^{13}C/^{15}N-Enriched L-Dopa as a Triple-Resonance NMR Probe to Monitor Neurotransmitter Dopamine in the Brain and Liver Extracts of Mice

Hisatsugu Yamada,*[a] Tetsuro Kameda,[b] Yu Kimura,[b, c] Hirohiko Imai,[d] Tetsuya Matsuda,[d] Shinsuke Sando,[e] Akio Toshimitsu,[b, f] Yasuhiro Aoyama,*[g] and Teruyuki Kondo*[a, b]

In an attempt to monitor μм-level trace constituents, we applied here ^{1}H-{^{13}C-^{15}N} triple-resonance nuclear magnetic resonance (NMR) to ^{13}C/^{15}N-enriched L-Dopa as the inevitable precursor of the neurotransmitter dopamine in the brain. The perfect selectivity (to render endogenous components silent) and μм-level sensitivity (700 MHz spectrometer equipped with a cryogenic probe) of triple-resonance allowed the unambiguous and quantitative metabolic and pharmacokinetic analyses of administered L-Dopa/dopamine in the brain and liver of mice. The level of dopamine generated in the brain (within the range 7–76 μм, which covers the typical stimulated level of ~30 μм) could be clearly monitored ex vivo, but was slightly short of the detection limit of a 7 T MR machine for small animals. This work suggests that μм-level trace constituents are potential targets of ex vivo monitoring as long as they contain N atom(s) and their appropriate ^{13}C/^{15}N-enrichment is synthetically accessible.

Multiple-resonance NMR is a powerful technique,[1–4] by which particular protons in the sequence ^{1}H-^{13}C-^{15}N (^{1}H-{^{13}C-^{15}N} triple resonance) or ^{1}H-^{13}C (^{1}H-{^{13}C} double resonance) can be detected highly selectively as a result of magnetic coherence transfer $^1H \rightarrow ^{13}C \rightarrow ^{15}N \rightarrow ^{13}C \rightarrow ^1H$ or $^1H \rightarrow ^{13}C \rightarrow ^1H$. Although the application of multiple-resonance NMR (double resonance in many cases,[2,3] mostly dealing with main metabolic sources such as glucose, amino acids, and fatty acids, and triple resonance in some[4]) to metabolic analysis is by no means rare, little is known, to the best of our knowledge, about its applicability to hormone-like trace (μм-level) constituents. In the present work, we applied triple-resonance NMR to ^{13}C/^{15}N-enriched L-Dopa (L-3,4-dihydroxyphenylalanine) as the inevitable precursor of neurotransmitter dopamine (2-(3,4-dihydroxyphenyl)-ethylamine) in the brain.

Dopamine plays important roles in motivation, reward, and motor control,[5] and problems with its metabolism can trigger several neurological/psychological disorders such as Parkinson's disease, schizophrenia, and depression.[6,7] Scheme 1 summarizes the L-Dopa-to-dopamine metabolism and its inhibition. The brain is not directly accessible by dopamine, which cannot pass through the blood–brain barrier (BBB). Instead, dopamine is generated in situ in the brain upon decarboxylation of its precursor, L-Dopa which is BBB-permeable, by the enzyme AAAD (aromatic L-amino acid decarboxylase). Dopamine thus generated in the brain undergoes rather rapid deactivation upon oxidative deamination by the enzyme MAO (monoamine oxidase, types A and B).

In clinical practice, L-Dopa as the precursor of dopamine is often administered together with inhibitors of enzymes AAAD[8] and MAO-A and MAO-B[9] as codrugs to maintain appropriate high concentrations of dopamine in the brain. Another way to achieve high dopamine levels (~30 μм[10, 11a, b] or ~2 μм[11c])[12] is to electrically stimulate the brain. In this work, we took ^{13}C/^{15}N-enriched L-Dopa as a triple-resonance probe to monitor dopamine in mice with a stimulated level of ~30 μм in the brain taken as a criterion to evaluate the per-

