Mapping neuronal populations that induce behavioral state changes after pharmacological activation requires discrete localization of drug injection sites, and is limited by widespread diffusion of molecular drugs. Nanospheres with diameters of 50–100 nm can reduce diffusion significantly because of their relatively large sizes. The cholinergic agonist carbachol was radiolabeled with methyl$^{14}$C and incorporated within a latex nanosphere delivery system (LNDS). We quantitatively compared diffusion of $^{14}$C-carbachol within these nanospheres with that of free $^{14}$C-carbachol, demonstrating approximately ten-fold reduced radial diffusion by nanospheres 10 min to 24 h post-injection; approximately 90% of injected radioactivity was restricted to regions within approximately 100–150 µm and 1400–1500 µm respectively. Thus, incorporation of active agents such as drugs within nanospheres dramatically increases the precision of their delivery in-vivo (here about 1000-fold by volume).

**Key words:** Diffusion, Carbachol, Nanospheres, Autoradiography

**Introduction**

Microinjection techniques for the central administration of neuroactive compounds that affect various neurotransmitter systems have yielded much useful information regarding regulation of behavioral and physiologic functions. Within the field of sleep physiology, the microinjection of cholinergic agonists into the feline anterodorsal pontine tegmentum induces an immediate and prolonged rapid eye movement (REM) sleep-like behavior. Despite the potency and specificity of this method, one serious disadvantage is the widespread extent of diffusion from the injection sites of free drug, and identification of neurons that induce behavioral state changes upon activation. Microinjection of neuroactive substances has been used to selectively activate or inhibit functionally defined pathways. Studies have shown that the injection of volumes as small as 100 nl produce extensive diffusion. This is important when mapping distributed systems such as the putative neuronal network associated with the generation of REM sleep.

To solve some of these problems, we developed a pharmaceutically active retrograde fluorescent probe offering advantages over presently available enzyme tracers and fluorescent dyes. More recently, a distinct but related family of latex nanosphere delivery system (LNDS) probes has been developed, capable of carrying a wide variety of active agents and fluorochromes. The relatively large size of these nanospheres (approximately 50–100 nm diameter) theoretically limits diffusion significantly compared with that of molecular-sized agents. Microscopic analysis of injection sites using such nanospheres with incorporated fluorochromes reveals extremely restricted spread of approximately 100 µm, suggesting that active pharmacologic agents incorporated within LNDS particles would exert their effects within a very small and well-defined region. In order to compare diffusion of free drug at a specific injection site with the microscopically more limited diffusion of these new probes more accurately, and in order to assure stability of pharmacologic agents within the LNDS polymer matrix following injection in-vivo, the cholinergic agonist carbachol was radiolabeled with methyl$^{14}$C and incorporated within latex fluorescent nanospheres (gift of C. Thies, Washington University). We compared diffusion of latex nanospheres containing $^{14}$C-carbachol with that of free radiolabeled $^{14}$C-carbachol.

**Materials and Methods**

Fourteen adult mice (C57B/6J) were used in these studies. Mice were deeply anaesthetized with Avertin, and pressure microinjections were performed within motor cortex using pulled glass micropipettes with tip diameters of approximately 50 µm. Each mouse received bilateral injections with 50 nl volume over 2 min at a depth of 500–550 µm. The motor cortex of one hemisphere was injected with free $^{14}$C-carbachol and the contralateral motor cortex was injected with LNDS nanospheres containing $^{14}$C-carbachol. All injections ($n=28$) contained equal concentrations of carbachol (0.8 µg 50 nl$^{-1}$), chosen to match that used previously with active pharmacologic effects. Radioactivity was constant at 0.026 µCi per 50 nl of injected volume.

After survival times of 10 min ($n=2$), 1 h ($n=4$), 4 h ($n=4$), 24 h ($n=2$), and 7 days ($n=2$), mice were
deeply anaesthetized with Avertin and decapitated. In order to avoid artificial spread of radioactivity by perfusion, fixation, or histological processing, brains were removed immediately, quick-frozen by immersion in liquid nitrogen, serially sectioned at 50 μm, thaw-mounted and dried, and exposed to Hyperfilm Betamax (Amersham, Arlington Heights, IL) at -80°C for 3 weeks. Autoradiographic [¹⁴C] Microscales (Amersham) with calibrated activities of 30–880 nCi gm⁻¹ were used as quantitative calibration standards. Autoradiographic images from serial sections were optically scanned using an automated Optronics P-1000 scanning drum densitometer at a resolution of 25 μm, and pixel density values representing tissue radioactivity values were quantitatively analysed and digitized for three-dimensional reconstruction using a VAX-based image analysis system and quantitative analysis software⁹ (Image Graphics Laboratory, Children's Hospital, Boston).

