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Synthesis and Proton NMR Spectroscopy of Intra-Vesicular Gamma-Aminobutyric Acid (GABA)*

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Abstract

We report the synthesis of vesicles containing gamma-aminobutyric acid (GABA), and their proton nuclear magnetic resonance (^1H NMR) spectra. These vesicles were constructed to more closely mimic the intracellular environment wherein GABA exists. For this study, these GABA-containing vesicles were examined under ^1H NMR as a potential platform for future studies on the differences between aqueous phantoms, *ex vivo* brain extracts, and *in vivo* magnetic resonance spectroscopy results. We found that intra-vesicular GABA faithfully yielded the chemical shifts and *J*-coupling constants of free aqueous GABA, alongside the chemical shift signals of the vesicle wall.

I. Introduction

While phantoms made in aqueous solution are widely used to corroborate the spectroscopic signals of brain metabolites, it has not been substantively studied how accurately such phantoms approximate their *in vivo* counterparts. For example, it recently has been determined that the T_2 relaxation time for gamma-aminobutyric acid (GABA) is 88 ms *in vivo* [1]. This is much shorter than a measured value of 260 ms within an aqueous phantom control, and also shorter than other detected metabolites, which range between 150 and 300 ms [2].

It is postulated that the reason stems from the cellular environment within which GABA exists [1]. Decreased rotational motion from cooler temperatures or higher viscosity is known to reduce T_2 ; but whether such an environment exists within an intracellular vesicle is unknown. If it does, its characterization would not only help more accurately quantify GABA *in vivo*, but also shed light on the intra-vesicular behavior of this inhibitory neurotransmitter.

Secondly, *in vivo* spectra will necessarily contain overlapping signals from lipids and other metabolites. As a means to distinguish these contents by proton nuclear magnetic resonance (^1H NMR) spectroscopy, *ex vivo* preparations have been studied [3]. However, synaptic

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vesicle fractionation requires differential centrifugation steps; and the extent to which extraction techniques alter or preserve intracellular vesicles cannot be easily corroborated.

A completely synthetic construct mimicking cellular structures of interest would be a helpful comparator. In the case of GABA (Fig. 1), the amount of neurotransmitter detected *in vivo* likely represents the intracellular content of GABAergic neurons [3,4], where GABA exists packaged within vesicles reported to measure from 29 to 36 nm in diameter [5]. A non-invasive and reliable way of measuring, and calibrating, intracellular GABA would be of great clinical interest.

To our knowledge, there are no prior reports in which synthetic GABA-containing vesicles have been characterized by NMR. Such a vesicle would be an important model for *in vivo* GABA measurements. Therefore, as a first step, we asked whether a synthesized vesicle containing GABA near physiological concentrations could, in fact, produce similar spectra to that of free GABA in aqueous solution.

II. Methods

A. Vesicle preparation

A thin film of dioctyl sodium sulfosuccinate (DSS) was prepared inside a 100-mL round-bottom flask by dissolving 0.178 g (0.4 mmol) DSS in 5 mL chloroform, with subsequent evaporation by nitrogen gas. The film was dried overnight under high vacuum, and then rehydrated with 30 mL of a 5-mM GABA solution in phosphate-buffered saline (PBS). The obtained DSS-GABA suspension was sonicated for 5 min in a sonication bath (Branson 2510 ultrasonic cleaner), followed by tip sonication for another 3 minutes at room temperature (Vibracell ultrasonicator). The resulting vesicle suspension was repetitively washed by PBS (50 mL $\times$ 3) to remove any extra-vesicular GABA using ultracentrifugation (Amicon Ultra centrifugal filter, cut-off pore size 10 kDa), and concentrated to desired volume. Vesicles remained stable for up to 4 weeks at 4 °C.

B. Intra-vesicular GABA concentration confirmation using high-performance liquid chromatography (HPLC)

The vesicle suspension was pretreated with dansyl chloride (Sigma Aldrich) at 80 °C in the dark and centrifuged. The supernatant was subjected for analysis (HPLC UV detector wavelength at 285 nm) [6]. Vesicle sizes were determined by dynamic light scattering (Malvern Zetasizer Nano).

