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Dorsomedial pontine neurons with descending projections to the medullary reticular formation express orexin-1 and adrenergic α_{2A} receptor mRNA

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ABSTRACT

Neurons located in the dorsomedial pontine rapid eye movement (REM) sleep-triggering region send axons to the medial medullary reticular formation (mMRF). This pathway is believed to be important for the generation of REM sleep motor atonia, but other than that they are glutamatergic little is known about neurochemical signatures of these pontine neurons important for REM sleep. We used single-cell reverse transcription and polymerase chain reaction (RT-PCR) to determine whether dorsomedial pontine cells with projections to the mMRF express mRNA for selected membrane receptors that mediate modulatory influences on REM sleep. Fluorescein (FITC)-labeled latex microspheres were microinjected into the mMRF of 26–34-day-old rats under pentobarbital anesthesia. After 5–6 days, rats were sacrificed, pontine slices were obtained and neurons were dissociated from 400 to 600 μm micropunches extracted from dorsomedial pontine reticular formation. We found that 32 out of 51 FITC-labeled cells tested (63 ± 7% (SE)) contained the orexin type 1 receptor (ORX1r) mRNA, 27 out of 73 (37 ± 6%) contained the adrenergic α_{2A} receptor (α_{2A}r) RNA, and 6 out of 31 (19 ± 7%) contained both mRNAs. The percentage of cells positive for the ORX1r mRNA was significantly lower ($p < 0.04$) for the dorsomedial pontine cells that were not retrogradely labeled from the mMRF (32 ± 11%), whereas α_{2A}r mRNA was present in a similar percentage of FITC-labeled and unlabeled neurons. Our data suggest that ORX and adrenergic pathways converge on a subpopulation of cells of the pontine REM sleep-triggering region that have descending projections to the medullary region important for the motor control during REM sleep.

Rapid eye movement (REM) sleep is a stage of sleep characterized by a combination of cortical and hippocampal activation, rapid eye movements, atonia of postural muscles with superimposed muscle twitches, and highly variable respiratory rate, heart rate and blood pressure. Many neurotransmitter receptor agonists and antagonist, including a cholinergic agonist, carbachol [1,4,7,11], $GABA_A$ receptor antagonist, bicuculline [3,21,24,26,38] and the peptide orexin (ORX) [14,37], can effectively trigger REM sleep-like state when injected into the dorsomedial pontine reticular formation. Other agonists have suppressant effects on REM sleep when injected into the same pontine region, e.g., agonists of α_2 adrenergic and type 1A and 2 serotonergic receptors [2,11,23,28,30]; reviewed in Ref. [16]. Some cells in the dorsomedial pontine reticular formation are immunoreactive for type 1 and/or type 2 ORX receptors [5,8,20] and are excited by ORX *in vitro* [6], and some cells express α_{2A} adrenergic receptors [29]. Importantly, it appears that stimulation of dorsomedial pontine type 2 ORX receptors promotes REM sleep and its atonia [14,37], whereas stimulation of either type 1 ORX receptors or α_2 adrenergic receptors suppresses REM sleep [2,22,30,35]. Therefore, we investigated the expression and co-expression of type 1 ORX and α_{2A} adrenergic receptor mRNAs in dorsomedial pontine cells that have axonal projections to the medial medullary reticular formation (mMRF), a region also important for the control of REM sleep and its motor atonia.

Among the efferent projection sites from the dorsomedial pontine REM sleep-triggering region [3,25,27], the mMRF received particular attention as potentially important for the generation of REM sleep and/or its motor atonia [15,18,28,31,36]. This very robust pathway is likely to be glutamatergic [17,19] and targets the medial magnocellular, ventral gigantocellular and medullary raphe nuclei [9,10,13,17,25,27]. Lesions of the mMRF region abolish or attenuate REM sleep and its atonia [12,31]. Since the atonia-controlling pathways can be modulated by both ORX and α_2 adrenergic receptors [2,14,22,30,35,37], it is of interest to determine whether these effects can be exerted on those cells within the dorsomedial pontine REM sleep-triggering region that have axonal projections to the mMRF. Here, we used single-cell reverse transcription and polymerase chain reaction (RT-PCR) to determine whether cells of the pontine REM sleep-triggering region that have axonal projections to the mMRF express mRNA for adrenergic α_{2A} receptor (α_{2A}r) and/or

