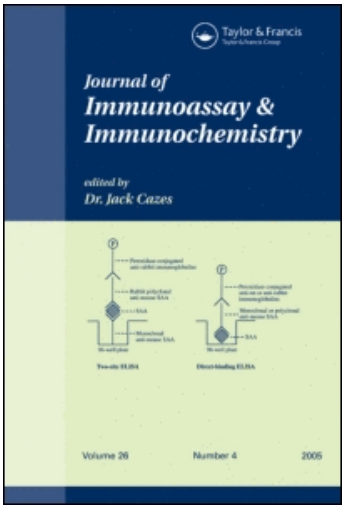

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The Fate of Perfluoro-Tagged Metabolites of L-DOPA in Mice Brains

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ABSTRACT

A novel compound for use in magnetic resonance (MR) imaging was created by covalently bonding multiple ^{19}F atom tags to L-DOPA. Tagging L-DOPA permits bypassing the rate-limiting factor in the biosynthesis of dopamine (DA), the conversion of tyrosine into L-DOPA. The next step in the biosynthetic pathway, the removal of the carboxyl group on the molecule by the enzyme L-aromatic acid decarboxylase (AADC), happens rapidly after L-DOPA is taken up into neurons. In order to be useful as a tool in MR imaging, the novel compound and/or its perfluoro-tagged metabolites must accumulate in vesicles in dopaminergic neurons. We administered L-DOPA with a nine ^{19}F atom tag

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(250 mg/kg) to mice pups, waited 1.5 or 3 hr, and used high pressure liquid chromatography (HPLC) to examine neural tissue samples for tagged L-DOPA and tagged DA. The isomer of L-DOPA with the tag bonded at the 5 position yielded the highest conversion to tagged DA at 1.5 hr after an i.p. injection. This study provides the first direct evidence that L-DOPA, tagged with nine fluorine atoms, is taken up into mammalian brain dopaminergic neurons where it is converted to perfluro-tagged DA. The use of these tagged compounds may make it feasible to investigate the uptake and conversion of important neurotransmitter in vivo with fluorine imaging.

Key Words: Dopamine; DOPA; HPLC; Neurotransmitter; Fluorine imaging; Mice.

INTRODUCTION

A novel compound for use in magnetic resonance (MR) imaging was created by covalently bonding multiple ^{19}F atom tags to L-DOPA. Tagging L-DOPA permits bypassing the rate-limiting factor in the biosynthesis of dopamine (DA), the conversion of tyrosine into L-DOPA. The next step in the biosynthetic pathway is the removal of the carboxyl group on the molecule by the enzyme L-aromatic acid decarboxylase (AADC). Using an enzymatic assay of crude lysate from *E.coli* transformed with the plasmid pKKAADCII, we have demonstrated that the 5-isomer of tagged L-DOPA converts into tagged DA in vitro.[1,2] In order to be useful in MR, the novel compound must accumulate in vesicles in dopaminergic neurons.

Amine neuron transporters utilize ion gradients as the driving force to bring molecules across the plasma membrane to achieve intracellular accumulation of neurotransmitters and their precursors. The transporter for dopamine, (DAT) exhibits ion dependence with an apparent stoichiometry of transport of $2Na^+ : 1Cl^- : 1DA$, suggesting that DA transport is an electrogenic process. Electrophysiological analyses have directly confirmed the electrogenic nature of DAT activity and demonstrated an increase in DAT velocity with hyperpolarization.[3,4] The third transmembrane domain in DAT appears to be crucial for DA uptake.[5] The vesicular monoamine transporter (VMAT) is used for all biogenic amines, concentrating DA into vesicles by catalyzing a H+/monoamine antiport.[6]

AADC accomplishes decarboxylation of DA, as well as serotonin. Huang and colleagues investigated stereo (D and L), geometrical (E and Z), and regional specific (2-, 4-, and 6-[^{18}F] fluoro) analogs of beta-fluoromethylene-*m*-tyrosine using positron emission tomography (PET). Transportation through the blood brain barrier (BBB) and AADC rates showed strict

structural dependency, with the E-isomer exhibiting higher specificity than the Z geometrical counterpart for ability to localize in central DA structures; and the 6-[^{18}F]fluoro substituted isomer was favored over the 2- and 4- versions.[7] Stereospecificity for activation of D_2 receptors has been reported, as have preferred conformations for enzyme active sites.[8,9]

