374.15: Empirical Testing Of A Diffusion Model For Central Injection Of Peptides Important In Feeding And Obesity

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Background

Many experimental manipulations of behavior in the neurosciences rely on site-specific application of agents that bind to endogenous receptors within the central nervous system. However, uncertainty over the diffusion of applied agents from the site of injection often makes interpretation of such experiments problematic. As concentration of a test substance is important in eliciting an effect, injection volume, distance from intended target and time since injection might affect the biological activity of the injectate. While many attempts to examine diffusion of injectate within the brain have been performed, many studies do not consider local uptake of injectate by endogenous receptors. We present here a study designed to empirically test a diffusion model (Fig 1) proposed by Nicholson [1] and modified by Nicholson and Tao [2] using the peptide hormone neuropeptide-Y (NPY), an endogenous signaling peptide important in the control of ingestive behavior [3, 4].

The Nicholson model (Fig 1) incorporates measures of the diffusion constant (*D*) for the tissue, tissue tortuosity (λ, the physical limitations on movement imposed by the substrate), the extracellular space fraction (α) in which diffusion occurs, uptake by receptor binding (k'), the initial concentration and volume of injectate (V), and the time (t) since delivery of peptide. In the equation, the corrected apparent diffusion constant $D^* = D/\lambda^2$. The equation yields a concentration (C, in mM) of injectate at a given time t and distance r from the point of release. This model predicts that receptor uptake, elapsed time and distance of target from injection site should most strongly affect diffusion, while volume of injection should have little effect. To test this model, we conducted a series of studies using NPY injected into the rat paraventricular hypothalamic nucleus (PVH), an area which contains a dense concentration of NPY receptors [5, 6]. We tested (1) the effects of injection volume on diffusion, (2) the effect of volume on behavioral response to peptide, and (3) the actual diffusion of a known quantity of peptide, and compared our results with predictions from the model.

Methods

For all experiments, adult male Sprague-Dawley (SD) rats were fitted with a unilateral stainless steel guide cannula stereotaxically directed at the PVH (AP: -1.9; LM: -.05; DV: -7.3; all measures = mm from bregma) for injection of peptide. In all cases cannula placement was verified post-injection and data from animals with misplaced cannulae were excluded from analysis. Values for constants in the Nicholson equation were based on published estimates for diffusion of small molecules through the brain as follows: $D = 2.28 \times 10^{-5}$ [7], $\alpha = 0.2$ [7], $\lambda = 1.77$ [2], and k' was constrained at 0 to assume zero uptake (the null hypothesis).

Experiment 1: To determine volume effects on peptide diffusion, approximately 20,000 CPM [125]I-labeled NPY in 0.25 (n = 4) or 1.0 µl (n = 4) vehicle was injected into the rat PVH. After 1 h, animals were sacrificed by decapitation, and unfixed brains were sectioned using a cryostat. Total radioactivity (CPM) of every 100 µm tissue section from 1.5 mm anterior to 1.5 mm posterior to the injection site was analyzed using a gamma counter.

Experiment 2: To study the effect of injection volume on NPY-induced food intake, a second group of rats (n = 10) received PVH injections of saline or 0.5 µg NPY dissolved in 0.25, 0.5, or 1.0 µl vehicle in a repeated measures study. Food intake (FI) over 4 h and latency to feed (LTF) were monitored.

Experiment 3: To compare diffusion of peptide with predictions from the model, [125]I-labeled NPY was mixed with unlabeled NPY to a final concentration of 0.234 mM NPY with a specific activity of approximately 40,000 cpm / µl. A third group of rats (n = 9) was given a single 0.5 µl injection of this mixture, containing a total of 117 pmol NPY in 0.5 µl vehicle. This treatment was intended to reflect a typical NPY dosage and injection volume used in our lab for feeding studies. To control for speed of injection, a Stoelting automated microstepper was used to deliver all peptide in this experiment at a fixed speed of 1 µl / min. Tissue was collected 1 h post-injection and CPM for every 100 µm section from 1 mm anterior to 1 mm posterior to injection site was determined as described above. Points were graphed and fitted using a standard linear regression to estimate X and Y intercepts.

Fig. 1: Nicholson Diffusion Model

$$C = \frac{V}{\alpha(4D^*t\pi)^{3/2}} \times e^{\left(-\frac{r^2}{4D^*t} - k't\right)}$$

C = Concentration (mM) at given time and distance
V = Dose injected (mM cm^3)
α = Volume fraction of extracellular space (unitless)
D^* = Apparent diffusion constant (cm^2×s^{-1}), corrected for tortuosity
t = Time (s) following injection
r = Distance (cm) from injection site
k' = Linear uptake constant (s^{-1})

Figure 1: Nicholson diffusion model, as modified by Nicholson and Tao [2]. Estimated values for constants used in this study are defined in the Methods.

Fig. 2: Volume Effects on NPY Diffusion (t = 60 min)

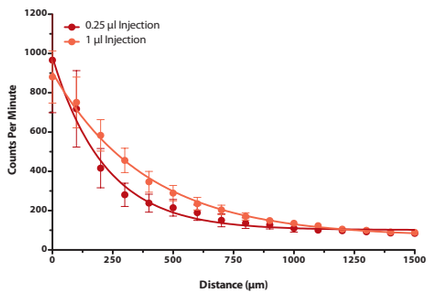

Figure 2: Diffusion over 60 min of 20,000 CPM [125]I-NPY in 0.25 or 1µl vehicle injected into the PVH. No significant differences were found, suggesting NPY volume does not affect diffusion.

