette should be marked on the outside of the cuvette so that the heights of the solutions using in the subsequent steps can be verified easily in order to ensure that all of the surface exposed to the sample is also exposed to the antibodies.

To the stirred cuvettes, add a solution of *N*-hydroxysuccinamide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (NHS/EDAC, 100 μL, prepared from 50 mg/mL NHS and 167 mg/mL EDAC in MES buffer). The shelf-life of this solution is less than 4 h, so it must be prepared fresh for each batch of samples. Stirring is continued for 8 h. Then another 100 μL NHS/EDAC solution is added and stirring continues for another 8 h. This process promotes the coupling of amines and acids into amides.

The acid groups on the walls of the cuvettes act as anchoring sites for the resulting amino acid chains. Amines in the solution act as terminators to chain growth. Therefore, the ratio of amines to amino acids will dictate mean chain length.

After the coupling reaction, the cuvettes are rinsed once with distilled water and once with phosphate buffered saline (PBS, pH 7.2) without Tween 20. Tween would interfere with the blocking of the plastic surface in the next step. If the immunoassay is not to be performed immediately, the cuvettes may be refilled with PBS 7.2 and covered.

If the initial sample volume was greater than 1.4 mL, the remaining sample may be reacted in the same cuvette as the first portion of the sample. The amines and amino acids conjugated to the cuvette walls in the first reaction will not be harmed by a second reaction. So, using the same cuvette, the remainder of the sample is added to the same cuvette and reacted in the same way as the first portion of the sample.

After the analyte is bound to the surfaces through amide linkages, blocking solution (rabbit gamma globulin at 2 g/L in PBS 7.2, shelf life 90 days at 4°C.) is added above the mark made previously at the top of the sample solution (~2.5 mL). The level of this solution also is marked so that subsequent solutions may be added between the two marks via squeeze bottle rather than by repeating pipette. Stir bars are not placed inside the cuvettes as they would scrape blocking from the bottom of the cuvette. Instead, several small stir bars (15 mm × 8 mm) are placed in the gaps between the cuvettes and the sides of the round beaker. The stir plate will cause these stir bars to knock against the cuvettes, imparting enough motion to keep the solution stirred. Stirring is continued for 90 min. Then, the cuvettes are rinsed twice with PBS (pH 7.2) and once with PBS/Tween (pH 7.2). Using Tween for all rinses or rinsing more than three times removes too much blocking.

To the blocked cuvettes is added serum solution (treated IgM solution to which has been added rabbit gamma globulin at the rate of 1 g/L; shelf life 90 days at 4°C.) to a level between the two marks. This ensures that all regions exposed to sample are also exposed to antibodies. But, antibodies are not exposed to regions without blocking that would result in excessive random adsorption. The extra rabbit gamma globulin is used to re-block regions that may have become uncovered, in order to reduce random adsorption. This solution is stirred 60 min in the same manner as for the blocking, using stir bars on the outsides of the cuvettes. After this incubation time, the cuvettes are rinsed three times with PBS/Tween.

After the primary antibodies are conjugated to the analytes, the secondary antibody solution (goat anti-mouse-IgM IgG-HRP, 1 mg/L, plus rabbit gamma globulin, 1 g/L, in PBS/Tween, shelf life 90 days at 4°C) is added to a height between the two lines on the cuvette. This is stirred 60 min in the same manner as for the blocking and primary antibody. After incubation, the cuvettes are rinsed five times with PBS/Tween. Then PBS (no Tween, pH 4.5) is added and allowed to stand 5 to 10 min.

After emptying the cuvette, substrate solution (2 mL, sodium citrate dihydrate, 14.7 g/L; urea hydrogen peroxide, 0.4 g/L; and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), 0.5 g/L; added sequentially to PBS 4.5 with stirring; shelf life 10 days at 4°C) is added to each cuvette. The pH greatly affects the velocity of color development, so the final solution should be adjusted to pH 4.5. The solutions are stirred externally 60 min by the method used for the blocking and antibody solutions. After this period of color development, the solutions are transferred to new, untreated spectrometer cuvettes in order to read the absorbances at 415 nm. Reduced volume cuvettes may be used. The structural material of the final cuvette may be selected for convenience.

