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Narcoleptic orexin receptor knockout mice express enhanced cholinergic properties in laterodorsal tegmental neurons

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Abstract

Pharmacological studies of narcoleptic canines indicate that exaggerated pontine cholinergic transmission promotes cataplexy. Since disruption of orexin (hypocretin) signaling is a primary defect in narcolepsy with cataplexy, we investigated whether markers of cholinergic synaptic transmission might be altered in mice constitutively lacking orexin receptors (double receptor knockout; DKO). We found that choline acetyltransferase (ChAT), vesicular acetylcholine transporter (VACHT) and the high-affinity choline transporter (CHT1) but not acetylcholinesterase (AChE) were significantly higher in samples from DKO compared to wild-type (WT) mice. This was region-specific since levels were elevated in samples from the laterodorsal tegmental nucleus (LDT) and the fifth motor nucleus (Mo5) but not in whole brainstem samples. Consistent with region-specific changes, we were unable to detect significant differences in Western blots for ChAT and CHT1 in isolates from brainstem, thalamus and cortex or in ChAT enzymatic activity in the pons. However, using ChAT immunocytochemistry, we found that while the number of cholinergic neurons in the LDT and Mo5 were not different, the intensity of somatic ChAT immunostaining was significantly greater in the LDT, but not Mo5, from DKOs compared to WTs. We also found that ChAT activity was significantly reduced in cortical samples from DKOs compared to WTs. Collectively, these findings suggest that the orexins can regulate neurotransmitter expression and that the constitutive absence of orexin signaling results in an up-regulation of the machinery necessary for cholinergic neurotransmission in a mesopontine population of neurons that have been associated with both normal REM sleep and cataplexy.

Keywords

acetylcholine; narcolepsy; ChAT; VACHT; CHT1

Introduction

The orexin neuropeptides (also named hypocretins) are synthesized in a subset of lateral hypothalamic neurons (de Lecea *et al.*, 1998; Sakurai *et al.*, 1998) with projection throughout the brain and spinal cord (Peyron *et al.*, 1997). These peptides act via two G-protein coupled receptors (Sakurai *et al.*, 1998), named orexin 1 receptor (OX1R) and orexin

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2 receptor (OX2R) having widespread central expression (Trivedi *et al.*, 1998; Marcus *et al.*, 2001). Both reverse and forward genetic approaches led to the discovery that disruption of orexin signaling results in a sleep disorder, similar to human narcolepsy with cataplexy, in mice (Chemelli *et al.*, 1999; Willie *et al.*, 2003) and dogs (Lin *et al.*, 1999). In humans, narcolepsy is characterized by excessive daytime sleepiness, abnormal REM latency, sleep paralysis, fragmented sleep and cataplexy, a sudden muscle atonia triggered by emotional stimuli (Anic-Labat *et al.*, 1999; Hungs & Mignot, 2001). The findings in animals rapidly led to the discovery that loss of orexin peptides, probably through the loss of orexin synthesizing neurons, is a primary defect in human narcolepsy with cataplexy (Peyron *et al.*, 2000; Thannickal *et al.*, 2000).

While it is not known how the loss of orexin signaling produces the symptoms of narcolepsy/cataplexy, orexin fibers innervate numerous regions historically linked to emotions, motivation and arousal including the locus coeruleus (LC), dorsal raphe (DR), laterodorsal tegmental (LDT) and pedunculopontine tegmental (PPT) nuclei, which also express orexin receptors. Neurochemical studies in the canine model from the pre-orexin era, have strongly supported the idea that an imbalance in monoaminergic and cholinergic transmission promotes cataplexy. For example, enhanced cholinergic transmission in the medial pontine reticular formation (mPRF) – an area including the nucleus pontis oralis (PnO), appears involved with both generating REM sleep (for review, see Kubin, 2001) and cataplexy (Reid *et al.*, 1994a; Reid *et al.*, 1994c). Since these regions receive significant cholinergic input from mesopontine cholinergic neurons in the LDT and PPT (Sato & Fibiger, 1986; Quattrochi *et al.*, 1989; Semba *et al.*, 1990; Semba, 1993), these data suggest that one adaptation to the loss of orexin signaling is dysregulation of these cholinergic neurons leading to increased release of ACh in the mPRF. Increased transmission could arise from numerous factors including altered synaptic control of the cholinergic neurons or their terminals, increased cholinergic receptor density at target sites, increased innervation of these target sites from either the same or a larger number of cholinergic neurons or an increased capacity for the release of ACh from cholinergic terminals. Indeed, evidence from the canine model suggested an increase in m2 receptor expression in the mPRF (Kilduff *et al.*, 1986) and there have been conflicting reports about the relative numbers of mesopontine cholinergic neurons in narcoleptic and normal canines (Nitz *et al.*, 1995; Tafti *et al.*, 1997).

We examined this issue using narcoleptic mice in which both orexin receptors were constitutively knocked out and which appear to be a phenocopy of orexin prepro-peptide knockouts (Kisanuki *et al.*, 2001; Willie *et al.*, 2003). We reasoned that if mPRF cholinergic transmission is increased in narcolepsy/cataplexy, the number of afferent cholinergic neurons might be increased and/or the expression of the enzymes necessary for cholinergic transmission might be altered in narcoleptic animals. We therefore compared the number of cholinergic cells in the LDT along with markers of cholinergic function in DKO and WT mice. These markers included: choline acetyltransferase (ChAT), which synthesizes ACh from choline and acetyl CoA; the vesicular acetylcholine transporter (VACHT), which transports ACh into synaptic vesicles; the choline transporter (CHT1) which mediates high affinity choline uptake into the cytoplasm; and acetylcholinesterase (AChE) which breaks down synaptically released ACh into choline and acetate.

Materials and Methods

Animals

All protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of New York Medical College and was compliant with NIH guidelines for ethical treatment of animals. Adult male mice, aged 35–60 days old, 19–25g,

were housed individually with unlimited access to food and water, on a 12:12 light-dark cycle, with 7:00 AM as the beginning of the light phase.

DKO breeder mice from the Yanagisawa laboratory were re-derived by embryo transfer at New York Medical College and the offspring from these re-derived mice were used for this study. DKO mice were originally created by breeding heterozygous progeny of homozygous single receptor knockouts (OX1R^{-/-} x OX2R^{-/-}). Production of the OX2R^{-/-} mice has been described in detail (Willie *et al.*, 2003) and the OX1R mice (Kisanuki *et al.*, 2000) will be characterized in detail in a future publication. The single receptor knockout mice, and hence the DKO mice used in this study had a mixed genetic background (C57BL/6J x 129/SvEv). For control mice we used wild type C57BL/6 mice (B6; Taconic, Hudson, NY, USA) or wild type mice having the same genetic background (C57BL/6J x 129/SvEv) as the DKO mice. The latter mice were termed “background control” (BC) mice and were produced by breeding the wild-type progeny from heterozygous OX2R parents.

Genotyping

To confirm genotypes, tail biopsies were obtained during brain slice preparation and subsequently analyzed by PCR. One set of primers were used to determine if the mouse was either a wild type or knockout for each orexin receptor. The three primers (Invitrogen, Carlsbad, CA) for OX1R consisted of a common primer (5' CTCTTTCTCCACAGAGCCCAGGACTC 3'), a knockout primer (5' TGAGCGAGTAACAACCCGTCGGATTC 3') and a wild type primer (5' gCAAGAATGGGTATGAAGGGAAGGGC 3'). The expected product sizes were ~320 base pairs for the wild type allele and ~500 base pairs for the knockout allele. The three primers for OX2R consisted of a common primer (5' CTGGTGCAAATCCCCTGCAA 3'), a knockout primer (5' GGTTTTCCCAGTCACGACGTTGTA 3') and a wild type primer (5' AATCCTTCTAGAGATCCCTCCTAG 3'). The expected product sizes were ~620 base pairs for wild type allele and ~310 base pairs for the knockout allele. These two sets of primers for different orexin receptors were processed separately and PCR was performed using 35 cycles of 30 s at 94° C, 30 s at 60° C and 1 min at 72° C, followed by one cycle at 72° C for 10 min. The result of each PCR reaction was then fractionated on a 2% agarose gel, and the PCR product was visualized by ethidium bromide staining.