[a] Dr. H. Yamada,$^+$ Prof. Dr. T. Kondo
Advanced Biomedical Engineering Research Unit
Center for the Promotion of Interdisciplinary Education and Research
Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510 (Japan)
E-mail: teruyuki@scl.kyoto-u.ac.jp
yamada.hisatsugu@tokushima-u.ac.jp

[b] T. Kameda, Dr. Y. Kimura, Prof. Dr. A. Toshimitsu, Prof. Dr. T. Kondo
Department of Energy and Hydrocarbon Chemistry, Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510 (Japan)

[c] Dr. Y. Kimura
Research and Educational Unit of Leaders for Integrated Medical System
Center for the Promotion of Interdisciplinary Education and Research
Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510 (Japan)

[d] Dr. H. Imai, Prof. Dr. T. Matsuda
Department of Systems Science, Graduate School of Informatics
Kyoto University, Yoshida-honmachi, Sakyo-ku, Kyoto 606-8501 (Japan)

[e] Prof. Dr. S. Sando
Department of Chemistry and Biotechnology, The University of Tokyo
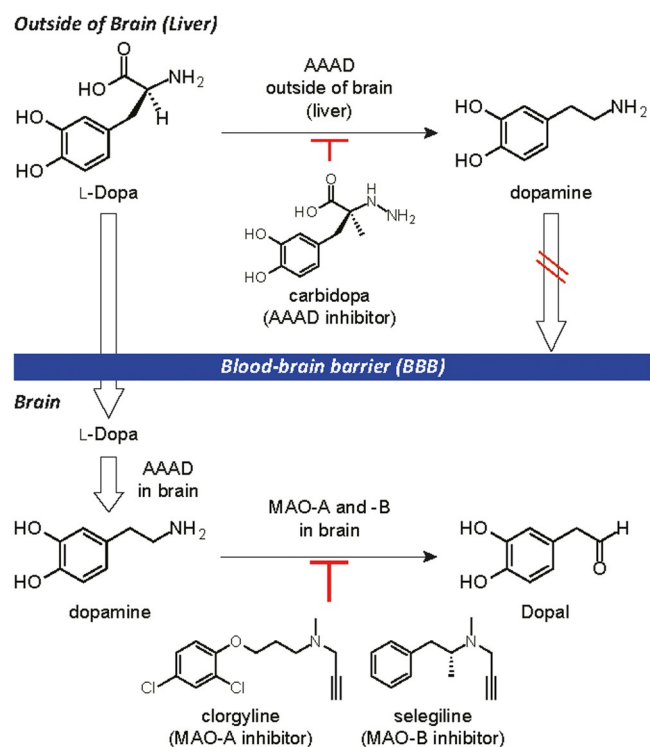7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8656 (Japan)

[f] Prof. Dr. A. Toshimitsu
Division of Multidisciplinary Chemistry, Institute for Chemical Research
Kyoto University, Gokanosho, Uji, Kyoto 611-0011 (Japan)

[g] Prof. Dr. Y. Aoyama
Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510 (Japan)
E-mail: aoyama.yasuhiro.78z@st.kyoto-u.ac.jp

[$^+$] Present address: Department of Life Systems, Institute of Technology and Science Graduate School, Tokushima University, Tokushima 770-8506 (Japan)

Scheme 1. L-Dopa-to-dopamine metabolism and its inhibition.

Figure 1. Structures of a) $^{13}C/^{15}N$-L-Dopa and b) $^{13}C/^{15}N$-dopamine and analysis of the L-Dopa-to-dopamine conversion. Time course of the change in c) ^{1}H-{^{13}C-^{15}N} NMR spectra and d) HPLC profiles of $^{13}C/^{15}N$-L-Dopa (0.5 mM) in 40 mM HEPES containing 80 mM NaCl, 100 μM pyridoxal phosphate, and AAAD (20 ng μL^{-1}), incubated at 37 °C for 0 or 60 min. The HPLC trace at the top is for the control run in the absence of AAAD at 60 min. e) Conventional ^{1}H (top) and ^{1}H-{^{13}C-^{15}N} (bottom) NMR spectra of a mouse liver lysate containing $^{13}C/^{15}N$-L-Dopa (0.5 mM) in 2 mM Tris-HCl, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, and 100 μM pyridoxal phosphate, incubated at 37 °C for 45 min. f) Inhibitory effects of carbidopa on the decarboxylation of $^{13}C/^{15}N$-L-Dopa. ^{1}H-{^{13}C-^{15}N} NMR spectra obtained in the presence of carbidopa (5 or 50 μM) as an AAAD inhibitor, under the same conditions as in e). The ^{1}H-{^{13}C-^{15}N} NMR spectra were obtained after 256 scans.