Fig 3: Volume Effects on Food Intake

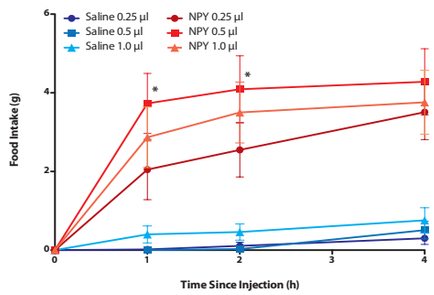

Figure 3: Food intake (g) following PVH injection of vehicle or 0.5 µg NPY in 0.25, 0.5 or 1µl vehicle. Significant difference between NPY 0.5 µl and NPY 0.25 µl groups indicated by *.

Fig 4: Volume Effects on Latency to Feed

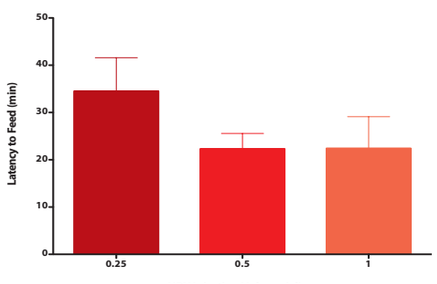

Figure 4: Latency to feed (min) following PVH injection of 0.5 µg NPY in 0.25, 0.5 or 1µl vehicle. Together Figs. 3 and 4 suggest that injection volume does not affect behavioral response to NPY.

Fig. 5: Actual Diffusion of 117 pmol NPY (t = 60 min)

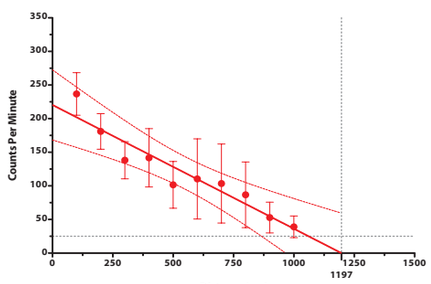

Figure 5: 60 min diffusion of 117 pmol [125]I-NPY in 0.5 µl vehicle injected into the PVH. Estimated X intercept is less than predicted in Fig. 6, suggesting receptor uptake limits diffusion of NPY.

Fig. 6: Theoretical Diffusion for 117 pmol NPY (t = 60 min, k' = 0)

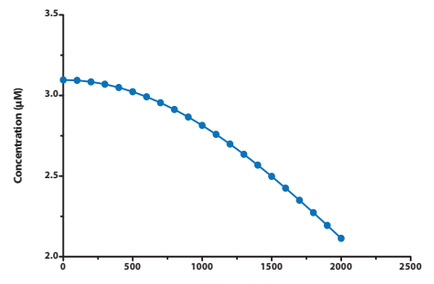

Figure 6: Theoretical 60 min diffusion of 117 pmol NPY in 0.5 µl vehicle injected into the PVH, assuming no uptake of peptide by receptors (k = 0). Note estimated X intercept is ≥ 2000 µm.

Results

Experiment 1: Approximately three quarters of injectate was found within 0.5 mm of the injection site after 60 min, with no significant differences in diffusion between injection volumes in sections up to 15 mm from the injection site (Fig. 2). **As predicted by the Nicholson model, the total volume of injectate does not appear to influence the overall diffusion of NPY.**

Experiment 2: While all concentrations of NPY elicited significantly higher FI than controls (Fig 3), rats receiving NPY in 0.25 µl ate significantly less than those receiving NPY in 0.5 µl at 1 and 2 hours (p = 0.026 and 0.050, respectively). Injection volume did not significantly affect LTF (Fig. 4), but there was a trend for higher LTF in the rats that received NPY in 0.25 µl. **These results show that there is no apparent bias in peptide activity due to injection volume**, and support the conclusion of Experiment 1 that volume of injected peptide does not strongly affect diffusion. However, the reduced FI and increased LTF for animals receiving NPY in 0.25 µl suggests that there are physical limits not addressed by this model; at too high a concentration an injected substance might too densely fill the extracellular space, increasing tortuosity, while at too low a concentration tissue distension from the volume injected may physically damage the target region, limiting responsiveness to injectate.

Experiment 3: At t = 60 m, linear regression showed 220.5 ± 26.63 CPM [125]I-NPY at the point of injection, with an estimated X intercept of 1197 µm (Fig. 5) indicating little to no spread of injectate 12 mm from the injection site. Using the same V and t values for this study, and constraining k' at 0 to assume no uptake, the Nicholson model predicts a much greater diffusion of peptide than observed here (Fig. 6). However, counts past 1 mm from the injection site approach the background limits of the gamma counter used (25 CPM, horizontal dashed line), suggesting that injectate beyond this point may not be reliably detectable. Also note that peptide recovery was lower in this study than in Experiment 1, as a larger proportion of peptide remained in the cannula after injection. Despite these caveats **the observed NPY diffusion strongly suggests that uptake of peptide by endogenous receptors significantly limits the actual spread of peptide following point injection.**

Conclusions

Experiment 1 supports the Nicholson model in that volume did not physically affect NPY diffusion. While **Experiment 2** supports this conclusion by showing that injection volume does affect the biological response to NPY, the reduced response to NPY at 0.25 µl suggests physical limits to this model. **Experiment 3** supports the model by indicating that endogenous receptor binding does appear to restrict diffusion to a significantly smaller area than that predicted if no uptake occurred.

Collectively, these results show that injections of neuropeptide primarily affect an area within a 1 mm radius centered on the target. In addition, these data suggest that attempts to limit diffusion by using reduced injection volumes may be unnecessary, and in extreme cases might negatively impact delivery of peptide to the target.

Acknowledgements

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References

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