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Mapping Neuronal Inputs to REM Sleep Induction Sites with Carbachol-Fluorescent Microspheres

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The cholinergic agonist carbachol was conjugated to latex microspheres that were fluorescently labeled with rhodamine and used as neuroanatomical probes that show little diffusion from their injection site and retrogradely label neurons projecting to the injection site. Microinjection of this pharmacologically active probe into the gigantocellular field of the cat pontine brain stem caused the awake cats to fall into rapid eye movement (REM) sleep indistinguishable from that produced by free carbachol. Three-dimensional computer reconstruction of the retrogradely labeled neurons revealed a widely distributed neuronal network in the pontine tegmentum. These pharmacologically active microspheres permit a new precision in the characterization and mapping of neurons associated with the control of behavioral state and of other cholinergic networks.

THE EVOLUTION OF TECHNIQUES for the central administration of pharmacological agents to mimic, enhance, or block the various neurotransmitters involved in the regulation of behavioral and physiologic functions has been dramatic in recent years. Within the field of sleep physiology, for example, the microinjection of cholinergic agonists into the brain stem pontine tegmentum of adult cats elicits immediate and prolonged rapid eye movement (REM), or desynchronized (D), sleep signs and "D sleep"–like behavior (*1*).

However, these cholinergic agonists show unacceptably wide diffusion within tissue. They can diffuse a mean radial distance of 1 mm from the injection site 1 hour after injection, even when volumes as small as 100 nl are used (*2*). Such widespread diffusion precludes discrete localization of injection sites and confounds the identification of neuronal populations that, once activated, generate the observed changes in behavioral state.

Similarly, neuroanatomical tracers also diffuse widely and do not identify a discrete population of neurons projecting to the injection site, limiting the understanding of how activation might be triggered under physiological conditions. This is particularly important when mapping the brain stem network associated with D sleep. The locus within the anterodorsal pontine tegmentum that produces D sleep when activated by cholinergic agonists has no cholinergic neurons (*3*). Inputs to this activation zone must, therefore, come from elsewhere and may project from several relatively distant neuronal groups. Because D sleep signs are enhanced by blockade of β-adrenergic receptors (*4*), it is also important to determine whether noradrenergic neurons project to this cholinoceptive zone.