formance of the present method. We use the selectivity and sensitivity of triple-resonance high resolution NMR with a cryogenic probe to perform quantitative metabolic/pharmacokinetic analysis of L-Dopa/dopamine in the extracts of brain and liver of mice, showing that the stimulated dopamine level (~30 μM) in the brain can be detected ex vivo. This work also illustrates where we are on the path to direct in vivo MR spectroscopic (MRS) monitoring of this neurotransmitter system.

$^{13}C/^{15}N$-enriched L-Dopa ($^{13}C/^{15}N$-L-Dopa, Figure 1a) with a ^{1}H-^{13}C-^{15}N sequence involving the asymmetric center was obtained starting from $^{13}C/^{15}N$-glycine in an optical yield of 94% ee, as detailed in the Supporting Information. $^{13}C/^{15}N$-enriched dopamine ($^{13}C/^{15}N$-dopamine, Figure 1b) was also prepared from K$^{13}C^{15}N$. One-dimensional (1D) ^{1}H-{^{13}C-^{15}N} triple-resonance spectra (^{13}C-decoupled) of $^{13}C/^{15}N$-L-Dopa and $^{13}C/^{15}N$-dopamine showed a single peak at 3.85 ppm or at 3.14 ppm for the methine proton (^{1}H-^{13}C-^{15}N) of L-Dopa or the methylene protons ($^{1}H_2$-^{13}C-^{15}N) of dopamine, respectively. The enzymatic decarboxylation of $^{13}C/^{15}N$-L-Dopa ($\delta = 3.85$ ppm) to dopamine ($\delta = 3.14$ ppm) in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer containing the decarboxylation enzyme AAAD was completed in 1 h, as revealed by the triple-resonance analysis (Figure 1c) in accord with the results of high-performance liquid chromatography (HPLC) monitoring (Figure 1d).

Decarboxylation and its inhibition in complex biological mixtures such as liver lysate could also be readily monitored by triple-resonance. $^{13}C/^{15}N$-L-Dopa (0.5 mM) in crude mouse liver lysate was incubated for 45 min. After workup, the mixture was subjected to NMR analysis. The conventional ^{1}H NMR spec-
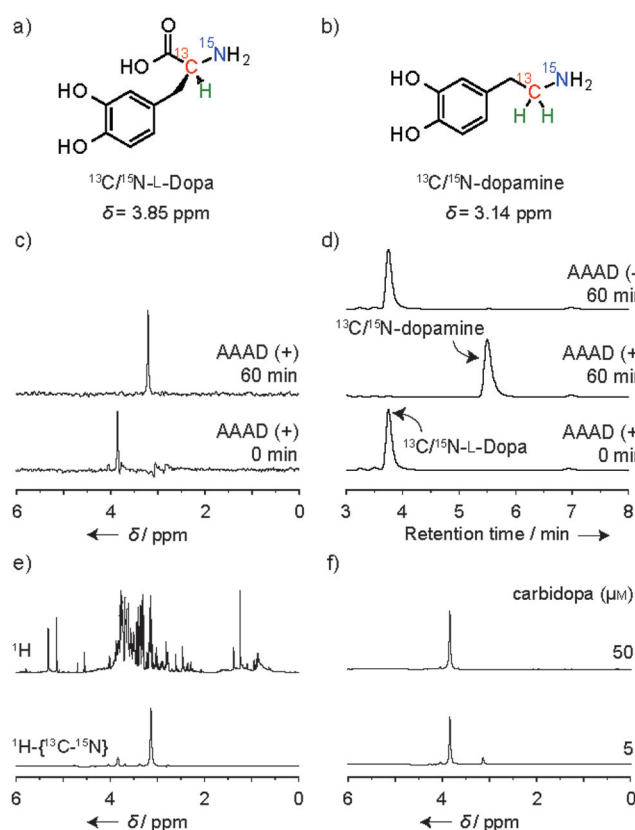
trum (Figure 1e, top) was completely useless; all ^{1}H-containing molecules in the lysate represent their signals. The ^{1}H-{^{13}C-^{15}N} triple-resonance spectrum (Figure 1e, bottom) exhibited two signals at 3.85 ppm for L-Dopa and 3.14 ppm for dopamine in a ratio of 1:7 (12% and 86% of L-Dopa used, respectively), indicating that most of the L-Dopa had undergone decarboxylation by endogenous AAAD contained in the liver lysate to give dopamine. In the presence of carbidopa (BBB-impermeable), a potent AAAD inhibitor that is clinically used as a codrug together with L-Dopa (referring to Scheme 1), the build-up of dopamine was effectively suppressed (~85%) even at [carbidopa] = 5 μM (0.01 equiv of L-Dopa (0.5 mM)) (Figure 1f, bottom) and completely suppressed at [carbidopa] = 50 μM (0.1 equiv) (Figure 1f, top). Triple resonance can thus completely suppress noise signals arising from endogenous components in complex biological mixtures to allow the unambiguous and quantitative metabolic analyses of $^{13}C/^{15}N$-enriched substrates therein.