Isolation of RNA

Coronal mouse brainstem slices were prepared as previously described (Burlet *et al.*, 2002). Briefly, 300µm thick brainstem slices were made using a vibrating microtome (Leica VT1000, Bannockburn, IL) in ice cold artificial cerebral spinal fluid (ACSF). The ACSF contained the following (in mM): NaCl (124), KCl (5), NaH₂PO₄ (1.2), CaCl₂ (2.7), MgSO₄ (1.2), NaHCO₃ (26), D-Glucose (10) (Fisher Scientific, Pittsburgh, PA). ACSF was oxygenated with 95% O₂ /5% CO₂ (TechAir, White Plains, NY). The ACSF also contained 100µM N(G)-nitro-L-arginine methyl ester (L-NAME) to inhibit nitric oxide synthase (NOS) activity to minimize the amount of RNA damage by free radicals. Slices were then transferred to a stage with oblique transillumination and viewed with a stereo-microscope to enable visualization of the target structures. A 1.00 mm diameter tissue puncher (Stoelting, Wood Dale, IL) mounted on a manual micromanipulator was then used to excise tissue from the LDT and the Mo5. The remaining portion of the brain slices were immersion fixed in 4% PFA, were re-sectioned at 30µm and processed for ChAT immunohistochemistry in order to confirm punch accuracy (figure 1B,C). Tissue from punches were collected from 4–5 mice (either DKO or wild type B6 age-matched mice), were pooled and then were flash frozen in liquid nitrogen and store at -80° C until isolation. Whole brainstems from DKO and wild type B6 and BC mice, which included the caudal medulla to the rostral end of the superior colliculus, were dissected from the skull while submerged in ice-cold ACSF, frozen in liquid

nitrogen and then stored at -80°C . To prepare the RNA, frozen samples were thawed, homogenized and total RNA was extracted using the RNeasy Lipid Tissue Mini kit (Qiagen, Valencia, CA). RNA quality and quantity was then determined by spectrophotometry.

Reverse transcription and RT-PCR

A 20 μl volume of the RNA solution, with 1 μg of total RNA from each sample was reverse transcribed using random primers and the Improm II Reverse Transcription (RT) kit (Promega). The samples were incubated with random primers for 5 min at 70°C , 5 min at 4°C and 1 hr at 37°C followed by 70°C for 15 min. The resulting cDNA was aliquotted and stored at -20°C .

For quantification of starting mRNA, 2 μl of the RT product with SYBR green I reagent, 25mM MgCl_2 and the relevant primers, made to a final volume of 20 μl was loaded into a Roche lightcycler (Roche, Basel, Switzerland). Primers (Invitrogen, Carlsbad, CA) for ChAT, VACHT, CHT1, AChE and cyclophilin A (table 1) were loaded at a constant concentration (10 μM) for their respective runs. Every run on the lightcycler contained two blanks, scales for the specific gene (see below), and the unknown samples, which were each loaded in triplicate with primers for the gene of interest.

Lightcycler protocols were denaturation at 95°C for 8:00 minutes, cycling 40 times at 95°C (5 s), 56°C (10 s) and 72°C (14 s). Optimization of this protocol was conducted during the production of the scales.

Measuring target message

In order to measure the absolute amount of starting sequence in each sample, standards were generated from RT product isolated from wild-type B6 brainstems. To do this, the RT product was loaded with primers of the genes of interest and amplified using a conventional PCR for 35 cycles to create standards of known concentrations. The product was then run on a gel to determine the specificity of the amplification. If there was a single band of the predicted size, the amplification was considered specific and the concentration of the PCR product was determined through spectrophotometry (Beckman Coulter DU 530, Fullerton, CA). Conventional PCR (Eppendorf, Mastercycler Gradient, Westbury, NY) was used in order to guarantee enough amplification to be measured by spectrophotometer. Known amounts of each amplified target sequence were created by serial dilution to generate a scale for each gene of interest that was amplified along with the unknown sample in the RT-PCR. These scales establish a correlation between the target cDNA fluorescence and the concentration. The threshold values from the unknown sample were then compared to the threshold values of serially diluted scales to determine the starting amount of the unknown. ChAT message scales ranged from $1.3 \times 10^{-2} \mu\text{g}/\mu\text{l}$ to $1.3 \times 10^{-5} \mu\text{g}/\mu\text{l}$. VACHT message scales ranged from $8.9 \times 10^{-2} \mu\text{g}/\mu\text{l}$ to $8.9 \times 10^{-5} \mu\text{g}/\mu\text{l}$. CHT1 message scales ranged from $1.2 \times 10^{-2} \mu\text{g}/\mu\text{l}$ to $1.2 \times 10^{-5} \mu\text{g}/\mu\text{l}$. AChE message scales were from $4.0 \times 10^{-3} \mu\text{g}/\mu\text{l}$ to $4.0 \times 10^{-6} \mu\text{g}/\mu\text{l}$. Cyclophilin A message scales were from $2.6 \times 10^{-3} \mu\text{g}/\mu\text{l}$ to $2.6 \times 10^{-6} \mu\text{g}/\mu\text{l}$. This method of creating scales was adopted from previous studies (Li & Wang, 2000; Wong & Medrano, 2005).

To verify primer specificity, lightcycler products were run on 2% agarose gels and visualized with ethidium bromide. Size markers (conventional DNA digest molecular weight markers, ϕX174 and λ DNA dephosphorylated markers, Promega, Madison, WI) were loaded with each gel to confirm the correct size of the amplicons. Amplification specificity was also confirmed by melting curve analysis. For each experiment, the same amount of sample cDNA was loaded into each lightcycler tube and mRNA concentration values were then computed. Data from six experiments (with 4–5 mice per genotypes per experiment)

were averaged for each gene investigated and compared between DKO and WT for LDT and Mo5 samples. Statistical significance was determined by two-tailed Student t-test with $\alpha = 0.05$ and data are presented as mean $\pm$ standard error of the mean (SEM).

Western Analysis

To determine if protein levels from whole brainstem (caudally from the medulla to the rostral end of the superior colliculus), cerebral cortex and thalamus varied by genotype, tissue was harvested from DKO (n = 8) and wild type BC (n = 8) mice and western blot analysis was performed on samples from each animal. Tissue from the cerebral cortex and thalamus were harvested from 400 μ m sagittal brain slice sections. The gross dissection of the cerebral cortex and the thalamus included the somatosensory and motor cortices (~0.12mm to ~2.88mm, lateral coordinates, Paxinos) and posterior, ventromedial, ventrolateral, ventroposteromedial and paracentral thalamus nuclei (~0.60mm to ~1.92mm lateral coordinates, Paxinos), respectively. All tissue was homogenized, vortex-mixed rigorously and incubated on ice for 15 minutes in homogenate buffer (50mM Tris-HCl at a pH of 7.4, 0.5% triton X-100, and 1 Roche protease-inhibitor cocktail tablet). The samples were then centrifuged at 12,000 rpm for 15 minutes at 4°C and the supernatant was isolated. Protein concentrations in the extracts were determined using the Bradford assay (Simonian & Smith, 2006). Aliquots of the extracts, normalized for total protein content (30 μ g per well), were separated on a 10% SDS-PAGE gel run for 55 minutes at 200V and transferred to Immobilon PVDF membrane (Millipore, Billerica, MA). All antibodies were ordered from Millipore. The membranes were blocked with 5% milk in TBST for two hours and then probed overnight in 4°C with polyclonal antibodies of various concentrations: rabbit anti-high affinity choline transporter (1:1000 Millipore, catalog # AB5966); and goat anti-choline acetyltransferase (1:500 Millipore, catalog # AB144P). After a series of washes (3x, 15 minutes each), the membranes were incubated with secondary antibodies for an hour and a half at room temperature (donkey anti-goat IgG HRP 1:5000, Millipore catalog # AP180 for goat anti-ChAT; and rabbit anti-goat IgG 1:5000, Millipore catalog # AP132P, for the anti-CHT1) and washed again (3x 15 min). The antibody/antigen complexes were visualized using Chemiluminescence HRP substrate (Millipore, catalog # WBKL 50050). The membranes were then washed multiple times with TBST and blocked for one hour with 5% milk. After the nonspecific block, the membrane was incubated overnight in 4°C with rabbit anti-cyclophilin A (1:10,000 Millipore, catalog # 07-313). After a series of washes, the membranes were incubated with goat anti-rabbit IgG HRP (Millipore, 1:10,000) for an hour and a half. The antibody/antigen complex was visualized with Chemiluminescence HRP substrate (Millipore). After each ECL incubation, the membrane was briefly exposed to film (Denville Scientific, catalog # E3018). Cyclophilin A was used as the house keeping protein to correspond what was found with the RNA study. ChAT bands were approximately 68–70kDa, CHT1 bands were approximately 60 kDa and cyclophilin A bands were 20kDa.