We have developed a new retrograde

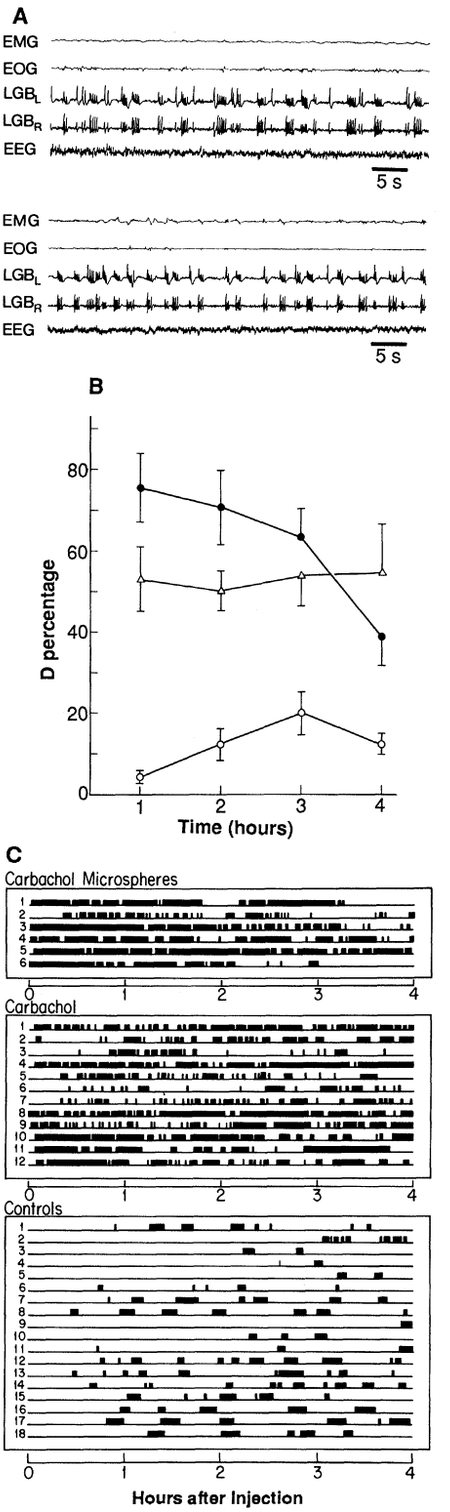

Fig. 1. (**A**) Polygraphic recordings illustrate D sleep induced by free carbachol (top) and that induced by carbachol microspheres (bottom). (**B**) The D% at each hour of recording time is significantly greater for both carbachol microspheres ($n = 6$; $F = 117.87$; $P < 0.0001$) and carbachol ($n = 12$; $F = 113.74$; $P < 0.0001$) than for controls ($n = 18$) (repeated measurement analysis of variance with Scheffe test). Values and error bars represent mean ± SEM. ●, Carbachol microspheres; △, carbachol; ○, controls. (**C**) Temporal distribution of D sleep episodes is shown for each trial by horizontal black bars.

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probe that has extremely limited diffusion and high pharmacological potency, which offers advantages over conventional techniques. To restrict diffusion (*5*) and to retrogradely label neurons projecting to the specific injection site, carbamylcholine chloride (carbachol) was conjugated to fluorescent latex microspheres (*6*), which have been used as a retrogradely transported fluorescent tracer. Carbachol is a mixed muscarinic-nicotinic cholinergic agonist that is useful in behavioral studies for activating cholinoceptive neurons (*7*). Using carbachol conjugated to fluorescent microspheres, we have mapped inputs to brain sites that produce a behavioral state change from waking to D sleep after cholinoceptive activation by carbachol.

Four adult male cats (3.5 to 5.0 kg) were prepared for surgery, anesthetized with pentobarbital (35 mg per kilogram of body weight, intraperitoneally) and implanted with electrodes for recording electroencephalogram (EEG), electrooculogram (EOG), electromyogram (EMG), and ponto-geniculo-occipital (PGO) waves. Bilateral stainless steel guide tubes were stereotaxically aimed [2.0 to 2.5 mm posterior (P), 1.5 to 2.0 mm lateral (L), −4 mm vertical (V), $\theta = 38°$ from vertical] toward the anterodorsal pontine reticular formation (*8*) and cemented in place with dental acrylic. This electrode-guide tube assembly permits recording in freely moving, unrestrained cats under normal conditions (*9*).

In order to establish baseline control values, electrophysiologic activity was recorded during four sessions for each of the four cats ($n = 16$), between 3 and 4 weeks after surgery. We then inserted stainless steel injection cannulae through the fixed guide tubes and performed extracellular pressure microinjections of 250 nl over 30 s. Twenty injections were made in four unanesthetized awake cats at six sites (two unilateral and four bilateral); these trials consisted of experimental and control injections of carbachol alone ($n = 12$; 4 μg/250 nl); carbachol-fluorescent microspheres ($n = 6$; 13 μg/250 nl); microspheres without carbachol ($n = 1$; 13 μg/250 nl); and a control solution consisting of the final dialysate without microspheres concentrated 100:1 from the carbachol-fluorescent microsphere conjugation ($n = 1$). We analyzed the conjugation with labeled [methyl-^{14}C]carbachol and demonstrated binding of carbachol to the surface of microspheres (*10*). Pharmacologic effects of microinjections were assessed by computer scoring (*11*) of polygraphic recordings of behavioral states for 4 hours immediately after each injection.