In vitro and ex vivo spectra were obtained with a 700 MHz (16.4 T) NMR spectrometer equipped with a high-sensitivity

cryogenic probe after 256 scans (~7 min), where the detection limit, i.e., the lowest concentration to give S/N = 3, of $^{13}C/^{15}N$-dopamine lies at around 4 μM. Triple-resonance spectra of $^{13}C/^{15}N$-dopamine as phantom samples (500 μL) were also obtained with an MR machine for small animals operating at 7 T (300 MHz) without a cryogenic probe (Supporting Information); the detection limit after 3600 scans (1 h) turned out to be ~1 mM.

We proceeded to the ʟ-Dopa/dopamine metabolic analysis in mice, focusing on the effects of inhibitors of decarboxylation (AAAD) and oxidation (MAO) enzymes. Mice (~16 g) were administered $^{13}C/^{15}N$-ʟ-Dopa (0.63 mmol kg^{-1}) with or without carbidopa (AAAD inhibitor) and MAO inhibitors [clorgyline and selegiline (BBB-permeable MAO-A and MAO-B inhibitors, respectively; see Scheme 1)]. After 1 h, brain and liver tissues were collected and, after workup, their triple-resonance spectra (256 scans) were obtained for doubly diluted (compared with the tissue weights) solutions, as shown in Figure 2, where the
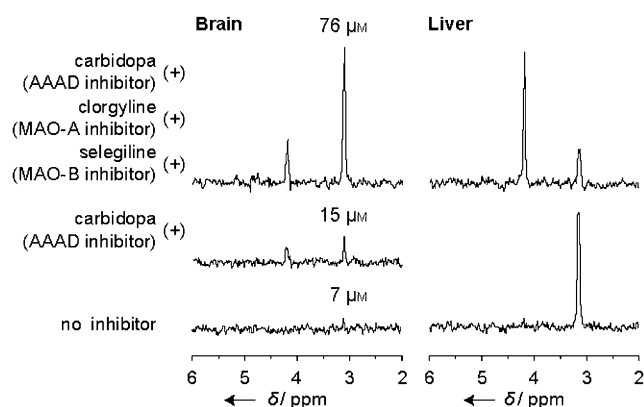