Band images were acquired and analyzed using ChemiImager 5500 (Alpha Innotech, San Leandro, CA, version 4.0.1). Density analysis was performed with the auto-background option enabled, black and white inversion (with white valued at 0 and black at 65535) and with the area for pixel density measurement held constant for each blot. The Integrated Density Value (IDV) from each region of interest, which was centered on each band of interest, was then used for analysis. The IDV for each target protein was normalized by dividing it by the IDV value for the corresponding cyclophilin band. The protein/cyclophilin A IDV ratios were displayed as a function of the cyclophilin A IDV and compared across genotypes. Data are presented as mean $\pm$ SEM and statistical significance was determined using a 3-way ANOVA (factors: genotype, nucleus and experiment) with Scheffe post-hoc testing to determine if there were significant differences between genotypes (DataDesk 6.2, Data Description, Inc., Ithica, New York, USA).

ChAT activity assays

To determine if we could detect differences in ChAT enzyme activity we isolated tissue samples from eight wild-type (BC) and eight DKO mice. We prepared tissue punches of the LDT from brain slices (300 μ m thick; pooled a 1mm punch from each side) and dissected a prism of tissue containing part of the PnO from the tissue slice just rostral to the slice used for LDT punches. We also harvested a piece of cortex from the frontal pole. Each sample was placed into a microfuge tube, flash frozen in liquid nitrogen and then stored on dry ice until analyzed. ChAT was measured in samples of frontal pole, PnO and LDT punches using a modification of the method of (Fonnum, 1975). Tissues were stored at -80° C, then thawed and homogenized by sonication in 19 volumes of 75 mM potassium phosphate buffer, pH 7.4. Equal 10 μ l aliquots of homogenate and buffer substrate solution (75 mM potassium phosphate solution, pH 7.4, containing NaCl 0.6 mM, MgCl₂ 40 mM, physostigmine 2 mM, bovine serum albumin 0.05%, choline iodide 10 mM, acetyl CoA, 0.87 mM, 3H-acetyl CoA, 5 μ Ci) were incubated for 30 min at 37°C. The reaction was stopped by addition of 50 μ l ice-cold water. Radiolabelled ACh was extracted by mixing the sample with 150 μ l of 3-heptanone containing 75 mg/ml sodium tetraphenylboron. Organic and aqueous phases were separated by centrifugation and 3H-ACh was determined in the organic phase by liquid scintillation spectrometry. Each sample was assayed in triplicate. Protein content in the samples was measured using the Pierce Micro BCA protein assay. Data is expressed as mean $\pm$ SEM in nmoles ACh formed/mg protein/hr. Statistical significance was determined by two-tailed Student's t-test with alpha = 0.05.

Immunocytochemistry

A total of seven pairs of mice (4 DKO and BC mice; 3 DKO and B6 mice), 30–60 day old, were anesthetized (isoflurane followed by 100 mg/kg pentobarbitol i.p.) and transcardially perfused with heparinized (1000 units/L) 0.9% saline followed by 80 ml ice cold 4% paraformaldehyde (PFA) in phosphate buffer solution (PBS). After perfusion, the whole brains were extracted and submerged in 4% PFA for 4 hours, then left for 24 hours in a solution of 30% sucrose in PBS. The brains were then blocked and sectioned (30 μ m) on a freezing microtome.

Sections to be processed for immunoperoxidase activity (from 4 pairs of DKO and BC mice) were rinsed 3 times in PBS for 5 minutes and incubated at room temperature for 30 minutes in 2% hydrogen peroxide in PBS. Sections were rinsed three more times in PBS then incubated for 1 hour in 2% mouse serum (Vector) in PBS with 0.3% triton-X 100. Sections were then incubated overnight in polyclonal goat anti-ChAT antibody (1:1000, Millipore) then transferred to a solution of biotinylated mouse-anti-goat IgG antibody (1:300, Jackson Immunoresearch, West Grove, PA). Tissue was processed with a Vector ABC kit as per the manufacturer's instructions and reacted with Sigma's FastDAB kit for five minutes (Sigma-Aldrich, St. Louis, MO). The precipitation reaction was stopped with three rinses of PBS and the sections were mounted on slides and coverslipped.

Tissue to be processed for immunofluorescence (from 3 pairs of DKO and B6 mice) was rinsed and incubated for 1 hour in 2% mouse serum PBS, incubated overnight in polyclonal goat anti-ChAT antibody (1:400, Millipore), rinsed three times in PBS, then placed in FITC-conjugated mouse anti-goat IgG (1:50, Jackson Immunoresearch), rinsed with PBS, then mounted on slides and coverslipped with non-fluorescent mounting medium (Harleco Krystalon, Gibbstown, NJ).

Cell Counting

To compare the number of ChAT positive cells in wild-type and DKO mice, cell counts were sampled from three immunoperoxidase labeled sections from matched rostral, middle

and caudal planes (separated by $> 60 \mu\text{m}$) containing the LDT and Mo5 from four wild-type BC and four DKO brains. Each section was traced to graph paper using a BX60 microscope (Olympus Center Valley, PA, 20x objective) equipped with a drawing tube. ChAT labeled cell profiles from each section (unilaterally) were plotted and counted by hand. Cell counts are reported as the mean number of cells per side $\pm$ SEM. The counts were statistically compared between genotypes by a two-tailed Student's t-test with $\alpha = 0.05$.

Immunohistochemical Quantification

All histological sections prepared for quantitative experiments were processed in pairs of a DKO and wild-type section to any minimize procedural differences. Digital images were acquired of the LDT and Mo5 with a 10x objective and 2.5x tube lens using a 12 bit cooled CCD CoolSnap camera (Roper Scientific, Tucson, AZ), mounted to a BX60 microscope (Olympus), using RSIImage software (Roper Scientific) set to acquire a raw grayscale image binned at 2×2 pixels. Every picture within a pair of mice was taken with the same camera exposure with the same microscope transillumination intensity for sections labeled with DAB and the same epi-illumination intensity for sections labeled with FITC. Pictures were saved as 16 bit grayscale TIFFs and analyzed with ImageJ v1.31 software. Somata within the nuclei were selected using the Image $\rightarrow$ Adjust $\rightarrow$ Threshold command and profiles were obtained using the Analyze $\rightarrow$ Analyze Particles command with Greg Joss' custom particle analyzer plug in and Wayne Rasband's area calculator plug in. The position, area, and average grayscale value of each particle was measured and copied to Microsoft Excel for analysis. Background intensity measurements were determined by measuring staining intensity in four surrounding square regions devoid of DAB or fluorescently labeled cells. Optical density was calculated from these data using the equation:

$$\text{Optical Density} = -\log[(\text{intensity} - \text{black})/(\text{incident} - \text{black})]$$

where intensity is the average grayscale value of one particle, incident is the intensity of the light passing through the tissue slice (the background), and black is the intensity of a pixel with no light passing through it, set to zero as the camera was calibrated before every picture. Fluorescence intensity was calculated as the difference between the particle intensity and the average background intensity. Statistical comparisons between genotypes were conducted using paired, two-tailed Student's t-test with $\alpha = 0.05$ for normalized optical density and fluorescence measures.