In previous studies, we investigated the conversion of tagged L-DOPA to DA using an in vitro AADC enzymatic assay to screen novel molecular configurations for bioavailability. Isomers of L-DOPA and DA with nine ^{19}F atom tags were exposed to AADC in crude bacterial lysate, and analyzed for evidence of conversion using high pressure liquid chromatography (HPLC). In the study reported here, we report the results of administering tagged L-DOPA to mice pups to investigate its conversion to DA at two points in time, 1.5 and 3 hr after administration of an i.p. injection. Supernatant from neural tissues was then analyzed using HPLC methods developed for enzymatic assay studies. Tissue was prepared using methods developed for immunoassays.[10]

EXPERIMENTAL

The bioavailability of L-5-(2-(nonafluoro-*t*-butoxy)acetyl-DOPA (PF-L-DOPA) in mammals was investigated by administering it to 10-day-old mice pups. The novel imaging compound was synthesized by Inovatia Laboratories, Inc. for use in fluorine MR studies during development. The structures for L-5-(2-(perfluoro-*t*-butoxy)acetyl)DOPA and 5-(2-(perfluoro-*t*-butoxy) acetyl)DA are shown in Fig. 1.

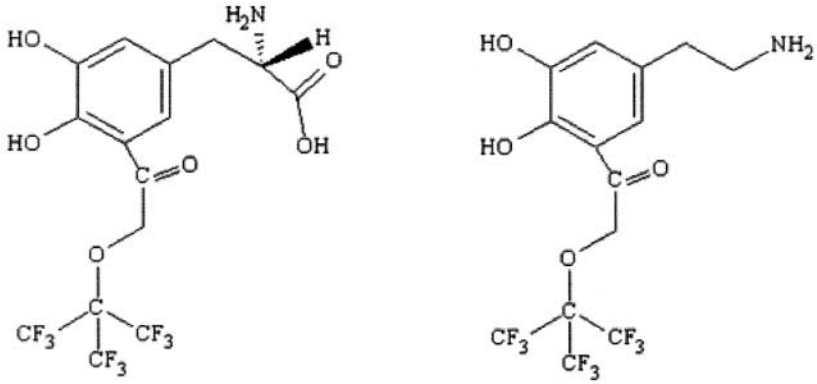

Figure 1. Structure of L-5-(2-(perfluoro-*t*-butoxy)acetyl)DOPA (left) and 5-(2-(perfluoro-*t*-butoxy)acetyl)DA.

Pups were injected i.p. with L-5-(2-(nonafluoro-*t*-butoxy)acetyl-DOPA (250 mg/kg) in a vehicle of phosphate buffered saline (200 μL) using sterile 0.5 cc disposable insulin syringes, and returned to their litters for a period of time (1.5 or 3 hr) to allow for uptake and conversion of the compounds. Pups were labeled with a non-toxic permanent marker to facilitate identification of individuals. At the end of the time interval, pups were again removed from the litter, and placed in chipped ice to partially anesthetize them prior to rapid decapitation. Brains were promptly removed for processing.

Whole brain tissue samples were placed into test tubes containing 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 and placed in another test tube to be centrifuged for an additional 12 min. The final samples had a volume of about 1.5 mL of fluid. The pellets were discarded. Samples were refrigerated overnight and shipped in a cooler with freezer packs to Inovatia Laboratories, LLC for HPLC analysis for L-DOPA, tagged L-DOPA (PF-DOPA), DA, and tagged DA (PF-DA).

Each sample was filtered through a 0.45 μm glass fiber filter before being analyzed by HPLC (HP 1090 M Series II), using a Discovery C18 column (250 mm × 4.6 mm, 5 μm, with guard column). The chromatograms were drawn from the absorbance at 280 nm, although the total spectrum from 200 to 600 nm is saved for each data point, on each chromatogram. The absorbance spectrum was used to confirm the identities of the compounds represented by the various peaks. A gradient solvent program was used, combining sodium acetate (0.01 M, pH 4.5) with methanol at a combined total of 0.2 mL/min. The program begins with the aqueous buffer alone for 1 min. Over the next 17 min, the methanol fraction increases linearly from 0% to 40%. Over the next 8 min, the methanol fraction increases from 40% to 60%, holding at 60% for an additional 6 min. Then, the methanol fraction decreases over the next 6 min back to 0%, using only the aqueous buffer for the last 5 min. Injections were 40 μL.