Figure 2. Effects of MAO inhibitors on the oxidative degradation of $^{13}C/^{15}N$-dopamine in mice. Weight-normalized ^{1}H-{^{13}C-^{15}N} NMR spectra (256 scans) for the extracts of brain (left) and liver (right) tissues of a mouse coadministered with $^{13}C/^{15}N$-ʟ-Dopa (0.63 mmol kg^{-1}) and carbidopa (63 μmol kg^{-1}) in the absence (middle) or presence (top) of clorgyline (MAO-A inhibitor, 63 μmol kg^{-1}) and selegiline (MAO-B inhibitor, 63 μmol kg^{-1}). The corresponding spectra in the absence of any inhibitors are shown at the bottom. The tissue extracts obtained were redissolved in D$_2$O and subjected to NMR analysis. The in-brain concentrations of dopamine were quantified via calibration and are shown.

signal intensities for the brain and liver samples are weight-normalized. In the absence of any inhibitors, a small amount of dopamine (3.14 ppm) was detected in the brain, while a much larger amount was found in the liver (bottom). ʟ-Dopa with a signal at 4.19 ppm[13] was hardly detected in the liver or the brain. Most of the ʟ-Dopa must have undergone wasteful decarboxylation by endogenous AAAD in the liver, and any dopamine generated remained therein since it could not pass though the BBB to reach the brain. In the presence of the AAAD inhibitor carbidopa (63 μmol kg^{-1}), the dopamine level in the brain increased, but only slightly (middle), probably because of its oxidative deamination by MAO to give dopal, which of course exhibited no ^{1}H-^{13}C-^{15}N signals. Indeed, when the MAO inhibitors clorgyline (63 μmol kg^{-1}) and selegiline

(63 μmol kg^{-1}) were both present, there was a 5-fold increase in the dopamine level in the brain, and a substantial amount of ʟ-Dopa that escaped decarboxylation remained in the liver (top).[14,15] The local concentrations of dopamine in the brain (~400 mg) were estimated by calibration using an authentic specimen to be 7 μM (in the absence of any inhibitors), 15 μM (with AAAD inhibitor), and 76 μM (with AAAD and MAO inhibitors). The inhibitor-dependent dopamine levels of 7–76 μM are consistent with those of 5–120 μM[9a] reported for rat based on HPLC analysis.

In this work, we investigated the usefulness of triple resonance for monitoring dopamine at a stimulated ~30 μM level. As shown above, a wide concentration range which includes this critical 30 μM could be easily accessed by a combination of ʟ-Dopa and inhibitors. Selectivity and sensitivity are key issues in applying NMR to complex biological mixtures. In this context, the present work may be summarized as follows: 1) Triple resonance showed "perfect" selectivity. The probability of the natural occurrence of the sequence ^{1}H-^{13}C-^{15}N is as low as $0.011 \times 0.0037 = 0.00004$ (0.004 %), where 0.011 and 0.0037 are the natural abundance of ^{13}C and ^{15}N, respectively, and the mole-based selectivity factor for the $^{13}C/^{15}N$-enriched target over endogenous components is $1/0.00004 = 25000$ (2.5×10^4). Thus, endogenous components may effectively compete with the enriched target at, for example, 10 μM, only when they are present in unnaturally high concentrations of 10×10^4 μM = 0.1 M. An implication of this observation is that selectivity is by no means a formidable issue to deal with for *any* μM-level trace constituents as long as they contain N-atom(s) and their appropriate double $^{13}C/^{15}N$-enrichment is synthetically accessible. 2) This perfect, noiseless selectivity of triple resonance gives rise to a μM sensitivity [4 μM, 256 scans under less time-consuming (in minutes), one-dimensional (monitoring of ^{1}H signals only) conditions]. This allows unambiguous and quantitative ex vivo metabolic/pharmacokinetic analyses of administered ʟ-Dopa and its metabolite dopamine, i.e., ratiometric monitoring of their decay/build-up profiles, which clearly shows that the stimulated level of dopamine in the brain can be monitored ex vivo. 3) Unfortunately, however, the key dopamine level of 30 μM is short of the detection limit (~1 mM, 3600 scans) of the 7T MR machine (noncryogenic probe) for noninvasive, i.e., in vivo, monitoring.[16] However, the gap between them is *only* a factor of ~30. This appears to be significant since an increase in sensitivity of this extent (~30-fold) may be achieved by combining an existing highest-field machine and a high-sensitivity cryogenic probe equipped with a triple-resonance coil.[17] In-brain dopamine may then become a real target of direct in vivo MRS with which we can record the dopamine spectra in the brain.