Results

To investigate whether message levels for cholinergic markers were different between wild type and DKO mice, we targeted mesopontine cholinergic neurons located in the LDT and Mo5 (Fig 1). The upper left panel of Fig 1A shows the atlas location of the LDT and Mo5. The bottom left panel shows a corresponding nissl stain and the bottom right panel shows the intense ChAT staining present in mouse LDT and Mo5. An adjacent section (Fig 1A, top, right), indicates the specificity of our immunoperoxidase staining since it was completely absent in tissue processed without the primary antibody. To isolate mRNA, we prepared brain slices and obtained tissue from punches aimed at the LDT and Mo5. The accuracy of these punches was confirmed by processing the remaining portion of these slices for ChAT immunocytochemistry (Fig 1B and C).

ChAT, VAcHt and CHT1 message levels were elevated in LDT and Mo5 from DKO mice

For each gene studied, we estimated the initial template concentrations in samples from the LDT and Mo5 as the average of six independent experiments. To control for the specificity

of each RT-PCR reaction, we examined the melting curve for the amplicon produced in each reaction (data not shown) and determined the size of the PCR product from each reaction (Fig 2A–D). These data indicate that a single amplicon of the predicted size (Table 1) was produced in each case.

We first compared the levels of ChAT message between samples from wild-type (B6) and DKO mice (reported as mean $\pm$ SEM). We found that the average starting concentration of ChAT message was significantly higher in DKO samples from both the LDT (DKO: $2.63 \pm 0.29 (10^{-4}) \mu\text{g}/\mu\text{l}$ vs. B6: $0.99 \pm 0.07 (10^{-4}) \mu\text{g}/\mu\text{l}$; $p < 0.05$; $n=6$; Fig 2A2 left) and Mo5 (DKO: $2.51 \pm 0.35 (10^{-4}) \mu\text{g}/\mu\text{l}$ vs. B6: $0.67 \pm 0.1 (10^{-4}) \mu\text{g}/\mu\text{l}$; $p < 0.05$; $n=6$; Fig 2A2 right).

A similar pattern was observed for VACHT message levels (Fig 2B). We found that the average starting concentration of VACHT message was significantly higher in DKO samples from both the LDT (DKO: $7.79 \pm 2.16 (10^{-5}) \mu\text{g}/\mu\text{l}$ vs. B6: $2.15 \pm 0.38 (10^{-5}) \mu\text{g}/\mu\text{l}$; $p < 0.05$; $n=6$; Fig 2B2 left) and Mo5 (DKO: $23.2 \pm 0.61 (10^{-5}) \mu\text{g}/\mu\text{l}$ vs. B6: $6.03 \pm 1.71 (10^{-5}) \mu\text{g}/\mu\text{l}$; $p < 0.05$; $n=6$; Fig 2B2 right).

Similarly, we found that the concentration of CHT1 message was significantly higher in LDT samples from DKO mice (DKO: $4.84 \pm 0.66 (10^{-6}) \mu\text{g}/\mu\text{l}$ vs. B6: $2.28 \pm 0.28 (10^{-6}) \mu\text{g}/\mu\text{l}$; $p < 0.05$; $n=6$; Fig 2C2 left). In the Mo5, the average CHT1 message concentration was numerically higher in samples from DKO mice however this difference did reach significance (DKO: $7.40 \pm 1.30 (10^{-6}) \mu\text{g}/\mu\text{l}$ vs. B6: $4.25 \pm 1.06 (10^{-6}) \mu\text{g}/\mu\text{l}$; $p > 0.05$; $n=6$; Fig 2C2 right).

The final marker for cholinergic synaptic transmission we examined was for the enzyme AChE. In contrast to the other markers, we found no significant differences in starting concentrations of AChE template across genotype in either the LDT (DKO: $8.76 \pm 2.14 (10^{-5}) \mu\text{g}/\mu\text{l}$ vs. B6: $9.39 \pm 3.08 (10^{-5}) \mu\text{g}/\mu\text{l}$; $p > 0.05$; $n=6$; Fig 2D2 left) or Mo5 (DKO: $5.22 \pm 2.54 (10^{-5}) \mu\text{g}/\mu\text{l}$ vs. B6: $2.83 \pm 1.62 (10^{-5}) \mu\text{g}/\mu\text{l}$; $p > 0.05$; $n=6$; Fig 2D2 right).

To determine if the observed pattern of differences in initial template concentration could be explained by a relative enrichment in message in our DKO samples, we compared the housekeeping gene cyclophilin A between DKO and wild-type (B6) samples. We found no significant difference in the starting concentration of cyclophilin A mRNA across genotype in either the LDT, (DKO: $7.64 \pm 1.60 (10^{-6}) \mu\text{g}/\mu\text{l}$ vs. B6: $11.3 \pm 2.36 (10^{-6}) \mu\text{g}/\mu\text{l}$; $p > 0.05$; $n=6$) or Mo5 (DKO: $4.67 \pm 2.49 (10^{-6}) \mu\text{g}/\mu\text{l}$; B6: $8.88 \pm 2.27 (10^{-6}) \mu\text{g}/\mu\text{l}$; $p > 0.05$; $n=6$).

Difference in message levels appeared localized to LDT and Mo5 regions

To determine if cholinergic message levels were increased throughout the brainstems of DKO mice, we compared message levels in homogenates of whole brainstems from DKO and wild-type (B6) mice (Fig 3). In these preparations, we found no significant differences between genotypes in average starting concentration of ChAT (B6: $9.21 \pm 2.86 (10^{-5}) \mu\text{g}/\mu\text{l}$ vs. DKO: $11.5 \pm 3.1 (10^{-5}) \mu\text{g}/\mu\text{l}$; $p > 0.05$; $n=6$; Fig 3A), VACHT (B6: $1.05 \pm 0.31 (10^{-4}) \mu\text{g}/\mu\text{l}$ vs. DKO: $9.65 \pm 0.56 (10^{-5}) \mu\text{g}/\mu\text{l}$; $p > 0.05$; $n=6$; Fig 3B), CHT1 (B6: $9.96 \pm 3.85 (10^{-6}) \mu\text{g}/\mu\text{l}$ vs. DKO: $12.0 \pm 3.9 (10^{-6}) \mu\text{g}/\mu\text{l}$; $p > 0.05$; $n=6$; Fig 3C) and AChE mRNA (B6: $4.21 \pm 0.38 (10^{-3}) \mu\text{g}/\mu\text{l}$ vs. DKO: $3.13 \pm 0.31 (10^{-3}) \mu\text{g}/\mu\text{l}$; $p > 0.05$; $n=6$; Fig 3D). Similarly, there was no difference in starting concentration of the housekeeping gene cyclophilin A (B6: $3.07 \pm 0.54 (10^{-4}) \mu\text{g}/\mu\text{l}$ vs. DKO: $4.07 \pm 0.38 (10^{-4}) \mu\text{g}/\mu\text{l}$; $p > 0.05$; $n=6$; data not shown).