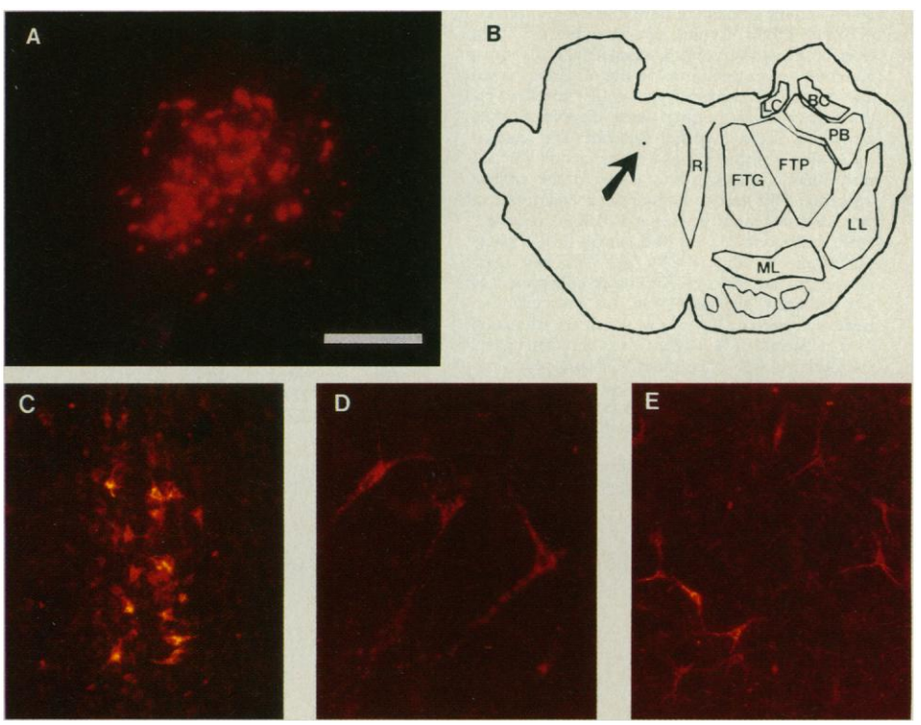

Fig. 2. (**A**) Carbachol-fluorescent microsphere injection site has a mean diameter of 200 μm and occupies a small well-defined volume of 0.004 mm^3. Bar, 100 μm. (**B**) Computer digitization of a single transverse section containing injection site (arrow), shown in (A). Nuclear boundary contours delineate dorsal raphe (R), FTG, FTP, LC, peribrachialis (PB), brachium (BC), lateral (LL), and medial (ML) lemniscus. Injection site is located at the lateral border of the FTG contiguous with the dorsomedial extension of the FTP. Retrogradely labeled fluorescent neurons are illustrated in (**C**) dorsal raphe (×120), (**D**) locus ceruleus (×232), and (**E**) FTG (×120).

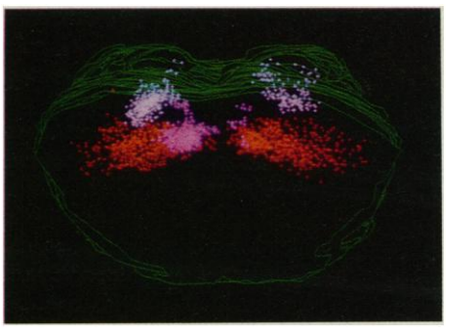

Fig. 3. Rostrocaudal (1.9 mm anterior to 5.0 mm posterior) three-dimensional computer reconstruction of serially registered transverse brain stem section contours (green) containing retrogradely labeled digitized cell items within three cholinergic nuclei. Pedunculopontine tegmentum (orange) demonstrates bilateral labeling with highest density along its anteromedial aspect. Peribrachialis (white) is intensely labeled ipsilaterally with less prominent contralateral labeling caudally. Dorsolateral tegmentum (violet) demonstrates prominent ipsilateral labeling with relatively few contralateral labeled cells (VAX-based DEC graphics display system, Image Graphics Laboratory, Children's Hospital, Boston).