Repeating the Immunoassay

If desired, the immunoassay may be repeated in the same cuvettes with the same samples by removing the proteins (antibodies and enzymes) that remain from the first immunoassay. PBS (pH 3, 3 mL) is added to each cuvette. The cuvettes are covered and shaken, then allowed to stand 15 min. Finally, the cuvettes are shakes again briefly, then rinsed sequentially with PBS/Tw, PBS (pH 7.2), and distilled water. The cuvette may then be re-analyzed starting from the blocking step.

Detecting Tagged 5-HTP vs. Untagged 5-HTP

A double blind pilot study tested whether the assay could discriminate between tagged 5-HTP and 5-HTP in samples prepared from whole brain tissues. Three eggs were injected with tagged 5-HTP (5 μg) and three with 5-HTP (5 μg), both in dilute PBS (100 μL), on Incubation Day 17. Neural tissue samples were prepared on Incubation Day 19.

Distribution of Tagged 5-HTP Among Organs

Four eggs received tagged 5-HTP (5 μg) in dilute PBS (100 μL) on Incubation Day 14 and again on Incubation Day 16. Three eggs received dilute PBS (100 μL) on the same days. A reference sample was created by adding tagged 5-HTP to brain tissue recovered from one of the three eggs that had received only PBS. Tissue samples were prepared on Incubation Day 19.

Distribution of Tagged 5-HTP Among Brain Regions

Eggs received tagged 5-HTP (5 μg) in dilute PBS (100 μL) on Incubation Day 14. Before sample preparation, brains regions were identified and separated as occipital lobe, cerebellum, frontal lobe, and midbrain. Due to the very small size of midbrains, equivalent amounts of tissue were compared from various brain regions. For example, an occipital lobe, a right frontal lobe, or the cerebellum were compared to a pooled sample of three midbrains. The midbrain consisted of about 250 μL of tissue.

Uptake Time Study

On Incubation Day 14, twenty-four eggs were injected with tagged 5-HTP (5 μg) in dilute phosphate buffered saline (100 μL). At 24-hour intervals following the injection, six developing chicks that had received tagged 5-HTP were removed from their shells and immediately sacrificed for immunoassay. Tissues from three midbrains were pooled to create a single sample, so each day provided two samples. The results of the immunoassays were averaged. A liver sample was taken randomly from a single chick on each day.

Repeating an Immunoassay in the Same Cuvette

Ten immunoassays were repeated using the same cuvettes as used in the first immunoassay. The cuvettes were treated with a high-salt solution to remove the previous antibodies. New antibody and substrate solutions were prepared. The results of the second immunoassay were compared to those of the first on a matched-pair basis.

RESULTS

Detecting Tagged 5-HTP vs. Untagged 5-HTP

The three tagged 5-HTP samples tested positive with an average absorbance of 0.346 ($\sigma = 0.020$). The average absorbance for the 5-HTP samples was only 0.186 ($\sigma = 0.049$). The *t*-statistic for the difference of these means yielded $\alpha = 0.0033$. The immunoassay was shown to be capable of detecting gross differences between injection conditions.

Distribution of Tagged 5-HTP Among Organs

The samples were processed on two different days, so separate controls were needed. The first set used a spiked tissue sample as the positive control and the pooled brain tissues from two eggs injected with PBS as the negative control. The second set used a reagent blank (no tissue) as the negative control and a spiked cuvette (2 μg tagged 5-HTP, no tissue) as the positive control.

The first set consisted of samples from five eggs. Three of the eggs were controls, having received PBS only. From these, the brain tissues from two eggs were pooled as one sample and used as the negative control. The brain tissue from the third control was spiked with tagged 5-HTP (5 μg) after harvesting, as the positive control. These were compared to the combined livers of the two eggs that had received tagged 5-HTP, the combined heart tissues of the same two eggs, and the individual brain tissues from the same two eggs.