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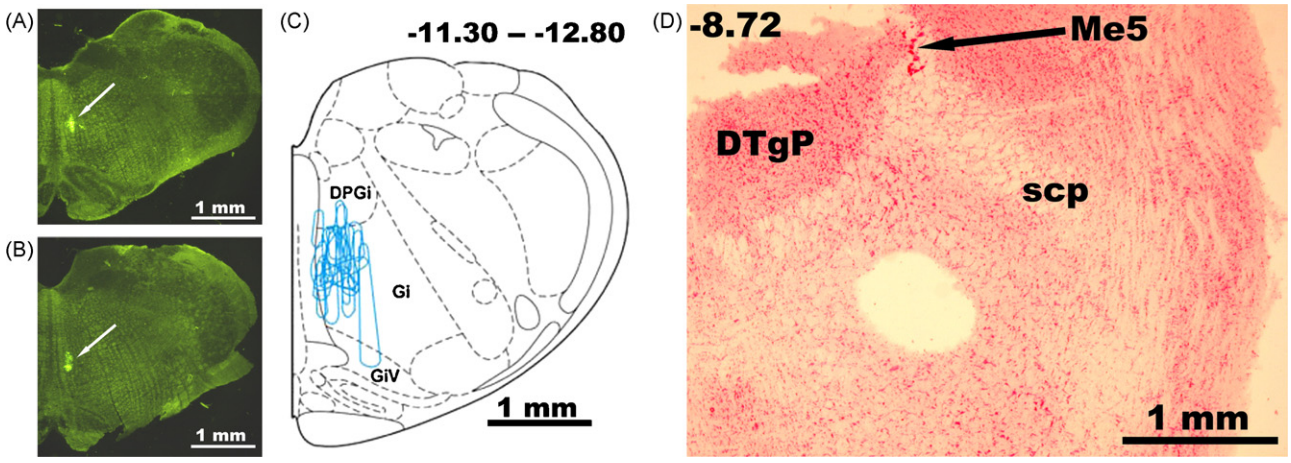

Fig. 1. FITC-labeled bead injection sites in the medial medullary reticular formation (A–C) and a typical location of a 600 μm micropunch of tissue taken from a pontine slice from a rat injected 5–6 days earlier with FITC-labeled beads (D). (A and B) Location of FITC deposit at two antero-posterior (AP) levels in one rat. (C) Injection sites as those shown in (A and B) from all animals superimposed on one standard cross-section located in the middle of the rostro-caudal span of the injected area that extended from AP-11.3 to AP-12.8 from bregma. Abbreviations: DPGi, dorsal paragigantocellular region; DTgP, dorsal tegmental region, pericentral; Gi, gigantocellular region; GiV, gigantocellular region, pars ventralis; Me5, mesencephalic trigeminal nucleus; scp, superior cerebellar peduncle.

type 1 ORX receptor (ORX1r). A preliminary report has been published [34].

Fifteen Sprague–Dawley rats were housed under a 12 h/12 h light–dark cycle with food and water available *ad libitum*. All surgical and animal handling procedures followed the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Fluorescein (FITC)-labeled latex microspheres (LumaFluor, USA) (100–450 nl; mean volume 248 ± 40 (SE)) were microinjected into the mMRF of 26–34-day-old pentobarbital-anesthetized rats. After 5–6 days, rats were sacrificed under deep isoflurane anesthesia (4%), and 1–2 coronal, 400 μm slices were cut from the level of the caudal inferior colliculus. The medulla was also extracted, fixed in formalin, and then sectioned and mounted to visualize the FITC-labeled microspheres injection site (Fig. 1A–C). Cells from the pontine reticular formation were dissociated as described previously [32,33]. Briefly, following enzymatic digestion with papain, micropunches (400–600 μm in diameter) were cut from the dorsomedial pontine reticular formation. The pontine slices from which the punches were extracted were fixed in formalin, cut into 20 μm sections and mounted to verify the punch locations (Fig. 1D).

