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The α_2-Adrenoceptor Agonist Dexmedetomidine Converges on an Endogenous Sleep-promoting Pathway to Exert Its Sedative Effects

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Background: **The authors investigated whether the sedative, or hypnotic, action of the general anesthetic dexmedetomidine (a selective α_2-adrenoceptor agonist) activates endogenous nonrapid eye movement (NREM) sleep-promoting pathways.**

Methods: **c-Fos expression in sleep-promoting brain nuclei was assessed in rats using immunohistochemistry and *in situ* hybridization. Next, the authors perturbed these pathways using (1) discrete lesions induced by ibotenic acid, (2) local and systemic administration of γ-aminobutyric acid receptor type A ($GABA_A$) receptor antagonist gabazine, or (3) α_2-adrenoceptor antagonist atipamezole in rats, and (4) genetic mutation of the α_{2A}-adrenoceptor in mice.**

Results: **Dexmedetomidine induced a qualitatively similar pattern of c-Fos expression in rats as seen during normal NREM sleep, *i.e.*, a decrease in the locus ceruleus (LC) and tuberomammillary nucleus (TMN) and an increase in the ventrolateral preoptic nucleus (VLPO). These changes were attenuated by atipamezole and were not seen in mice lacking functional α_{2A}-adrenoceptors, which do not show a sedative response to dexmedetomidine. Bilateral VLPO lesions attenuated the sedative response to dexmedetomidine, and the dose–response curve to dexmedetomidine was shifted right by gabazine administered systemically or directly into the TMN. VLPO lesions and gabazine pretreatment altered c-Fos expression in the TMN but in not the LC after dexmedetomidine administration, indicating a hierarchical sequence of changes.**

Conclusions: **The authors propose that endogenous sleep pathways are causally involved in dexmedetomidine-induced sedation; dexmedetomidine's sedative mechanism involves inhibition of the LC, which disinhibits VLPO firing. The increased release of GABA at the terminals of the VLPO inhibits TMN firing, which is required for the sedative response.**

α_2-ADRENOCEPTOR agonists (*e.g.*, clonidine, dexmedetomidine, xylazine) are anesthetic agents used widely in clinical and veterinary settings for their sedative, or hypnotic, and analgesic effects. One of the highest densities of α_2-adrenoceptor has been detected in the pontine locus ceruleus (LC),[1] a key source of noradrenergic innervation of the forebrain and an important modulator of vigilance.[2,3] The sedative effects of α_2-adrenoceptor activation have been attributed to the inhibition of this nucleus.[4,5]

Dexmedetomidine (marketed as Precedex by Abbott Laboratories, Abbott Park, IL) is the pharmacologically active dextroisomer of medetomidine and displays specific and selective α_2-adrenoceptor agonism. It was approved by the US Food and Drug Administration in 1999 as a short-term medication ($<$ 24 h) for sedation of patients in the intensive care unit, and it has also been shown to act as a general anesthetic.[6] Its specificity for the α_2-adrenoceptor, and relative selectivity for the α_{2A}-adrenoceptor subtype (which is responsible for its sedative properties[7]), provides for a more effective sedative and analgesic agent than clonidine and makes it an ideal probe with which to investigate anesthetic mechanisms.

After dexmedetomidine binds to α_2-adrenoceptors in the LC, transmembrane signaling results in activation of an inwardly rectifying potassium channel facilitating a K^+ efflux and inhibition of voltage-gated Ca^{2+} channels. The resulting hyperpolarization decreases the firing rate of LC neurons and allows presynaptic inhibition of their terminals.[8–10] Hyperpolarization of noradrenergic LC neurons appears to be a key factor in initiating the anesthetic mechanism of action of dexmedetomidine.[5,11]

Qualitatively, dexmedetomidine induces a sedative response that exhibits properties similar to natural sleep, unlike other anesthetics. Patients receiving dexmedetomidine experience a clinically effective sedation yet are still easily and uniquely arousable, an effect not observed with any other clinically available sedative.[12] Data from our recent functional magnetic resonance imaging (fMRI) study in human volunteers indicate that the blood oxygen level dependent (BOLD) signal, which positively correlates with local brain activity, changes during dexmedetomidine sedation in a similar fashion to that seen during natural sleep, whereas changes induced by the benzodiazepine midazolam were markedly different.[13] We postulate that a dexmedetomidine-induced decrease in firing of noradrenergic LC neurons leads to loss of consciousness, at least in part, *via* activation of an endogenous sleep-promoting pathway.