Study One

Three mouse pups were injected with tagged L-DOPA and three control pups were injected with just PBS. Pups were randomly selected and injected at 15 min intervals over the day, to facilitate observation of the mother's behavior towards the pups after injections. Pups injected with PF-L-DOPA

remained still for as long as 130 min before resuming more species typical behaviors. One of the goals of this first study was observing maternal behavior toward pups injected with the novel compounds. She engaged in licking pups after injections, focusing particular attention on the injection site. She isolated the recently injected pup from its littermates. After 3 hr, samples of neural tissue were prepared as described previously.

Study Two

The experiment was repeated with nine mice pups, three controls injected with PBS were sacrificed at 3 hr, three pups injected with tagged L-DOPA were sacrificed at 3 hr, and three pups injected with tagged L-DOPA were sacrificed at 1.5 hr. This study was undertaken to investigate the time course for the conversion of tagged L-DOPA to tagged DA, information that will facilitate administering the compounds for in vivo fluorine imaging.

RESULTS

Study One

Significantly more L-DOPA and DA were present in the brains of the injected pups than in the brains of the control pups (Fig. 2). The retention time and UV spectra for tagged and untagged L-DOPA, and tagged and untagged DA, are quite similar and could not be distinguished in these samples. The pups injected with tagged L-DOPA had higher levels of DOPA and DA in neural tissue, presumably tagged versions of the compounds.

Study Two

The three full chromatograms with enlargements are shown in Figs. 3–5. All three samples have the same peak just before 10 min. The area of that peak in the chromatogram from neural tissue samples taken 1.5 hr after injection of tagged L-DOPA, is more than five times as greater than in the other samples. The other peaks in the sample appear small by comparison because of the automatic scaling feature of the graphing software. The 6-isomer of tagged DA is absent from the 1.5-hr sample, suggesting that this isomer of L-DOPA is not converted to any significant degree in vivo to tagged DA. The peak at 19 min in the control sample was previously observed in lysate from *E. coli* modified to express AADC.

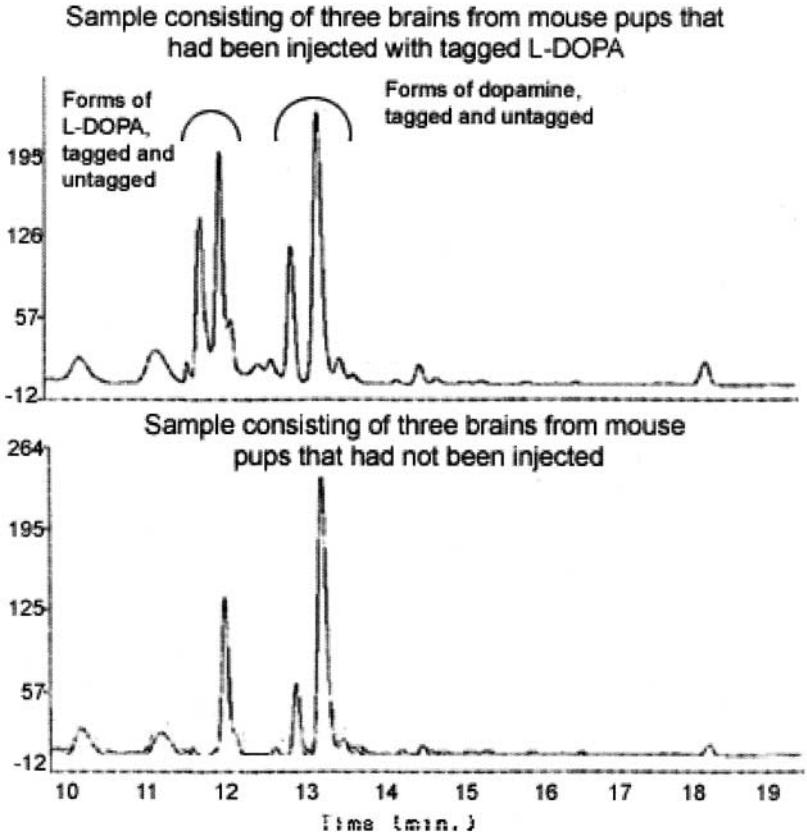

Figure 2. Tagged L-DOPA was converted to tagged DA in mice pup brains.