We found that carbachol-fluorescent microspheres were as effective as carbachol alone in inducing D sleep. Carbachol-fluorescent microspheres induced D sleep that was electrographically indistinguishable from that induced by free carbachol (Fig. 1A). The mean latency to D sleep onset after administration of carbachol-fluorescent microspheres was 9.0 ± 3.1 min compared with 9.3 ± 3.6 min after carbachol alone; both were significantly different ($P < 0.0001$; repeated measurement analysis of variance) from control recordings (71.2 ± 19.2 min). The mean percentage of recording time in D sleep (D%) was insignificantly greater after injection of carbachol-fluorescent microspheres (55.8 ± 9.6%) than after injection of carbachol alone (47.4 ± 8.3%), but both were significantly increased ($P < 0.0001$) compared to control injections (12.2 ± 3.1%) (Fig. 1B). The D% was actually greater during the first 2 hours (75.8 ± 8.4% and 71 ± 9.3%) for carbachol-fluorescent microspheres than for carbachol (51.3 ± 8.1% and 50.3 ± 5.6%). In contrast, by the fourth hour, D% was greater with carbachol (54.7 ± 7.2%) than with carbachol-fluorescent microspheres (39.3 ± 12.1%). The effect of carbachol-fluorescent microspheres on the temporal distribution of D sleep episodes (Fig. 1C) demonstrates a significant difference (*t* test with Bonferroni correction; $P < 0.05$) compared to controls, but not compared to free carbachol ($P > 0.05$).

The retrograde labeling of neurons by this pharmacologically active probe anatomically defined the neuronal network projecting to

our discrete injection sites within the pontine tegmentum. Observations from one effective injection site (2.53 P, 1.62 L, −4.13 V) (Fig. 2, A and B), histologically identified at the junction of the dorsolateral gigantocellular tegmentum (FTG) and dorsomedial paralemniscal tegmentum (FTP), indicate that labeled inputs to this cholinoceptive trigger zone for D sleep are localized within discrete nuclear boundaries. Labeling of the dorsal raphe (DR), locus ceruleus (LC), and FTG (Figs. 2, C through E) revealed a contiguous topographical distribution of cells within these neuronal groups, which are of particular interest because their state-dependent firing properties suggest involvement in D sleep generation (*12*).

Retrogradely labeled cells found in nuclei known to contain cholinergic neurons (*13*) are shown in Fig. 3; the pedunculopontine tegmentum (PPT, Ch5), the parabrachialis (PbN, Ch5), and the dorsolateral tegmentum (DLT, Ch6) contain prominent contralateral (PPT) and ipsilateral (PbN, DLT) projection neurons (*14*).

Our documentation of these labeled input patterns illustrates the widely distributed network involved in the generation of D sleep. Although we have not yet defined the chemical identity of these projection neurons, it is clear that the identified cholinoceptive trigger zone for D sleep receives prominent inputs from known cholinergic nuclei (PPT, PbN, and DLT); such a pathway could mediate activation of the putative D sleep generator network under both physiological and pharmacological conditions (*15, 16*). The labeled inputs from known aminergic sources (DR and LC) provide a possible substrate for the reciprocal suppression of the D sleep generator that has been suggested by studies with extracellular recording (*17*), pharmacologic stimulation (*18*), and selective lesioning techniques (*19*).

Determination of the chemical nature of the afferent projections will now be possible by combining the retrograde transport capability of carbachol-fluorescent microspheres with immunohistochemical identification of choline acetyltransferase for cholinergic neurons, and tyrosine hydroxylase for noradrenergic cell groups. It may also be possible to use simultaneously an anterograde labeling approach with phaseolus vulgaris leucoagglutinin to identify output termination patterns from the microsphere injection sites.

This pharmacological activation of microspheres opens new avenues for a "behavioral anatomy" of the mammalian brain that recognizes the practical complexity of neuronal population dynamics.

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