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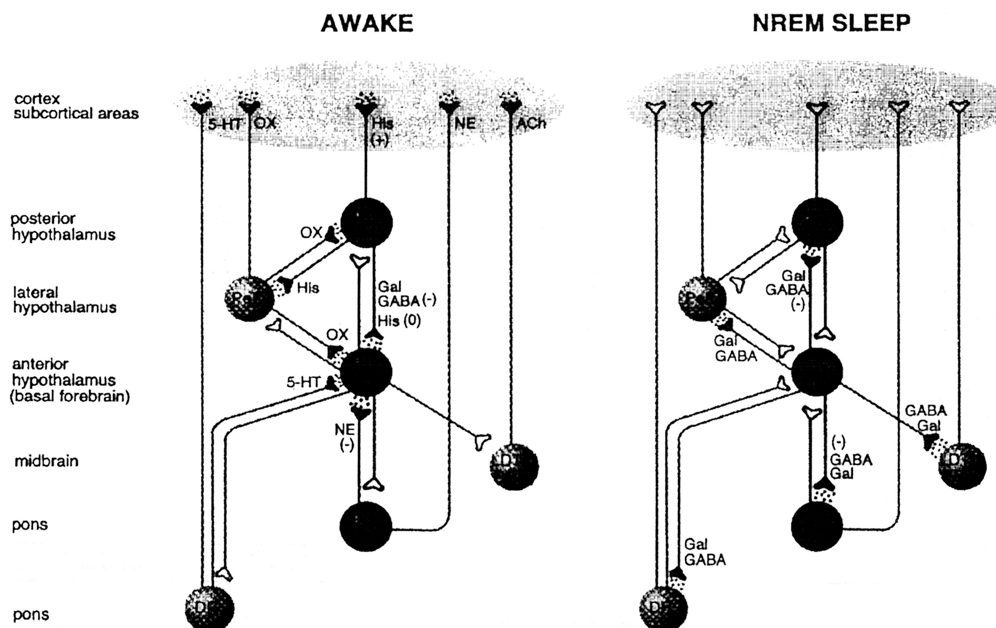

Fig. 1. Cartoon of a simplified nonrapid eye movement (NREM) sleep-promoting pathway activated by dexmedetomidine. An inhibition of firing of noradrenergic neurons in the locus coeruleus (LC), which accompanies endogenous NREM sleep[30,31] and is the site of initiation of α_2-adrenergic anesthesia,[4,5] releases a tonic noradrenergic inhibition of the ventrolateral preoptic nucleus (VLPO). The activated VLPO[14] is believed to release γ-aminobutyric acid (GABA) into the tuberomammillary nucleus (TMN),[17,20,39–42] which inhibits its release of arousal-promoting histamine into the cortex and forebrain to induce loss of consciousness.[19,22] A number of pathways are involved in NREM sleep; the sleep-active VLPO[14] projects to all the ascending monoaminergic, cholinergic, and orexinergic arousal nuclei,[17,20] which project to the cortex, forebrain, and subcortical areas where they release neurotransmitters of arousal to promote wakefulness.[14] This study concentrates on the TMN as representative of all of these arousal centers inhibited by the VLPO during sleep, but it seems likely that others may also be inhibited by dexmedetomidine. The LC widely innervates the brain, but only projections associated with NREM sleep are shown here. Although the TMN densely innervates the VLPO, histamine does not influence VLPO firing rates *in vivo*,[15] but TMN neurons also contain galanin and GABA, which could influence VLPO activity.[21] A simplified version of this circuitry, the portion of the pathway highlighted in black, is the focus of this investigation. Ach = acetylcholine; DR = dorsal raphe nuclei; His = histamine; 5-HT = serotonin; LDTg = laterodorsal tegmental nuclei; NE = norepinephrine; OX = orexin (hypocretin); PeF = perifornical area; PPTg = pedunculopontine tegmental nuclei.