DISCUSSION

The ^{19}F atom tags appear to have good potential for use in fluorine MR studies. NMR and MR data for the nine ^{19}F atom tags reveals a single peak that is theoretically detectable in vivo with a high field magnet (Fig. 6). In order for multiple ^{19}F atom tags to be useful as a tool in MR, the fluorine atom tags must be concentrated into vesicles to achieve sufficient signal-to-noise to be detectable. This study reports the first direct evidence that L-DOPA, with nine fluorine atom tags, crosses the BBB of mammals, is taken up into dopaminergic neurons, and converted to tagged DA.

DA would not be detectable at all, given the low sensitivity of non-proton MR, if it was homogeneously distributed throughout the brain. However,

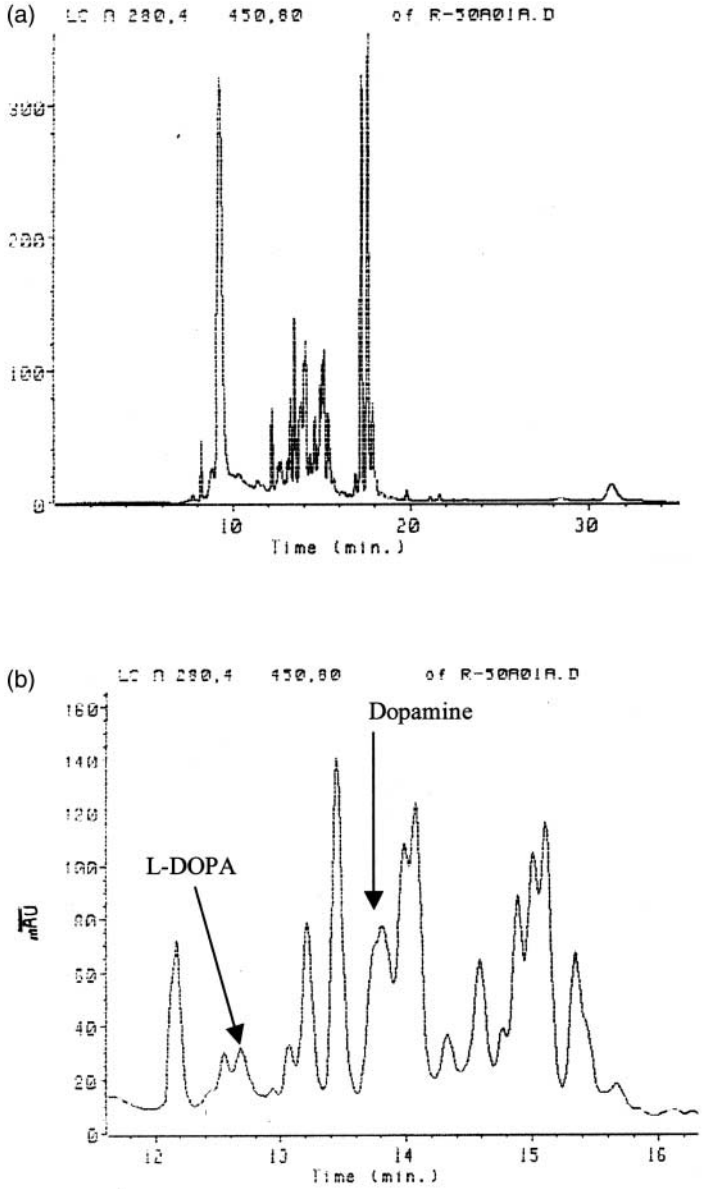

Figure 3. (a) Control sample of three mice brains at 3 hr; (b) magnification showing the location of endogenous L-DOPA and DA.