Since the DKO mice were on a mixed genetic background, we also measured whole brainstem message levels of these markers in wild-type BC mice. We found that neither ChAT (BC: 12.0 ± 0.86 (10^{-5}) $\mu\text{g}/\mu\text{l}$ $p > 0.05$; $n=4$), VACHT (BC: 1.54 ± 0.19 (10^{-4}) $\mu\text{g}/\mu\text{l}$; $n=4$; $p > 0.05$;) or CHT1 (BC: 15.2 ± 0.31 (10^{-6}) $\mu\text{g}/\mu\text{l}$; $p > 0.05$; $n=4$) levels were statistically different from those obtained from the B6 mice. Thus, the differences in ChAT, VACHT and CHT1 message between B6 and DKO mice are unlikely to be attributable to genetic background.

Results from the qRT-PCR analysis are summarized in Figure 4, where the template concentrations measured for the DKO samples are normalized to the wild-type B6 samples. On average we found 2.6-fold more ChAT message, 3.6-fold more VACHT message and 2.1-fold more CHT1 message in LDT samples from DKO mice than from B6 mice. In contrast, there was no difference in the amount of AChE or cyclophilin A ($p > 0.05$) in these samples. In the Mo5 samples, we found 3.7-fold more ChAT message and 3.8-fold more VACHT in samples from DKO mice compared to B6 mice. CHT1 message was greater in the DKO samples, but the difference did not reach significance at the 0.05 level and there were also no differences between AChE and cyclophilin A levels across genotype. In samples from whole brainstem, there were no significant differences in any of the message species studied across genotype. Collectively, these data indicate that there is a localized elevation in message levels for key molecules necessary for the synthesis and transmission of acetylcholine in mice constitutively lacking functional orexin receptors.

Differences in ChAT and CHT1 protein levels between genotypes were not detectable by Western blot analysis

We next attempted to determine if there were corresponding differences in ChAT and CHT1 protein levels by western blot analysis by comparing the ratio of target protein to cyclophilin A band intensities across samples. In this series of experiments we were able to compare samples from DKO mice to those from wild-type BC mice having the same genetic backgrounds. We compared protein homogenates isolated from the whole brainstem, the thalamus (which receives its major cholinergic input from the LDT/PPT) and the cortex (which receives its major cholinergic input from the basal forebrain groups).

In each comparison, we were unable to detect significant differences between genotypes in these ratios for ChAT (Fig 5A) or CHT1 (Fig 5B) protein. For brainstem westerns probed for ChAT and cyclophilin A (Fig 5A1) we found that the average ratio for BC samples (0.41 ± 0.05 ; $n=8$) was not significantly different from the average ratio for DKO samples (0.45 ± 0.09 ; $n=8$; $p > 0.05$; Fig 5A2). Similarly for the thalamus, the average ratio for BC samples (0.55 ± 0.06 ; $n=6$) was not significantly different from DKO samples (0.54 ± 0.07 ; $n=6$; $p > 0.05$; Fig 5A2). For neocortical samples, we also were unable to detect a significant difference between BC samples (0.48 ± 0.07 ; $n=8$) and DKO samples (0.63 ± 0.16 ; $n=8$; $p > 0.05$; Fig 5A2).

A similar pattern was observed for the CHT1 western blots (Fig 5B1). We were unable to detect a significant difference in brainstem samples (BC: 0.70 ± 0.06 ; $n=8$ vs. DKO: 0.69 ± 0.04 ; $n=8$; $p > 0.05$; Fig 5B2), thalamic samples (BC: 0.65 ± 0.11 ; $n=4$ vs. DKO: 0.81 ± 0.26 ; $n=4$; $p > 0.05$; Fig 5B2) or cortical samples (BC: 0.97 ± 0.11 ; $n=7$ vs. DKO: 1.15 ± 0.18 ; $n=7$; $p > 0.05$; Fig 5B2).

ChAT activity measures

We also tested for possible genotypic differences in ChAT enzyme activity, which might be expected if ChAT protein levels were different but not detectable by Western blots. In this experiments, we compared tissue samples from eight wild-type BC mice and eight DKO

mice. From each of the 16 mice we prepared tissue punches centered on the LDT, a tissue prism from the PnO and a piece of frontal cortex. The PnO was chosen since this subregion of the mPRF region receives input from LDT/PPT cholinergic neurons and it is a region where cholinergic agonists have long been known to promote REM sleep and cataplexy, in narcoleptic canines. The cortex was chosen since it receives its major cholinergic input from basal forebrain cholinergic neurons, which, while not the focus of this work, is an important target for orexin actions regulating cortical signs of arousal (see discussion). We found that the results from the LDT tissue punches were too variable to measure accurately, as many samples were too small to have detectable ChAT activity or protein. We did detect ChAT activity from each PnO and cortex sample (Fig 5C). However, we did not detect a difference between genotypes in ChAT activity from the PnO samples (BC: 2.55 ± 0.42 nmoles/mg protein/hr, $n = 8$ vs. DKO, 2.36 ± 0.22 nmoles/mg protein/hr, $n = 8$; $p > 0.05$). In contrast, we found that ChAT activity was significantly lower in the DKO samples from the frontal cortex (BC: 2.74 ± 0.28 nmoles/mg protein/hr, $n = 8$ vs. DKO: 1.86 ± 0.20 nmoles/mg protein/hr, $n = 8$; $p < 0.05$). Collectively, results from the western blots and ChAT assay suggested that either our measured changes in message in the LDT and Mo5 are not translated into changes in protein level or that the protein differences were too localized for our assay methods to detect. In addition, our ChAT enzyme assay suggested that there might be a lower capacity for cortical ACh biosynthesis in DKO mice.

Immunocytochemistry revealed similar numbers of ChAT-labeled neurons but more intense ChAT staining in LDT neurons from DKO mice

To address the possibility that genotypic difference in mesopontine ChAT levels may be very localized, we conducted ChAT immunoperoxidase and immunofluorescence labeling of tissue sections from WT and DKO brains. We first compared the numbers of LDT and Mo5 neurons between DKO and wild-type (BC) mice. To estimate whether there were differences in numbers of cells between genotypes, we plotted and counted all labeled somata in three matched sections (30 μ m thick) from one side of the brain chosen from the rostral, middle and caudal portions of each nucleus from four DKO mice and four BC mice. Fig 6A shows the plotted ChAT+ neurons in the Mo5 and LDT from matched mid-sections through the LDT from a BC (left) and DKO (right) brain. We found that the average number of ChAT+ LDT cells per mouse was not different ($p > 0.05$) between BC (311 ± 15 , $n = 4$) and DKO (331 ± 32 , $n = 4$; $p > 0.05$) mice (Fig 6B left histogram). Similarly, there was no difference in the number of ChAT+ neurons in the Mo5 sample from BC (334 ± 30 , $n = 4$) and DKO (353 ± 12 , $n = 4$; $p > 0.05$) mice (Fig 6B right). Thus, a difference in number of cholinergic neurons between normal and DKO mice is unlikely.

We next investigated whether there were differences in the intensity of ChAT staining between wild-type mice and DKO mice. In the first series of experiments, we compared four BC and four DKO mice processed as pairs using immunoperoxidase staining with a shortened DAB reaction to prevent saturation. After staining, we performed a particle analysis (see methods) to measure the optical density (OD) of the ChAT immunoreactive somata in the LDT and Mo5. Figure 7 illustrates images of the LDT from co-processed BC (Fig 7A1) and DKO (Fig 7A2) sections. It is apparent from the micrographs that the staining was darker in the DKO neurons. Staining intensity is quantified in Fig 7B which shows the distribution of somatic optical densities for this section pair and indicates that DKO neurons were darker than WT neurons with little overlap in their distributions. The mean $\pm$ SEM of OD from four such co-processed sections are illustrated in Figure 7C. In three of four reactions, average LDT neuron staining intensity from the DKO mouse clearly exceeded that of the BC (Fig 7C1). In contrast, identical measures made in the Mo5 from the same tissue sections indicated that the ODs were not different between genotypes (Fig 7C2). In the one paired reaction where the mean OD was lower in the LDT from the DKO than from the BC,

the OD was also lower in the Mo5 from the DKO, suggesting the staining conditions might explain this variation. Indeed, if the mean ODs from the LDT were normalized by the mean Mo5 OD from the same tissue sections, all four paired reactions show increased LDT staining in DKOs (Fig 7C3). After normalization, the LDT neurons from DKO mice showed a $37 \pm 14\%$ ($p < 0.05$) increase in staining over these neurons from wild-type BC mice.