In addition to a variety of techniques, based on HPLC,[18a] biological (enzyme-linked immunosorbent assay, ELISA) affinity (for the analysis of urine),[18b] microdialysis,[18c] electrochemical techniques,[18c] and chemical sensing,[18d] a couple of methods have recently been developed to monitor dopamine in the brain. One is positron emission tomography (PET) using ^{11}C-raclopride, which competitively binds to the dopamine receptor to enable the [dopamine]-dependent emission of

gamma rays.[19] The other is MRI using a protein-engineered heme-based contrast agent which reversibly binds to dopamine, thereby changing the relaxivity, and thus gives [dopamine]-dependent images.[10] Both methods are highly sophisticated, but are indirect and involve complicated complexation processes. MRS is much simpler and can directly monitor the targets and their transformations with minimal noise signals which may arise from nonspecific binding, etc. Currently, the metabolic analysis of ^{13}C-glucose in the brain has received increasing attention.[3] The present work shows a way to detect μm-level trace constituents and has shed light on the issues to be overcome for in vivo imaging. Further work is now underway along these lines with an ultimate goal of detection of hypodopaminergy in related diseases.

Experimental Section

1) General methods, 2) preparation, 3) monitoring of the L-Dopa-to-dopamine conversion and subsequent dopamine oxidation, and 4) phantom MRS are included in the Supporting Information.

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[12] The reported values for rat are ~35 μm (electrochemical),[11a] a) ~30 μm (electrochemical),[11b] b) ~27 μm (MRI),[10] and ~2 μm (electrochemical).[11c]

[13] The workup procedure involves treatment with 10% trichloroacetic acid (TCA). ^{13}C/^{15}N-L-Dopa was independently shown to be stable when treated with 10% TCA under the same conditions and exhibited methine proton resonance at 4.19 ppm, which is shifted downfield by 0.34 ppm (probably as a result of changes in the protonation state) from that (3.85 ppm) for TCA-untreated L-Dopa.

[14] The liver spectrum (Figure 2, top, right) shows a notable amount of dopamine (3.14 ppm), indicating that 0.1 equiv of carbidopa is not enough to completely suppress in vivo decarboxylation of L-Dopa in the liver, although enzymatic in vitro decarboxylation of L-Dopa can be completely inhibited by 0.1 equiv of carbidopa (Figure 1 f).

[15] Interestingly, the L-Dopa level in the brain is higher with carbidopa and MAO inhibitors than with carbidopa alone.

[16] The sensitivity of MR machines for small animals with a wider bore diameter, a longer sample-to-coil distance, and more pronounced inhomogeneity of the magnetic field is known to be considerably lower than that of spectrometers dealing with solution samples in 5 mm tubes. Under these circumstances, recent attention has been directed to specific signal amplification techniques such as hyperpolarization and CEST (chemical exchange saturation transfer). For example, a) A. Viale, S. Aime, *Curr. Opin. Chem. Biol.* **2010**, *14*, 90–96 and references therein; b) A. R. Lippert, K. R. Keshari, J. Kurhanewicz, C. J. Chang, *J. Am. Chem. Soc.* **2011**, *133*, 3776–3779; c) S. Viswanathan, Z. Kovacs, K. N. Green, S. J. Ratnakar, A. D. Sherry, *Chem. Rev.* **2010**, *110*, 2960–3018 and references therein.

[17] The commercially available, highest-field MR machine for small animals operates at 21 T (Bruker, Billerica, MA, USA). The NMR sensitivity increases in proportion to $B_0^{3/2}$, where B_0 is the static magnetic field. A cryogenic probe is known to enhance sensitivity by a factor of ~5 compared with noncryogenic probes. Thus, a 21 T (900 MHz) machine with a cryogenic probe is expected to be more sensitive than a 300 MHz (7 T) machine without a cryogenic probe by a factor of $(21/7)^{3/2} \times 5 = 26$.

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