C. ^1H NMR spectroscopy

Samples were placed in a standard 5-mm NMR tube, and high-resolution spectra were acquired on a Varian 400 MHz spectrometer using water suppression (WET). Parameters were: flip angle 45°, relaxation delay 10 s, spectral width 8021.8 Hz, and 32 repetitions. Spectra were post-processed with a 1.0-Hz line-broadening function, and referenced to water at 4.7 ppm. The process was repeated for a 1-mM GABA solution in PBS alone.

III. Results

The final internal concentration of GABA by HPLC was 2.44 ± 0.29 mM. The mean vesicle diameter was 37 nm (Fig. 2), close to the sizes reported in tissue [5].

Fig. 3 displays the near-identical spectra acquired from free aqueous (*top*) and intra-vesicular (*bottom*) GABA. For aqueous GABA, the GABA C2 protons resonated as a triplet, centered at 1.839 ppm; the C3 protons resonated as a multiplet, centered at 1.442 ppm; and the C4 protons resonated as a triplet, centered at 2.559 ppm.

For intra-vesicular GABA, the C2 protons resonated as a triplet, centered at 1.839 ppm; the C3 protons resonated as a multiplet, centered at 1.442 ppm; and the C4 protons resonated as a triplet, centered at 2.560 ppm. The *J*-coupling values were identical, and are summarized in Table 1.

IV. Discussion

There were no chemical shift or *J*-coupling differences between aqueous and intra-vesicular GABA. Additionally, the greater signal-to-noise ratio within the vesicular GABA sample appeared to agree with the higher GABA concentration (2.44 mM) measured within the synthesized vesicles by HPLC.

These results indicate GABA-containing vesicles successfully replicate the chemical shifts characteristic of GABA. The lack of chemical shift and line-width changes also confirms that the diamagnetic susceptibility of the vesicle wall has an extremely small effect, and does not appreciably alter the B_0 field inside.

From X-ray diffraction studies, vesicles synthesized from DSS have a lipid bilayer thickness of 19.5 Å [7–9]. Molecular dynamics simulations of a closely related surfactant, sodium bis-3,5,5-methyl-1-hexyl sulfosuccinate, result in a similar thickness of 19.2 Å [10]. Due to packing differences and a shorter carbon chain length, DSS vesicles possess a shallower wall than those constituted from the unsaturated phospholipid 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (38 Å) typical of cell membranes [11,12]. Nevertheless, packaging GABA within vesicles represents a step toward the synthesis of more advanced and realistic phantoms. Given the intricacies of synaptic vesicle fractionation *ex vivo* [13], such phantoms would offer more robust comparisons to *in vivo* ^1H NMR data.

With the close reproduction of expected chemical shifts, further experiments on optimizing these vesicles are certainly warranted. Once optimized, these synthesized vesicles should serve as a useful substrate in T_2 experiments. In particular, this intermediate environment will provide a promising platform to study in further detail the intra-vesicular behavior of GABA, and help define the mechanisms underlying the discrepancy in T_2 relaxation times between *in vitro* and *in vivo* conditions.

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Figure 1.

Structure of gamma-aminobutyric acid (GABA). The numbers correspond to the carbons in the molecule, ordered by standard nomenclature.

Figure 2.
Distribution of vesicle diameters (nm).

Figure 3. Spectra from aqueous gamma-aminobutyric acid (GABA; *top*) and GABA-containing vesicles (*bottom*). The yellow numbers correspond to protons on the carbons identified in Fig. 1.

TABLE I

Coupling Constants Between Aqueous and Intra-Vesicular GABA

Carbon #	Coupling constant <i>J</i> (Hz)	
	<i>Aqueous GABA</i>	<i>Intra-vesicular GABA</i>
2–3	7.3	7.3
3–4	7.4	7.4