Defining the spiked sample as positive and the pooled brain tissue from controls as negative, the absorbances were grouped as positive or negative. The only positive samples were the spiked sample and the pooled livers from the two eggs that had received tagged 5-HTP. The average absorbance was 0.295 ($\sigma = 0.010$). The negative group consisted of the pooled brain tissues from the two control eggs, the pooled heart tissues from eggs that had been exposed to tagged 5-HTP, and the individual brain tissues from eggs that had been exposed to tagged 5-HTP. The average absorbance was 0.230 ($\sigma = 0.022$). The *t*-statistic for the difference of means yielded $\alpha = 0.009$. The results are illustrated in Fig. 1. If the tagged molecule was simply circulating in the blood system of the developing chick, it might appear in organs with considerable blood flow in them. The tag would also be expected to accumulate in cord and liver samples, considering the route of administration of the

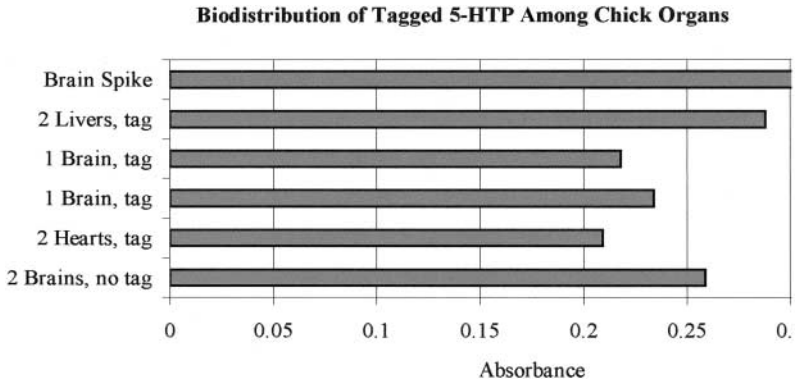

Figure 1. Only the absorbances of the pooled tissue from two tagged livers and the spiked brain tissue exceeded the absorbance of the pooled brains from chicks that had not received the tag.

compound into the egg, and hence through the cord, and the function of the liver. We thus compared these tissues to another with considerable blood content, the heart, in order to investigate whether the tag being detected with the immunoassay was in blood or tissue compartments of the samples. These samples demonstrated a threshold effect for the immunoassay. The results are illustrated in Fig. 1.

The second set of samples was taken from four eggs: the liver tissues of two PBS-only eggs, the liver tissues of two tagged 5-HTP eggs, the brain tissues from the same tagged 5-HTP eggs, and the cord tissues from the same tagged 5-HTP eggs. The absorbances of the negative controls averaged 0.148 ($\sigma = 0.050$). The two control spikes (2 μg tagged 5-HTP without tissue) had an average absorbance of 0.219 ($\sigma = 0.003$). The *t*-statistic for the difference of means for the negative and positive controls was $\alpha = 0.091$. The brain, liver, and cord tissue samples gave an average absorbance of 0.464 ($\sigma = 0.119$). The *t*-statistic between these samples and the negative controls was $\alpha = 0.021$. The t-statistic between these samples and the two positive controls was $\alpha = 0.035$. The absorbance from the PBS-only liver tissue was 0.098, exactly 1σ below the mean of the negative controls. The results are illustrated in Fig. 2.

Distribution of Tagged 5-HTP Among Brain Regions

The samples harvested according to brain region were analyzed in the same batch as the second set of organ comparisons, so the same positive

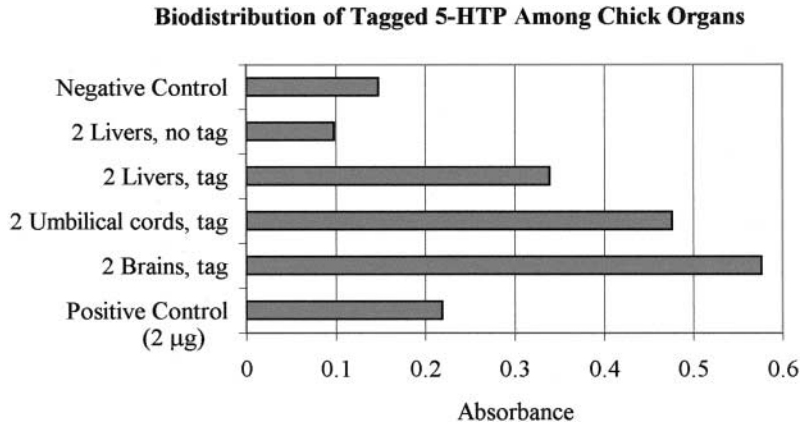

Figure 2. Tags were detectable in liver, cord, and brain tissue from exposed chicks. Liver tissue from unexposed chicks gave no response.