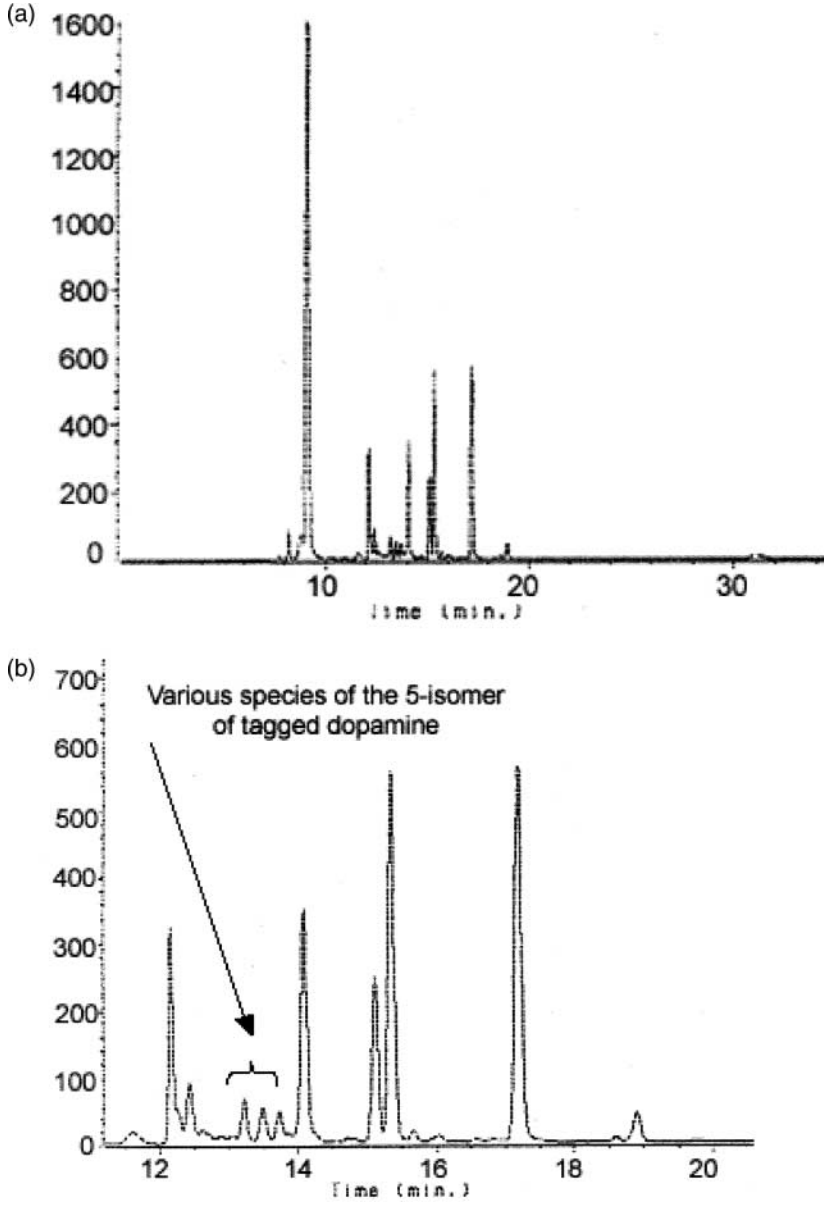

Figure 4. (a) Three mice brains at 1.5 hr after injection with tagged L-DOPA; (b) magnification showing various species of the 5-isomer of tagged DA.