Two types of analysis were performed: qualitative inspection for relative radial diffusion within all cases, and quantitative analysis of radial diffusion within two cases each at 10 min, 1 h, 4 h and 24 h. We analysed only the largest appearing LNDS injection sites at each time point, as diffusion was restricted to two or fewer sections in the other LNDS cases. Quantitative analysis was not performed in those cases to prevent underestimation of diffusion and to ensure the most conservative analysis. The image analysis system was used to generate contours at each of 15 radioactivity levels, and average diameters of these contours were calculated. In order to standardize comparison and to allow interpretation within the context of standard diffusion theory,¹⁰ radial distance values were compared at the 1/e point (37% of peak radioactivity level). Three-dimensional reconstructions of both free carbachol and LNDS injection sites were produced from one representative case to aid visual comparisons of their respective volumes of diffusion.

**Results**

There was dramatically less radial diffusion from the nanosphere injection sites than from the free drug sites at all observation times. Nanosphere sites tended to remain extremely localized over the 24 h allowed for diffusion, while the free carbachol sites enlarged considerably during the first 10 min to 1 h. The differences were readily and directly visible from the primary autoradiograms (Fig. 1A). Nanosphere injection sites displayed extremely small, punctate central regions of intense radioactivity with only a relatively small surrounding area of low level signal. Free carbachol injection sites displayed a large central area of relatively high level radioactivity with an extensive gradient of autoradiographic density extending from that zone.

Quantitative assessment reinforced these subjective impressions. Figure 1 demonstrates the two-dimensional distribution of free carbachol and nanosphere injection sites 10 min after injection. The 1/e point of free [¹⁴C]-carbachol diffusion is approximately 1400 μm, ten times larger than the 1/e point for the LNDS site (approximately 140 μm). Ninety percent of the peak level of radioactivity of a free [¹⁴C]-carbachol

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**FIG. 1.** One-and two-dimensional distributions of free [¹⁴C]-carbachol (FREE) and [¹⁴C]-carbachol incorporated within latex nanospheres (LNDS), 10 min after microinjection into mouse motor cortex. A. Autoradiogram showing bilateral injection, significant radial dispersion of the free carbachol, and restriction of the LNDS site to a punctate zone with little surrounding activity. B. Quantitative densitometry from autoradiogram in A showing the mean radial diffusion distances of free [¹⁴C]-carbachol at standardized radioactivity levels, and the '1/e radius' of diffusion. C. Quantitative densitometry from the contralateral LNDS injection site.
injection site is present 500 \( \mu m \) from the center almost immediately, and \(^{14}C\)-carbachol activity can be detected more than 1.6 mm from the focus of the injection. In comparison, 90\% of the peak level of radioactivity of the LNDS injection extends less than 50 \( \mu m \) from the center, and less than 10\% of the peak level can be detected 150 \( \mu m \) from the focus of injection.

Over time, the free \(^{14}C\)-carbachol injection site expanded in radius to 2–2.5 mm at the 1/e point at 1 h, then decreased in size by 4 and 24 h to approximately 1 300 \( \mu m \) and 1 000 \( \mu m \) respectively (Fig. 2). This diminution in size of the region containing \(^{14}C\)-carbachol is probably due to dilution, metabolic degradation, and vascular clearance of the \(^{14}C\)-carbachol within the surrounding tissue. In one case of LNDS injection analysed at 1 h, local spread extended to approximately 500 \( \mu m \). In all other cases of LNDS injections analysed at 1 h, diffusion was restricted to less than 100 \( \mu m \) (2 sections or less) and not quantified. In all cases at 4 and 24 h, diffusion was limited to approximately 100 \( \mu m \), indicating no significant dispersion or degradation of the LNDS particles over the times studied.