To determine if the mouse background strains might contribute to these differences we conducted two types of experiments. First, we compared ChAT immunoperoxidase staining from a B6 and 129/SvEv (129) mouse pair representing the inbred background strains of the DKOs. There was complete overlap in the distributions of immunoperoxidase intensity in the Mo5 and LDT (Fig 7D). Next we compared the intensity of ChAT staining between paired sections from three wild-type B6 and three DKO mice. In this series of experiments we used indirect ChAT immunofluorescence to also examine whether the previous OD differences observed in the LDT could be due to subtle differences in the peroxidase reaction conditions. An example of the distributions of LDT ChAT fluorescence from one pair of sections is shown in Fig 7E and indicates there was greater ChAT intensity from the DKO sample. The mean $\pm$ SEM of this somatic fluorescence for LDT neurons is illustrated in Fig 7F1 for each genotype along with the comparable measures from Mo5 (Fig 7F2). In all three pairs, somatic cellular fluorescence intensity was greater in LDT neurons from the DKOs than that from B6 mice. Nevertheless, in the same tissue sections, there were no genotypic differences in fluorescence intensity for Mo5 neurons (Fig 7F2), supporting the findings from the immunoperoxidase reactions. Normalization of LDT intensity values with those from Mo5 indicated ChAT staining in LDT neurons from the DKO mice was $42 \pm 17\%$ ($p < 0.05$) brighter than that from the B6 mice. Collectively, evidence from our immunocytochemistry studies strongly indicate that there are no differences in the number of cholinergic LDT or Mo5 neurons between genotypes but that there is greater expression of ChAT protein in LDT neurons from DKO mice compared to both BC and B6 wild-type strains.

Discussion

Using qRT-PCR, we found that ChAT, VACHT and CHT1 message levels were elevated approximately two to four-fold in mesopontine tissue samples from the LDT and Mo5 regions of DKO mice compared to those from B6 mice. Since this pattern was not observed in samples from whole brainstem, which includes additional cholinergic cell groups, these increases appear localized to mesopontine regions. Results from our ChAT immunocytochemistry experiments indicate that these elevated message levels are translated into increased protein expression in the LDT region. Since this ChAT immunoreactivity was greater compared to both BC and B6 wild-type mice, the observed elevation in cholinergic markers likely results from the absence of orexin receptors rather than differences in genetic background between B6 and DKO mice. Our inability to detect genotypic differences in ChAT or CHT1 protein levels from homogenates of whole brainstem, thalamus or cortex is also consistent with a restricted increase in these cholinergic properties since multiple cholinergic sources contribute to the innervation of these regions. Similarly, our inability to detect a difference in ChAT activity from PnO samples may reflect the presence of ChAT-expressing processes arising from sources other than the LDT (Jones, 1990). Since the number of ChAT labeled LDT neurons was not different between DKO and wild-type BC mice, it is likely that the higher message levels observed in DKO samples reflect an up regulation of cholinergic properties within LDT neurons developmentally destined to become cholinergic. Collectively, these findings suggest that in the absence of orexin receptor signaling, there is an increase in expression of the machinery necessary for cholinergic neurotransmission in a restricted population of mesopontine cholinergic neurons. Since these neurons have been associated with both the production of normal REM sleep

and cataplexy, we hypothesize that the capacity for cholinergic transmission is increased in these neurons and that this promotes cataplexy by enhancing cholinergic outflow to REM- atonia generating targets.

We also found that ChAT activity was significantly lower in cortical samples from DKO mice compared to those from wild-type BC mice, even though our Western blots did not detect differences in ChAT protein. The difference between the Westerns and ChAT activity measures may reflect a tissue sampling difference since ChAT activity was measured from more rostral cortical regions that included little somatosensory cortex due to the difference in slicing methods. While not the main focus of this study, this finding suggests a new role for orexin receptors in maintaining cortical cholinergic function although additional experiments will be necessary to elucidate the basis of this genotypic difference. One possibility is that orexin signaling maintains ChAT activity arising from the terminals of basal forebrain cholinergic neurons, which provide the major cholinergic input to the cerebral cortex (For review see Wainer & Mesulam, 1990). Orexin directly excites these neurons (Eggermann *et al.*, 2001) to release ACh in the cortex (Fadel *et al.*, 2005) and can mediate the arousal/attention-related increase in cortical ACh release evoked by natural stimuli (Frederick-Duus *et al.*, 2007). Reduced cortical ChAT activity may reflect a reduced capacity for sustained cortical cholinergic transmission and thereby contribute to the inability to sustain long periods of arousal in narcolepsy.

Regulation of neurotransmitter properties by orexin receptors?

The mechanism(s) by which the absence of orexin receptors results in localized alteration in cholinergic properties remains to be determined. One intriguing possibility is that signaling through the OX1R and/or OX2R influences neurotransmitter phenotype, either developmentally or in an ongoing, activity-dependent manner. Although little is known about the factors regulating cholinergic properties of mesopontine neurons, regulation of the cholinergic phenotype has been extensively studied in the developing sympathetic nervous system and spinal cord where it has been established that Ca^{2+} influx (Walicke & Patterson, 1981) and synaptic targets (Schotzinger *et al.*, 1994) play key roles in determining transmitter expression (For review see Spitzer *et al.*, 2004). A consistent finding is that decreasing Ca^{2+} influx or the frequency of Ca^{2+} spiking favors the cholinergic phenotype, while increasing Ca^{2+} influx suppresses or restricts this phenotype. The importance of Ca^{2+} influx via NMDA receptors and L-type Ca^{2+} channels has been recently illustrated in the developing hypothalamus where persistent blockade of Ca^{2+} influx via glutamate receptors and L-type Ca^{2+} channels produces a dramatic increase in the number of cholinergic neurons and spontaneous cholinergic synaptic transmission (Belousov *et al.*, 2001; Belousov *et al.*, 2002). Orexin receptors regulate both these pathways in LDT cholinergic neurons by stimulating glutamatergic synaptic inputs (Burlet *et al.*, 2002) and increasing intracellular Ca^{2+} via direct depolarization and enhancement of Ca^{2+} entry through L-type Ca^{2+} channels (Kohlmeier *et al.*, 2004; Kohlmeier *et al.*, 2008). Moreover, orexin receptors can also interact with downstream signaling pathways that regulate the cholinergic properties of hypothalamic neurons. For example, inhibition of PKC inhibits orexin-mediated elevation of intracellular Ca^{2+} in hypothalamic (van den Pol *et al.*, 1998) and LDT neurons (Kohlmeier *et al.*, 2004; Kohlmeier *et al.*, 2008) and enhances expression of cholinergic properties in hypothalamic neurons (Belousov *et al.*, 2002; Liu *et al.*, 2008). Inhibition of the ERK/MAPK pathway also enhances expression of cholinergic properties in hypothalamic neurons and although not studied in central neurons, orexin receptors activate this pathway in cell lines (Ammoun *et al.*, 2006; Ramanjaneya *et al.*, 2008; Tang *et al.*, 2008). Hence in DKO mice, the absence of orexin receptor-dependent Ca^{2+} influx and activity in these pathways might inhibit the factors that normally limit cholinergic gene expression in LDT neurons.