Cells contained in the micropunch were mechanically dispersed, plated, and those with FITC-labeled beads were identified using an inverted phase contrast microscope equipped with fluorescent light source and FITC filters (IMT-2, Olympus, Japan) (Fig. 2A). Individual cells, labeled and unlabeled, were collected, their intracellular contents treated with DNAse and then subjected to reverse transcription, as described previously [32]. Aliquots of the resulting cDNA (each corresponding to ~1/3 of the total volume from each cell) were used in a two-stage, semi-nested PCR (Fig. 2B–D). The first round of amplification (35–37 cycles) was performed using a conventional thermal cycler (PCR Sprint; Thermo Hybaid, UK) and the second round with a real-time cycler (LightCycler; Roche Diagnostics, USA). Primers were designed using Vector NTI software (Invitrogen, USA). The criteria for primer specificity, reaction quality control and the strategy to optimize PCR conditions were described previously [32]. The primers used in this study included those for the ORX1r (accession: NM_013064) and the adrenergic α_{2A}r (accession: M62372). The sequences of the ORX1r primers were: 5′-GTGTCGGTGTCAGTGGCAGT-3′ (sense, first and second PCR rounds), 5′-TGAGGGTCGCTTCCAGTTCC-3′ (antisense, first PCR round), and 5′-GAAGAGCCGTGTGCGATTGG-3′ (internal antisense, second PCR round). The primer sets used for the α_{2A}r have been published previously [32]. The position and size of the melting curve peaks obtained in the second PCR round provided an initial assessment as to whether the expected cDNA was generated (Fig. 2C). Selected PCR products were then separated on ethidium bromide-stained 2% agarose gels to further verify that they were of the expected size (Fig. 2D). To control for false positive results, 8 non-reverse transcribed single-cell samples from two rats were amplified with all primers. To control for mRNA contamination of the medium, one sample of the fluid from above the plated cells was collected at the end of each cell collection session and subjected to the RT-PCR procedures identical to those with single-cell samples. None of those control reactions was positive.

The standard errors (SE) for estimation of the proportions of cells positive for distinct mRNAs were determined based on the size of each cell population and the corresponding statistical tests used the assumption of a random bimodal distribution (Analyse-It Software, Leeds, UK). Statistical comparisons of the percentages of cells expressing different mRNAs were conducted using Fisher exact test (Analyse-It Software). Differences were regarded significant at $p < 0.05$.

Ninety-seven FITC-labeled cells were collected from 15 rats, and an additional 32 unlabeled cells were collected from a subset of 9 rats from the same group of 15 animals (7 ± 0.9 (SE) and 4 ± 0.9 cells per animal, respectively). Following histological verification, 5 single-cell samples from 2 rats (4 FITC-labeled and 1 unlabeled) were excluded due to improper location of the punch. From the remaining 93 FITC-labeled and 31 unlabeled cells, 31 labeled and 2 unlabeled cells were tested for the presence of both the ORX1r and α_{2A}r mRNAs. cDNA samples from the remaining 62 labeled cells were tested for the presence of one mRNA species only, 20 for the ORX1r mRNA and 42 for the α_{2A}r mRNA. Similarly, for the remaining 31 unlabeled cell samples, 17 were tested for the ORX1r mRNA only and 12 for the α_{2A}r mRNA only. The remaining cDNA from these single-cell samples (~2/3 of the total volume) was used for testing and optimizing various primer sets.

Within the FITC-labeled population, 32 out of 51 cells tested (63 ± 7%) contained the ORX1r mRNA, 27 out of 73 (37 ± 6%) contained α_{2A}r mRNA (Fig. 3), and 6 out of 31 tested (19 ± 7%) contained both mRNAs. The proportion of cells positive for the ORX1r mRNA was significantly lower ($p < 0.04$) among the dorsomedial pontine cells that were not retrogradely labeled from the mMRF (6 out of 19, or 32 ± 11%). The proportion of FITC-labeled cells positive for the adrenergic α_{2A}r mRNA did not differ significantly from that for