and negative controls were used: 0.219 ($\sigma = 0.003$) and 0.148 ($\sigma = 0.050$). The brain tissue portions were pooled as necessary to provide equivalent tissue per sample. The absorbances were 0.116 (occipital), 0.146 (cerebellum), 0.192 (frontal), and 0.316 (mid-brain). If the controls are treated as a linear regression, the quantities found in occipital lobe and cerebellum would be not detectable (less than the negative control value). The frontal lobe sample absorbance would correspond to 0.249 μg ($\sigma = 0.224$ μg). The mid-brain sample absorbance would correspond to 0.943 μg ($\sigma = 0.431$) per midbrain. The results are illustrated in Fig. 3. The tagged molecule is accumulating in regions of the brain known to have numerous serotonin varicosities, which provides strong support that this novel molecule is being taken up into and stored in vesicles.

Uptake Time Study

The amount of tag detected in midbrains of chicks injected on Incubation Day 14 rises over three days before declining, indicating substantial bioaccumulation in vivo. Results of this study are shown in Fig. 4. Although the organs were harvested on consecutive days, the immunoassays were performed together so the absorbances would be comparable. Between harvesting and analysis, the digested and preserved samples were stored at -10°C.

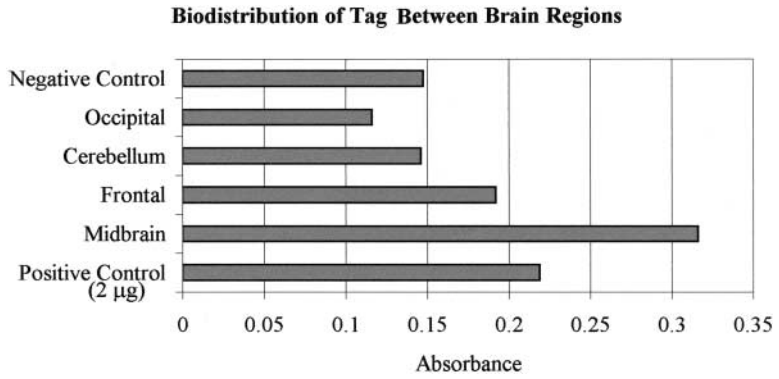

Figure 3. The tag was most detectable in the midbrain region. Only the absorbances from the midbrain and frontal lobe samples exceeded that of the negative control.

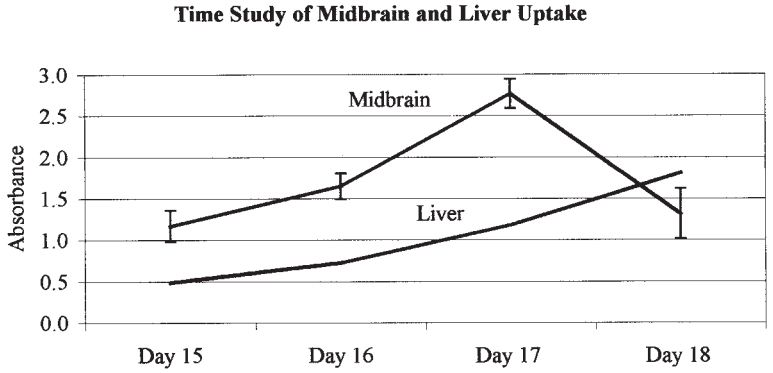

Figure 4. The liver and midbrain showed different uptake rates. Each vertical bar represents the standard deviation between two samples composed of three midbrains each.

Repeating an Immunoassay in the Same Cuvette

The absorbances obtained from the first and second immunoassays in the same cuvettes are shown in Fig. 5. A linear regression of these data produced a slope of 1.38 ($\sigma = 0.13$ or 9%). The intercept was -0.035. If this relationship were used as a calibration curve, a value at the middle of the curve would yield a predicted value having a relative standard deviation of only 7%.