Nonrapid eye movement (NREM) sleep appears to involve distinct neuronal pathways in at least three discrete regions of the rodent brain. It is hypothesized that "sleep-promoting"[14] γ-aminobutyric acid (GABA)-containing neurons in the ventrolateral preoptic nucleus (VLPO) in the anterior hypothalamus and basal forebrain are under inhibitory control by norepinephrine and serotonin from the LC and raphe nucleus, respectively.[15,16] A decrease in noradrenergic firing disinhibits galanin and (γ-aminobutyric acid-mediated (GABAergic) VLPO neurons, which are thus activated. VLPO neurons innervate the LC and the tuberomammillary nucleus (TMN),[17] a posterior lateral hypothalamic cell group known to play a key role in promoting arousal,[18,19] alongside other ascending arousal-promoting monoaminergic, cholinergic, and orexinergic nuclei.[17,20] The TMN is known to be the only neuronal source of histamine and to also contain GABA and galanin.[21] At the level of the LC, this VLPO innervation has an inhibitory effect, further decreasing firing in the LC (and consequently reinforcing sleep by further decreasing the LC's tonic inhibition of VLPO). Descending projections from the VLPO, containing GABA and galanin,[17] inhibit neurons in the TMN, which releases histamine into the cortex, forebrain, and subcortical regions during wakefulness;[19,22] histamine release in these higher centers is thought to be responsible, at least in part, for loss of wakefulness. If a NREM sleep-promoting pathway (fig. 1) is important for dexmedetomidine-induced sedation, it follows that although dexmedetomidine does not bind directly to the γ-aminobutyric acid receptor type A ($GABA_A$) receptor, GABA is mechanistically important downstream from dexmedetomidine's binding site in the LC. (We report elsewhere on how putatively $GABA_A$ receptor-mediated anesthetic agents interact with this pathway.[23])

Hypothesizing that dexmedetomidine exerts its sedative effects by converging on an endogenous NREM sleep-promoting pathway, we first evaluated at a cellular level the brain response to dexmedetomidine by way of c-Fos protein immunostaining during dexmedetomidine anesthesia. We then tested the role of downstream GABAergic pathways in mediating dexmedetomidine anesthesia by perturbing the sleep-promoting circuitry using discrete central nervous system (CNS) administration of a $GABA_A$ receptor antagonist and discrete neuronal lesioning.

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Materials and Methods

Animals

This experimental protocol was approved by the Home Office of the United Kingdom (London, UK), the Ethics Review Committee of Imperial College of Science, Technology and Medicine (London, UK), and the Institutional Animal Care and Use Committees of Beth Israel Deaconess Medical Center and Harvard Medical School (Boston, Massachusetts), and all animal procedures were carried out in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986. Adult male Fischer rats (weight, 250–300 g; n = 66) and adult male C57B6 mice (weight, 25–30 g; n = 24) purchased from B&K Universal (Grimston Aldbrough Hull, UK) and adult male D79N transgenic mice (weight, 25–30 g; n = 24; C57B6 genetic background; dysfunctional α_{2A}-adrenoceptor gene, kindly donated by Professor Lee Limbird, Ph.D., Department of Pharmacology, Vanderbilt University, Nashville, Tennessee) were given access to food and water *ad libitum* and housed under controlled conditions (12 h of light starting at 7:00 PM; 20–22°C) in an isolated ventilated chamber. All efforts were made to minimize animal suffering and reduce the number of animals used.

Drug Preparation

Gabazine hydrobromide (SR-95531, Tocris, Bristol, UK), dexmedetomidine (donated by Orion Pharmaceuticals, Espoo, Finland), and chloral hydrate (Sigma, Poole, UK) were prepared in normal saline (NS; 0.9% NaCl w/v) solution. Halothane (Fluothane, Zeneca Ltd., Cheshire, UK) was administered *via* an anesthesia machine. Atipamezole hydrochloride (5 mg/ml; Antisedan®, Pfizer, Sandwich, UK) was used as premixed solution. Pentobarbital (J.M. Loveridge Plc., BM-20111, Southampton, UK) was used as 20% solution (w/v) in NS.

Cannulae Implantation Surgery

Animals were anesthetized with halothane in a Plexiglas chamber (Röhm GmbH, Darmstadt, Germany), prepared for aseptic surgery, and secured in a stereotaxis frame. One hole for intracerebroventricular injection or two for TMN injections were drilled into the skull and filled with removable bone wax (Ethicon, Brussels, Belgium). Cannulae (22 gauge; 20 mm for TMN, 15 mm for intracerebroventricular; Tomlinson Tubes Ltd., Bidford-on-Avon, UK) were stereotaxically positioned (intracerebroventricular coordinates: ± 1.0 mm mediolateral, −5.2 anteroposterior, −9.1 dorsoventral from Bregma; TMN coordinates: −1.0 mm mediolateral, −1.0 mm anteroposterior, −4.0 mm dorsoventral),[24] and affixed with dental resin (Orthoresin, Dentsply, Surrey, UK).