While orexin receptor signaling may plausibly regulate expression of cholinergic properties in LDT neurons, it can't be the only relevant factor. Orexin has a similar influence on Ca^{2+} influx into non-cholinergic LDT neurons and adjacent dorsal raphe (Kohlmeier *et al.*, 2004; Kohlmeier *et al.*, 2008) and locus coeruleus neurons (unpublished observations). Nevertheless, we did not observe an increase in the number of ChAT+ neurons in the LDT region, nor did we observe ectopic ChAT expression in the adjacent locus coeruleus or dorsal raphe. We also found that message level for ChAT and VAcHT were elevated in DKO tissue samples centered on the Mo5 but that ChAT immunostaining was statistically not different in these neurons, suggesting that additional factors regulate the translation of elevated message into protein, as is well known in other systems (Han *et al.*, 2007; Pichiule *et al.*, 2007; Brooks *et al.*, 2009). Differences between the LDT and Mo5 neurons which may contribute to this effect include: 1) Orexin receptor distribution - LDT neurons express both OX1R and OX2Rs while Mo5 neurons appear to exclusively express OX1Rs (Marcus *et al.*, 2001) and 2) LDT and Mo5 neurons are expected to have different firing patterns during REM and cataplexy and hence, different demands for the synthesis ACh. Thus, factors related to the absence of orexin 2 receptors or ACh utilization might account for higher amounts of ChAT protein in LDT and regulate translation of increased cholinergic message levels into increased protein levels.

Implications for cholinergic transmitter release

Increased expression of ChAT, VAcHT and CHT1 implies that these neurons have an increased capacity to synthesize and package ACh into synaptic vesicles and that cholinergic synaptic transmission will be enhanced. Assuming that substrate is not limiting, increased expression of ChAT, a cytoplasmic enzyme, would increase the amount of axoplasmic ACh available for packaging into synaptic vesicles. Increased VAcHT, a synaptic vesicle protein, could result in a larger quantal size if more than a single VAcHT molecule is present per vesicle (Parsons *et al.*, 1993) or alternatively, may reflect a larger pool of cholinergic synaptic vesicles. Up regulation of CHT1 implies that extracellular choline will be retrieved more efficiently by the terminals following release. This is important since retrieval of choline derived from released ACh is thought to be rate limiting, especially under high demand conditions (Bazalakova & Blakely, 2006). High affinity choline uptake is regulated by several mechanisms. One involves the trafficking of CHT1 located in synaptic vesicles into the presynaptic membrane during synaptic activity in order to match uptake capacity with ACh release (Ferguson *et al.*, 2003; Ferguson *et al.*, 2004; Ferguson & Blakely, 2004). Evidence from genetic models of cholinergic dysfunction also indicate that the expression of CHT1 is robustly regulated and can compensate for perturbations in other parts of the cholinergic pathway (Bazalakova & Blakely, 2006). For example, strong up regulation of CHT1 message and protein is observed in ChAT+/- mice, which helps maintain normal cholinergic transmission even though these mice express of only half the normal amount of ChAT protein (Brandon *et al.*, 2004). Our finding that expression of CHT1, ChAT and VAcHT are all increased in the LDT region from DKO mice suggests that there is both increased capacity to synthesize and package ACh and increased utilization of ACh by these neurons. In contrast, the absence of an increase in either ChAT protein or CHT1 message in Mo5 neurons from DKO mice suggest that neither capacity nor ACh utilization is increased for these neurons even though ChAT and VAcHT message was elevated.

Unlike the other cholinergic markers, we found no significant difference in AChE message expression in the DKO mice. Although there are multiple splice variants of AChE, we designed primers for AChE-S (synaptic) variant, which is considered the major form of AChE located at the synapses since it can anchor to the synaptic membrane (Meshorer & Soreq, 2006). This finding suggests that mechanisms responsible for terminating cholinergic transmission are unchanged in the DKO.

Implications for Narcolepsy/Cataplexy

Studies of narcoleptic canines have led to a consensus that an imbalance between cholinergic and monoaminergic transmission contributes importantly to the generation of narcolepsy/cataplexy (Tafti *et al.*, 1997). Cholinergic agonists acting at m2 receptors in the REM-induction regions of the mPRF (Reid *et al.*, 1994b; Reid *et al.*, 1994c) elicit cataplexy in these dogs but not in normal dogs and there is increased m2 receptor binding in this region (Kilduff *et al.*, 1986). In addition, a large increase in ACh release in the mPRF was observed during epochs of cataplexy provoked by the food elicited cataplexy test. Importantly, this elevation was not produced during epochs of locomotor activity or feeding without cataplexy, nor was it produced in normal dogs under similar testing conditions (Reid *et al.*, 1994a). Since narcolepsy/cataplexy in these dogs results from a OX2R null mutation (Lin *et al.*, 1999), these findings imply that a key adaptation to the loss of signaling at OX2Rs is increased cholinergic transmission within the mPRF. Our findings indicate that mouse cholinergic LDT neurons are not increased in number and agree with a prior study (Tafti *et al.*, 1997) that was unable to replicate an earlier report of more cholinergic neurons in the LDT/PPT from narcoleptic canines (Nitz *et al.*, 1995). Thus, it is unlikely that increased cholinergic transmission results from there being more cholinergic neurons to innervate these reticular formation targets. In contrast, our findings suggest that along with potential changes in mAChR expression, cholinergic transmission could be augmented by an increased capacity to synthesize, package and release ACh by LDT neurons—some of which project to this region of the reticular formation (Satoh & Fibiger, 1986; Quattrochi *et al.*, 1989; Semba *et al.*, 1990; Semba, 1993). Future studies will need to examine the consequences of the adaptations described here on phasic and tonic ACh release in the mPRF and the role of pontine mAChRs in regulating these processes.

Assuming these changes in cholinergic markers reflect an altered capacity for cholinergic transmission, our data from DKO mice suggest a plausible mechanism to account for the enhanced levels of ACh in the mPRF associated with cataplexy in narcoleptic canines. Nevertheless, there are important differences between these models that need to be addressed in future experiments. For example, OX2R null mice show only rare cataplexy, unlike canines with inherited narcolepsy (Willie *et al.*, 2003). It will therefore be important to determine whether the up regulation in the cholinergic system requires the absence of one or both orexin receptors in mice. Moreover, it will be important to determine if there are expression changes in other transmitter systems like the catecholamines in the DKOs. Since several adaptations are undoubtedly necessary to produce narcolepsy/cataplexy (Mignot *et al.*, 1993), changes in these other systems may be critical for producing the cholinergic properties described here. In addition, while a role for pontine cholinergic transmission in the control of REM sleep and cataplexy is firmly established from feline and canine studies (for review, see Kubin, 2001), its role in REM regulation in rodent is more controversial and requires further examination (Lydic *et al.*, 2002; Coleman *et al.*, 2004; Pollock & Mistlberger, 2005). Moreover, only limited data is available indicating a role for brain cholinergic systems in mouse models of narcolepsy. Available data indicate that systemic atropine reduces cataplexy in the orexin ligand knockout mouse (Willie, 2005) and that systemic or pontine delivery of cholinesterase inhibitors promotes cataplexy-like behavioral arrests in DKO mice while a similar delivery of atropine suppresses these behaviors (Kalogiannis *et al.*, 2008). These data, in combination with our present findings, suggest that one important adaptation to the loss of orexin signaling is a localized up regulation of the molecular machinery for cholinergic transmission which supports enhanced cholinergic transmission in brainstem regions that gate cataplexy.