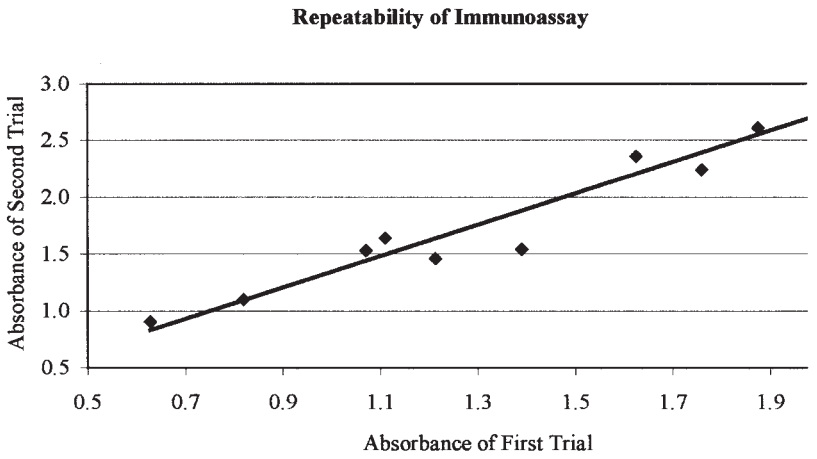

Figure 5. Immunoassays could be repeated in the same cuvettes, allowing replicates and the opportunity to overcome poor results that were a result of a bad batch of immunoassay reagent.

DISCUSSION

The larger objective into which this research feeds is the development of imaging agents for magnetic resonance to investigate the development of specific neural pathways and events related to the synthesis, release and re-uptake of neurotransmitters in vivo. The ability to investigate neurotransmitters using magnetic resonance methods, concurrent with functional and anatomic methods, would vastly enhance the arsenal of research tools for studying brain development and dysfunction in psychiatric disorders. This noninvasive approach would make it feasible to repeatedly image neural development in the same organism over time, employing such subjects in a variety of behavioral paradigms and as models of epigenetic disorders. This novel use of magnetic resonance technology is feasible if neurotransmitters, or their precursors, can be tagged with a sufficient number of fluorine atoms to provide adequate signal-to-noise for in vivo assessment.

The magnetic resonance of ^{19}F is about 83% of that of ^{1}H, making it a feasible imaging agent if a sufficient quantity of tagged compound is taken up into neurons. More importantly, compounds tagged with fluorocarbon moieties can be uniquely monitored, since very few organic compounds normally present in living tissues contain them. Further, trifluoromethyl moieties have been found to move more readily through cell membranes due to increased lipophilicity, so transport across the cell

membrane is generally enhanced, a characteristic exploited by several drugs.[10] A perfluoroalkyl tag was chosen for these advantages.

To simplify the task of synthesizing tagged L-5-HTP, heptafluorobutyric chloride (Aldrich) was chosen as the tag. The chirality of the L-5-HTP was preserved by, first, purchasing pure L-5-HTP, and then maintaining that chirality by carefully protecting the amine and acid groups during the synthesis. This allowed the bioavailability of modified 5-HTP to be assessed early in the research process, demonstrating that the target position on the 5-HTP, the 6-position, would not interfere with normal enzymatic processes, and that such substitutions were not overtly toxic in developing organisms. Further, an antibody to perfluoroalkyl moieties already existed and was available for detecting compounds in tissues. The first drawback of this *n*-alkane is that its fluorines are in three distinct chemical shifts, resulting in a somewhat diffuse signal in ^{19}F MRS. Second, it has only seven fluorine atoms per molecule; more fluorine atoms would yield a stronger signal. Synthesis of the new tag, 2-(nonafluoro-*t*-butoxy)acetyl chloride has been completed by Inovatia Laboratories, LLC, since the experiments reported here. The number of fluorine atoms has been increased from seven to nine, and the chemical shifts of the nine fluorine atoms are identical. The new tag has been attached successfully to L-5-HTP, L-DOPA, and dopamine.

In research reported previously, the bioavailability of the L-6-(heptafluorobutyryl)-5-HTP was investigated using a crayfish dominance paradigm.[11] The tagged compound mimicked the effect of untagged L-5-HTP on the aggressive behavior of subordinates. Crayfish were chosen for behavioral pharmacological studies of the compounds because they are not vertebrates, they are inexpensive, and they have readily observable behaviors that can be manipulated by administering monoamines.

In this research, an efficient and inexpensive model for determining biodistribution was developed in order to investigate whether the tagged compounds bioaccumulate in serotonergic neurons where they could be detected with magnetic resonance. The results of these studies using in ova chicks provide strong support for bioaccumlation. When eggs were injected with a single dose of 5 μg of tagged 5-HTP, between 0.5 μg and 1 μg of tagged molecules was detected in each 250 μL midbrain sample.