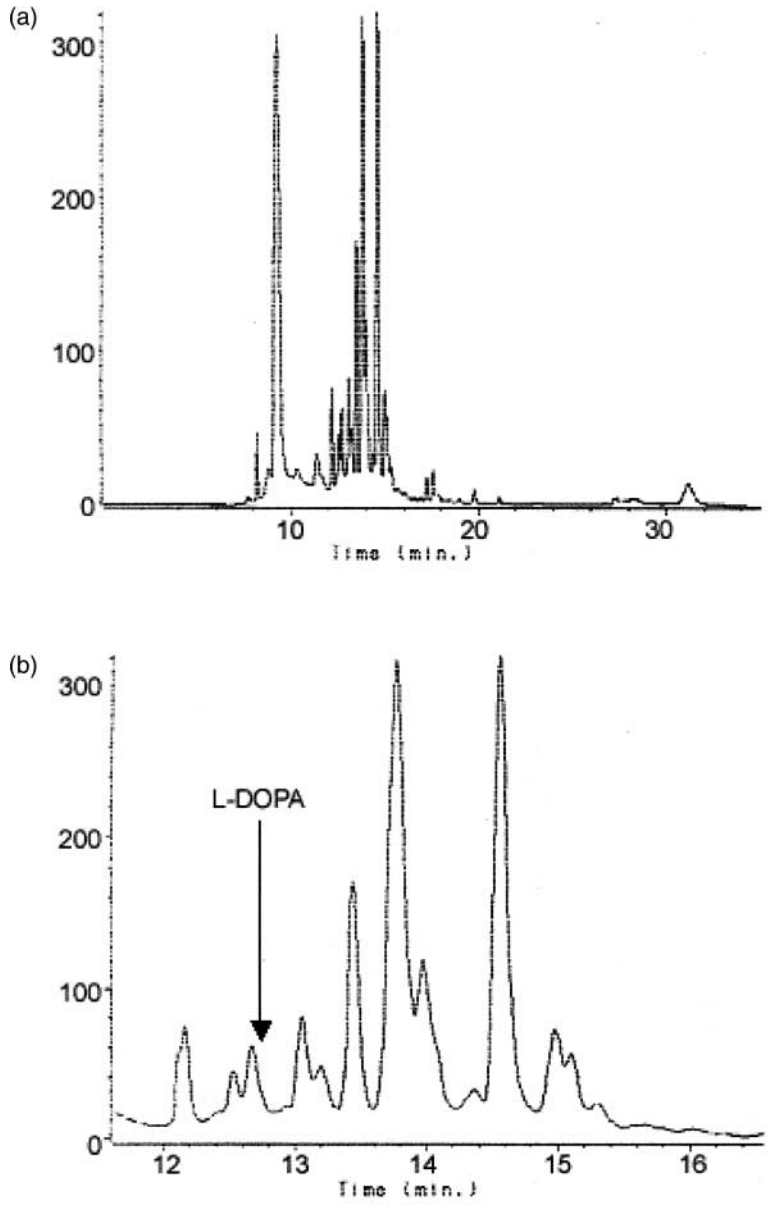

Figure 5. (a) Three mice brains at 3 hr after injection with tagged L-DOPA; (b) magnification showing L-DOPA peak.

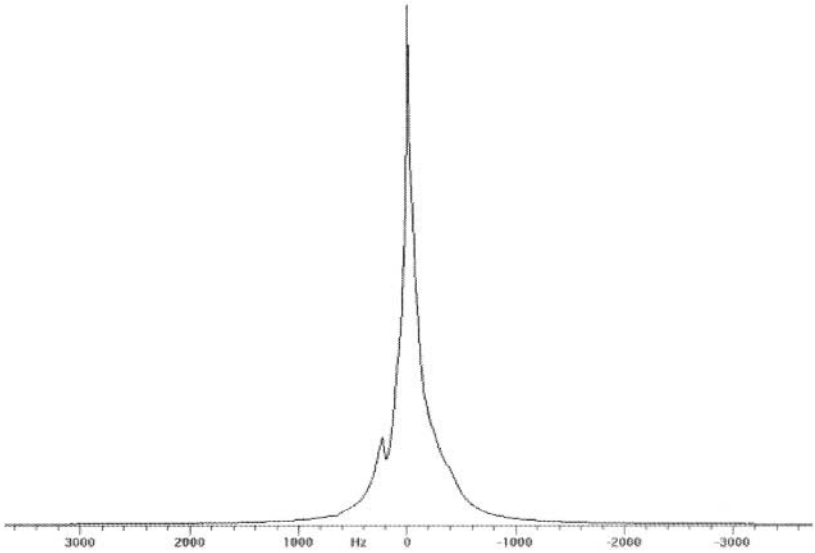

Figure 6. NMR of ^{19}F atom tag.

as has been repeatedly demonstrated, discrete neuronal pathways employing specific neurotransmitters exist within the brain. Dopaminergic and noradrenergic fibers display characteristic varicosities with a high density of vesicles. MR spectroscopy should be able to detect fluorine atoms concentrated within these vesicles.

In a high field magnet with adequate sensitivity, it may even be possible to detect processes associated with the synthesis, release, and re-uptake of fluorine tagged compounds. The turnover rate of a neurotransmitter is an important indicator of neuronal activity. In the brain, L-DOPA is normally only briefly present in the course of catecholamine synthesis. We can conceivably manipulate the levels of tagged compound in vivo through pharmacological treatments. Tyrosine hydroxylase inhibitor can be used to prevent the conversion of tyrosine to L-DOPA for a period of time. During this period of time, we can administer tagged L-DOPA, thus bypassing the rate-limiting factor in the synthetic pathway for DA.

The ability to monitor the epigenesis of catecholamine pathways during development would provide a powerful new tool for understanding complex interactions taking place between genes and the environment during the wiring of the brain. Many existing methods for tracing neural development sacrifice the organism, making it impossible to investigate subsequent development and behaviors. The new tool we are developing for use with MR could

make it possible to monitor structural and, perhaps, even the functional development of specific neuronal pathways in vivo. Events associated with DA transmission may be detectable as increased signal from active regions, when PF-L-DOPA is converted to PF-DA and concentrated into vesicles, replacing the natural DA in response to increased demand for the transmitter. This would lead to much greater specificity than monitoring global physiological correlates of general neuronal activity. Epigenesis of specific pathways under a variety of conditions, could then be related to adult behavior patterns.

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