From our readers

DIFFUSION MEASUREMENTS FOR DRUG DESIGN

To the editor – Stroh *et al.* recently proposed that diffusion measurements may be used to direct rational drug design[1]. They focused on brain-derived neurotrophic factor (BDNF), a neurotrophin nearly identical to nerve growth factor (NGF) in size (~27 kDa), shape and charge. Despite the similarities, BDNF distribution in brain is often more limited than that of NGF after central administration. Stroh *et al.* concluded that chemical modification of BDNF with polyethylene glycol (PEG) produces a conjugate with enhanced diffusion properties in rat brain slices, as compared with native BDNF[1]. Two problems are evident from these authors' data.

First, the free diffusion coefficient, D_f, measured for tetramethylrhodamine-labelled BDNF (R-BDNF) was far lower than expected. Tetramethylrhodamine conjugates are prone to aggregation[2,3] so this finding should have aroused concern. Correlations[2] predict a size of 360–630 kDa for R-BDNF based on the value of D_f (4.57×10^{-7} cm^2 s^{-1}) reported by Stroh *et al.*[1], strongly suggesting aggregation. Had R-BDNF been stable in solution, D_f should have been similar to that earlier measured by Stroh *et al.*[4] for NGF (12.6×10^{-7} cm^2 s^{-1}; predicted size[2] 17–30 kDa).

Second, the effective diffusion coefficient, D_b, determined for 2 kDa PEG in neostriatum by Stroh *et al.*[1] ($19.4 \pm 15.1 \times 10^{-7}$ cm^2 s^{-1}; mean ± s.d.) yielded a surprisingly low tortuosity ($= (D_f/D_b)^{1/2}$) of 1.02. A tortuosity of 1.88 had been reported previously for a slightly larger PEG in a hippocampal slice preparation[5]. Because tortuosity describes tissue hindrance to diffusion, a value of 1 suggests PEG can diffuse in brain, an environment containing many obstacles, with no more difficulty than it does in water. If true, this would be a very important finding, so we attempted to verify it using integrative optical imaging[2]. Our preliminary data indicate, however, that PEG diffusion is significantly hindered, both in neostriatum and neocortex (Table 1). Tortuosity measured with PEG appears similar to that obtained with 74 Da tetramethylammonium (1.54 and 1.62 in neostriatum[5] and neocortex[6], respectively). We suspect the accuracy of the Stroh *et al.* data[1] was compromised by the substantial variability in some of their measurements.

We agree that diffusion analysis can be used to screen drugs and chemically modified drug conjugates. However, we must stress that high signal-to-noise measurements and careful interpretation are keys to unlocking the power of diffusion analysis for rational drug design.

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Table 1 PEG diffusion parameters measured in free medium (0.3% agarose) and in rat brain slices.

Brain area	Dilute agarose D_f (10^{-7} cm^2 s^{-1})	Brain slice D_b (10^{-7} cm^2 s^{-1})	Tortuosity $(D_f/D_b)^{1/2}$
Neostriatum	19.8 ± 0.6 (n = 12)	8.7 ± 1.1 (n = 7)	1.50
Neocortex		7.8 ± 0.9 (n = 6)	1.59

D_f (determined at 37 ± 0.5 °C and corrected to 34 °C) and D_b (determined at 34 ± 1 °C), refer to the free and effective diffusion coefficients (mean ± s.d.), respectively. PEG refers to the hydrolysed *N*-hydroxysuccinimidyl ester form of fluorescein-PEG-NHS-2000 (Nektar Therapeutics), as in Stroh *et al.*[1], where D_f was 20.2×10^{-7} cm^2 s^{-1}.

AUTHOR'S RESPONSE

The aim of the brain-derived neurotrophic factor (BDNF) study in question[1] was not to provide a validated set of diffusion coefficients. Rather, in this study we sought to exploit our method featuring multiphoton microscopy to guide the design of an improved therapeutic agent. Therefore, the experimental design and presentation emphasize this broader goal.

Previous to this, we had published a related study of the nerve growth factor (NGF) with the aim of validating the new method using multiphoton microscopy to obtain tissue diffusion coefficients[2]. In that work we designed the experiments and performed the analysis to emphasize the values of diffusion constants, and to test that these constants were similar to historical data or within predicted ranges.

In contrast, our specific goal in investigating the free diffusion coefficient for BDNF was to compare relative values of diffusion behaviour for our preparations in the brain. In accordance with this goal, we neither designed the experiments nor conducted the appropriate analysis to test against a predicted free diffusion coefficient. Although we agree with the points made by Thorne *et al.*, and we appreciate the value of the new measurements of PEG diffusion that they provide, we believe that the data in Table 1 of ref. 2 demonstrated the relative differences in diffusion coefficient sufficiently to support the approach to protein modification that we pursued, even with the difficulties that Thorne *et al.* identify.

With regard to the specific comments of Thorne *et al.*, we considered the possibility that aggregation influenced the diffusion measurements. (The influence of aggregation on protein diffusion is a familiar issue for us: see Radomsky *et al.*[3]) Our NGF diffusion measurements, which Thorne *et al.* cite as a benchmark for comparison against the BDNF results, were likewise obtained using a tetramethylrhodamine (TMR) conjugate[1]; aggregation was clearly not an issue in that data. In addition, gel electrophoresis of our BDNF preparations did not reveal an unexpected, high-molecular-weight product (the conjugate migrated as expected), the TMR–BDNF conjugate was bioactive, and the material was extensively centrifuged before measurement. All of these observations led us to conclude that the labelled proteins were suitable for study.

Regarding the comments on polyethylene glycol (PEG), our goal was not to validate a brain diffusion constant for PEG. It was rather to contrast to the behaviour of the PEG–BDNF conjugate, and to provide evidence to the reader that we were monitoring the latter rather than free PEG diffusion in the brain. We feel that Table 1 and Figs 3d and e of the manuscript[1] convincingly demonstrate that we were not measuring the diffusion of free PEG in tissue, but the diffusion of a BDNF–PEG conjugate. We agree that the tortuosity reported for PEG in the correspondence by Thorne *et al.* (~1.6) is different from ours (~1.0), and more in line with past measurements using their technique. However, we note that Thorne *et al.* contrast their results from a single-photon methodology with our multiphoton results, which could perhaps account for some of the discrepancy, as suggested by some recent reports[4]. More measurements, and side-by-side comparisons, are probably needed to fully address this issue.

Although we agree that it is important to be careful in the interpretation of diffusion data, we do not find that variability in our measurements is an issue that compromises our ability to design an improved drug.

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