Estimates of concentrations of serotonin in brain tissue in the literature suggest that the quantity of tagged neurotransmitter found in the midbrain via immunoassay were reasonable values. Estimates of serotonergic varicosities in neural tissues range from 2.7×10^6 varicosities/μL to 7.1×10^6 varicosities/μL.[12,13] The mass of serotonin

per varicosity has been estimated to be between 4.5×10^{-17} and 7.0×10^{-17} g.[14,15] Using a molar mass of 176 g/mol for serotonin and the preceding values, the 5-HT concentration in the midbrain should fall between 6.9×10^{-7} and 2.8×10^{-6} mol/L. The molar mass of tagged 5-HTP is 416.25 g/mol, so the 0.5 μg to 1 μg of tagged neurotransmitter found in the 250 μL midbrain corresponded to 4.8×10^{-6} to 9.6×10^{-6} mol/L of tagged neurotransmitter consisting of 5-HTP, 5-HT, and 5-hydroxyindoleacetic acid (5-HIAA). Since 5-HTP is converted to 5-HT rapidly, the tags probably are associated with 5-HT and 5-HIAA. So, using the ratio of 1:1.4 for those compounds, the tagged 5-HT concentration may be estimated to be 2.0×10^{-6} mol/L to 4.0×10^{-6} mol/L, indicating that nearly all of the neurotransmitter in the serotonin pathway in the midbrains of the chicks was tagged, suggesting that the tagged compounds eventually replaced the endogenous ones.[16] These values may be due to the high lipophilicity of the tag, causing the tagged 5-HIAA to be cleared more slowly than untagged 5-HIAA.

Since each tag contained seven fluorine atoms, the 0.5 to 1 μg of tag found in the midbrain would represent a fluorine concentration of 3.4×10^{-5} to 6.7×10^{-5} mol/L. In a 2 mm voxel (8 μL), the quantity of fluorine with the seven atom tag would be 2.7×10^{-10} to 5.4×10^{-10} mol. The new tag has nine fluorine atoms. The quantity of fluorine in 8 μL of midbrain tissue (a 2 mm voxel) would be 3.5×10^{-10} to 6.9×10^{-10} mol, if the molecule with the improved tag bioaccumulates in the same manner as the tag investigated in this study. If the fluorine concentration is not high enough, the voxel size can be increased. A 4 mm voxel would contain between 2.8×10^{-9} and 5.52×10^{-9} mol of the new tag.

In addition to advances toward the ultimate goal of imaging neurodevelopment and activity, these studies have validated a novel immunoassay. Although the replicates inherent in microtiterplate formats were sacrificed in order to make the immunoassay work, the technique was shown to be reproducible via the novel approach of repeating the immunoassay on the same surfaces. The amino acids and amines are covalently bound to the walls of the cuvettes, while the proteins (antibodies, enzymes, and blocking) are only adsorbed. The adsorbed proteins can be removed reasonably easily, whereas the analytes can survive all but harsh chemical conditions or physical abrasion. The slope of the matched pair data showed that fresh substrate solution yields a higher response. The negative intercept showed that the fresh antibody solution resulted in a higher random adsorption. Both effects are intuitive for immunoassays. Since this assay was designed

for an application in which a large number of samples may not be possible, the ability to repeat an assay on the same tissue extract could be very valuable.

Biodistribution between types of tissue (cardiac, liver, and neural) and different regions of the brain have been confirmed. As would be expected, the highest concentrations of tagged 5-HTP were found in samples from the midbrain regions of developing chicks. A detection limit for the immunoassay was demonstrated to be about 1 μg. Since single midbrains are quite small and contained only 0.5 to 1 μg of tagged 5-HTP, midbrains were pooled to create a sample that could exceed the threshold of detection in this immunoassay. When these pooled samples were compared with samples from similar amounts of tissue from other regions of the brain, the midbrains contained more tag.

Compounds injected into the air space of an egg must diffuse across more biological membranes than those injected i.p. into a mammal. Once having entered the circulatory system of the developing chick, the compound must still cross the blood brain barrier and be concentrated into vesicles in serotonergic neurons in sufficient quantity to be useful as an imaging agent. The immunoassay revealed the time course of the uptake of the tagged 5-HTP into the neural tissues of the chick. Maximum concentration was found on the third day after administration. Fluorine has excellent MR properties.[17,18] Taken together these studies provide strong support for the feasibility of using multiple-fluorine-atom tags to the precursors of neurotransmitters for magnetic resonance spectroscopy and imaging.

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