Figure 3 demonstrates the three-dimensional distribution of \(^{14}C\)-carbachol surrounding free drug and LNDS injection sites at 10 min post-injection. The most dramatic differences are the approximately 1000-fold restrictions of both the central volume with greater than 90\% of peak activity levels and the '1/e boundary' where activity levels have decreased by 63\%. Integrating the total radioactivity within these boundaries, computer-generated quantitative values reveal that approximately 90–95\% of the total radioactivity injected is contained within the '1/e boundary' in both free carbachol and LNDS cases. These data indicate that incorporation of carbachol within LNDS particles limits the volume of distribution of \(^{14}C\)-carbachol in tissue approximately 1000-fold. This extremely limited diffusion and high degree of stability within the CNS typically restricts approximately 90\% of the drug to a well-defined zone of 50–150 \( \mu m \) in radius.

**FIG. 2.** Time course of diffusion following injection of free \(^{14}C\)-carbachol and \(^{14}C\)-carbachol incorporated within LNDS nanospheres. Each bar represents radial distance measurements from an individual injection site, using serial section autoradiograms and computer-automated contour generation to calculate radius from the center of the injection site to the '1/e contour'. The other LNDS cases exhibited diffusion radii restricted to two or fewer sections (100 \( \mu m \)), and were not quantified.

**FIG. 3.** Three-dimensional computer reconstructions from serial section autoradiograms, 'cut out' to allow visualization of three volume contours: red represents 90\% of the peak radioactivity level, green represents 37\% (1/e) of the peak activity level, and blue represents less than 5\% of the peak level. A. Free \(^{14}C\)-carbachol injection site at 10 min post-injection. B. Contralateral LNDS injection site at 10 min post-injection. Scale bars = 1 mm.

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Discussion

The degree of localization achieved by the microinjection of drugs contributes to the efficacy of these procedures in defining functional anatomic networks. Neuropharmacological studies using central microinjection techniques are based on the delivery of a finite volume and activity of drug at a specific anatomic site. The interpretation of such studies describing behavioral effects requires an understanding of the distribution of the active drug within the tissue under study. Widespread diffusion from a drug injection site limits this understanding of how observed behavioral state changes might be triggered under physiological conditions. The power and interpretation of such studies would be greatly enhanced if drugs of interest can be localized to a precisely defined injection site. Because LNDS particles have such limited diffusion, experiments will now be possible in which LNDS particles are injected into discrete sites of interest, pharmacological effects behaviorally and physiologically assessed, and circuit neurons associated with these effects anatomically identified.

In one such model system in which the cholinceptive induction of REM sleep is being studied, LNDS particles provide a potentially powerful tool to better understand and determine the relative efficacy of a specific brain injection site in eliciting pharmacologically induced behavior. The area within the anterodorsal pons that generates REM sleep when activated by microinjection of cholinergic agonists is found to be devoid of cholinergic neurons.11 Cholinergic inputs to this activation site must therefore come from elsewhere, and may project from relatively distant and separate neuronal groups. Within this cholinceptive pontine region, it has been shown that a non-linear relationship exists between cholinergic potency and distance from the optimal short REM latency injection site suggesting a variable, diffusion-based delay of drug to distant neuronal populations involved in REM sleep state production.12,13 However, the source of cholinergic input to a well-defined induction zone of cholinceptive ‘REM-on’ neurons remains to be determined. Using carbachol-fluorescent probes and immunohistochemical techniques, we are beginning to analyse with a new precision the anatomic and neurochemical signatures within this multicellular network involved in REM sleep generation. When combined with microelectrode recording to characterize activated neurons electrophysiologically with more precision, assessment of the injection locus in a functional context may now be possible.

LNDS particles will also be of great value in limiting delivery of photoactive agents to defined subpopulations of neurons in order to effect cell-type specific lesioning studies using deeply penetrating long-wavelength light energy.14,15 Such studies are now providing a model of neocortical injury and degenerative disease for developmental and transplantation studies.16 Applications exist toward selective ablation of specific cellular elements within a distributed network and of pathologic cells within otherwise normal structures.17 Highly restricted localization of the injection site of retrograde probes carrying photolytic chromophores provides additional selectivity to these methods.

Conclusion

A wide range of applications is now possible with this discrete localization of effective drug or photoactive agent injection sites using LNDS particles. These probes may significantly refine our understanding and provide new information regarding putative neuronal populations involved in the neurochemical basis of behavior, and allow increased selectivity in a variety of experimental approaches to distributed neuronal networks.

References


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