Abbreviations

ACh	acetylcholine
AChE	acetylcholinesterase
ACSF	artificial cerebral spinal fluid
ChAT	choline acetyltransferase
CHT1	high-affinity choline transporter
DKO	double orexin receptor knockout
DR	dorsal raphe
IDV	integrated density value
LC	locus coeruleus
LDT	laterodorsal tegmental nucleus
Mo5	motor nucleus of the fifth cranial nerve
mPRF	medial pontine reticular formation
Ox1R	orexin 1 receptor
Ox2R	orexin 2 receptor
PPT	pedunculopontine tegmental nucleus
qRT-PCR	quantitative real time- polymerase chain reaction
WT	mice with wild type allele for both orexin receptors

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Figure 1. ChAT immunostaining and tissue punch targets

(A) Representative coronal brain section (top right) at a rostro-caudal plane containing both the LDT and the Mo5 demonstrates selective DAB immunoperoxidase staining of cholinergic nuclei (bottom right). The ChAT antibody labeled only a small fraction of the neurons present as indicated by the Nissl stain (bottom left) and was specific as indicated by a control in which the primary anti-ChAT antibody was excluded (top right). Tissue punches were obtained from slices containing the LDT and Mo5. ChAT immunohistochemistry from a punched slice, confirming that the LDT (B) and the Mo5 (C) were excised.

Figure 2. Message levels for markers of cholinergic synaptic transmission were elevated in LDT and Mo5 samples from DKO mice

(A1) ChAT message amplicons were visualized by ethidium bromide and formed a single band of ~200 bp. Samples run in lane 1: Blank, 2–5: Scales, 6: DKO LDT, 7: B6 LDT, 8: DKO Mo5, 9: B6 Mo5. (2) ChAT message levels (mean ± SEM μg/μl, n = 6) were greater in DKO vs B6 samples from LDT (left) and Mo5 (right); * p < 0.05. (B1) VAcHT message amplicons were ~150 bp. Samples run in lane 1: Blank, 2–5: Scales, 6: DKO LDT, 7: B6 LDT, 8: DKO Mo5, 9: B6 Mo5. (2) VAcHT message levels (mean ± SEM μg/μl, n = 6) were greater in DKO vs B6 samples from LDT (left) and Mo5 (right); * p < 0.05. (C1) CHT1 message amplicons were ~150 bp. Samples run in lane 1: Blank, 2–5: Scales, 6: DKO LDT, 7: B6 LDT, 8: DKO Mo5, 9: B6 Mo5. (2) CHT1 message levels (mean ± SEM μg/μl, n = 6) were greater in DKO vs B6 samples from LDT (left) and Mo5 (right); * p < 0.05. (D1) AChE message amplicon were ~160 bp. Samples run in lane 1: Blank, 2–5: Scales, 6: DKO LDT, 7: B6 LDT, 8: DKO Mo5, 9: B6 Mo5. (2) AChE message levels (mean ± SEM μg/μl, n = 6) were not different between DKO vs B6 samples from either LDT (left) and Mo5 (right); p > 0.05. (μg/μl). Significance was determined by two-tailed Student t-test.

Figure 3. Message levels for markers of cholinergic synaptic transmission were not different in samples from whole brainstem

Message levels for each cholinergic synaptic marker from whole brainstem samples (mean $\pm$ SEM $\mu\text{g}/\mu\text{l}$) taken from wild-type (B6; left, $n = 6$) and DKO (right, $n = 6$) mice. Data for ChAT (A), VACht (B), CHT1 (C) and AChE (D) are illustrated. No significant differences were found between genotypes ($p > 0.05$).

Figure 4. Summary of message levels from DKO samples as fold-difference from B6 samples
 Message levels for ChAT, VAcHt and CHT1 were between 2 and 4 fold greater in DKO samples from LDT punches. Message levels for CHAT and VAcHt, but not CHT1 were between 2 and 4 fold greater in DKO samples from Mo5 punches. In contrast, no difference was seen in samples from whole brainstems suggesting the differences observed in punches were localized. Asterisks indicate the comparisons that were significant by comparison of absolute levels in each sample.

Figure 5. Genotypic differences in protein or ChAT activity attributable to mesopontine cholinergic neurons were not detected

(A1) Representative Western blots of samples isolated from whole brainstem and probed for ChAT (~ 68–70 kDa) and cyclophilin-A (~20 kDa). (A2) Mean $\pm$ SEM of the ChAT band (integrated density value) normalized by the cyclophilin-A band value for samples obtained from DKO (filled bars) and wild-type BC mice (BC; open bars) mice from whole brainstem (BS; n = 8 each genotype); thalamus (Thal; n = 6 each genotype) and cortex (Ctx; n = 8 each genotype). (B1) Representative Western blots of samples isolated from whole brainstem and probed for CHT1 (~60 kDa) and cyclophilin-A. (2) Mean $\pm$ SEM of the normalized CHT1 band for the same tissues in A2 (BS, n = 8; Thal, n = 4; Ctx n = 7). No differences were found between genotypes for any samples. (C) Mean $\pm$ SEM ChAT activity (nmol/mg protein/hr) for samples from the PnO samples and cortical (Ctx) samples obtained from BC mice (n = 8; open bars) and DKO mice (n = 8; filled bars). No difference between genotype was observed for pontine samples but there was significantly less ChAT activity in the cortical DKO samples.

Figure 6. LDT and Mo5 cholinergic neuron counts from BC and DKO mice

(A) Camera lucida drawings of a mid-LDT section from a wild-type (BC; A1) and DKO (A2) mouse with ChAT immunoreactive cell bodies plotted (dots). (B) The mean $\pm$ SEM number of ChAT immunoreactive cells counted on one side of three sections matched in the caudal, middle and rostral levels of the LDT (left) and the Mo5 (right) from wild-type (BC; $n=4$) and DKO ($n=4$) mice. No difference in cell numbers were found across genotype ($p > 0.05$).

Figure 7. ChAT Quantitative Immunohistochemistry

(A) Micrographs of DAB stained neurons in the LDT from a pair of co-processed sections from a wild-type (BC; 1) and DKO mouse (2). (B) Histogram showing the distribution of optical densities from each LDT revealed the distribution from the DKO mouse shifted toward greater optical density. (C) The mean $\pm$ SEM of optical density values from four such section-pairs for the LDT (C1) and Mo5 (C2). (C3) LDT values normalized by the corresponding Mo5 values indicates that ChAT staining was systematically greater in the LDT from DKO mice. (D) Distributions of LDT ChAT optical densities for co-processed sections from a wild-type (B6) and a wild-type (129) mouse. The distributions completely overlapped. (E) Distribution of background-subtracted ChAT fluorescence of LDT neurons

from co-processed sections from a DKO and wild-type (B6) mouse. (F) The mean $\pm$ SEM of ChAT fluorescence from three sets of co-processed sections from the LDT (F1) and Mo5 (F2) of wild-type (B6) and DKO mice. (F3) LDT fluorescence normalized by Mo5 values indicate that ChAT staining was greater in the LDT from DKO mice than in the LDT from B6 mice. Scale bar in A1 = 20 μ m and also applies to A2.

Table 1

Primer sequences for RT-PCR

<i>Gene</i>	Sequence	Pubmed ID	Amplicon size (bp)
ChAT	forward: 5'- AGCCTGAGGCCATCACCTTC-3' reverse: 5'- TTGGCTTGGTTGGGCCTCTA-3'	NM_009891 (2456–2656)	200
VAcHt	forward: 5'- GTGCGCCACGTATCAGTCTA-3' reverse: 5'- GTCCTTGCCCTGCACCTCAC-3'	AF019045 (5574–5724)	150
CHT1	forward: 5'- GCAAGGCACAGTGAAGAGAA-3' reverse: 5'- CTTAGCCCAAGACATGCAGA-3'	AJ01467 (1555–1705)	150
AChE	forward: 5'- AAGGGCTGGGATATAATACGAC- 3' reverse: 5'- CTTAGCCCAAGACATGCAGA-3'	NM_009599 (2016–2176)	160
Cyclophilin A	forward: 5'- TCCGACTGTGGACAGCTCTA-3' reverse: 5'- TTGAAGGGGAATGAGGAAAA-3'	NM_008907 (516–666)	150