Discrete Administration of Anesthetic Agents into CNS

Drugs were discretely administered into the TMN or intracerebroventricular space at least 4 days postsurgery. The α_2-adrenoceptor agonist dexmedetomidine (2–6 μg/10 μl) was microinjected into the intracerebroventricular space, or the $GABA_A$ receptor antagonist gabazine (0.2 μg/0.2 μl per side) was microinjected directly into the TMN using a Howard CMA microinjection pump (CMA/100, CMA/Microdialysis, Stockholm, Sweden) at rates of 5 μl/min and 0.4 μl/min, respectively. The needle was removed 2 min after the completion of the injection. The locations of the injections were later confirmed histologically.

Loss of Righting Reflex Assessment

Sedation was assessed using a loss of righting reflex (LORR) endpoint defined as the inability of animals to right themselves when positioned in a supine position in a warm environment. Dose–response data were fitted according to the method of Waud[25] to a logistic equation of the form

$$p = \frac{100\ D^n}{D^n + (ED_{50})^n} \qquad (1)$$

where p is the percent of the population anesthetized, D is the drug dose, n is the slope parameter, and ED_{50} is the drug dose for a half-maximal effect.

Electroencephalogram and Electromyogram Electrode Implantation

Rats were fitted with electroencephalogram and electromyogram electrodes according to methodology previously described.[26] Briefly, they were anesthetized with intraperitoneal chloral hydrate, 350 mg/kg, prepared for aseptic surgery, and secured into a stereotaxis frame. Four electroencephalogram electrodes were screwed into the skull, two electromyogram electrodes were placed under the nuchal muscles, and all leads were connected into a pedestal socket and affixed to the skull with dental cement. Animals were allowed to recover for at least 6 days.

Sleep Recording and Scoring

Sleep was scored and recorded according to methodology previously described.[26] Animals were acclimated to the recording apparatus 24–48 h before experiments began. Electroencephalogram and electromyogram signals were amplified by a polygraph (GRASS, West Warwick, RI) and digitized by the ICELUS program (G-Systems, Plano, TX) for LabVIEW (National Instruments, Austin, TX). An investigator blinded to treatment manually scored sleep–wake states in 12-s epochs. Wakefulness was identified by desynchronized electroencephalogram and high electromyogram activity, NREM sleep

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by high-amplitude slow-wave electroencephalogram and low electromyogram, and REM sleep by a regular θ component in the electroencephalogram power spectrum coupled with very low electromyogram activity.

Transcardial Perfusion and Tissue Sectioning

Animals were anesthetized with intraperitoneal chloral hydrate, 350 mg/kg, or intraperitoneal pentobarbital, 140 mg/kg, and transcardially perfused with NS (100 ml) or phosphate buffered saline (PBS; 100 ml; 0.1 M phosphate buffer, 0.9% NaCl; pH 7.4) followed by 10% formalin (500 ml; Sigma-Aldrich, Poole, UK) or 4% paraformaldehyde (500 ml; BDH, Poole, UK) in 0.1 M phosphate buffer. Whole brains were removed, postfixed in 10% formalin or 4% paraformaldehyde overnight, incubated in 20% sucrose overnight (or until tissue sank), and coronally cryosectioned (1:4 series, 30 μm).

Immunohistochemistry

Sections were double-immunostained using previously described methods.[14,17,27] All sections were stained for c-Fos (goat polyclonal antibody; 1:20,000; Santa Cruz, Insight Biotechnology, Wembley, UK; or 1:150,000; rabbit; Oncogene, CN Biosciences, Nottingham, UK) using secondary donkey antigoat IgG (1:200; Chemicon, Harrow, UK), VectaStain® Elite ABC solution (Vector, Peterborough, UK) visualized using 3,3-diaminobenzidine (DAB; Vector) with nickel ammonium sulfate for black staining. Next, they were separated into those containing the LC, VLPO, and TMN and then counterstained for dopamine β-hydroxylase (DBH; rabbit polyclonal antibody; 1:20,000; Affiniti Research Products, Exeter, UK), galanin (rabbit polyclonal antibody; 1:50,000; Bachem, St. Helen's, UK), or adenosine deaminase (ADA; rabbit polyclonal antibody; 1:20,000, Chemicon), respectively, using donkey antirabbit IgG (1:200; Chemicon, Harrow, UK) and visualized using DAB without nickel. Galanin staining was enhanced using tyramide amplification (TSA Biotin System, PerkinElmer, Hounslow, UK) according to manufacturer's protocol.

In situ *Hybridization*

According to previously described methodology,[28] to further verify the anatomic location of the VLPO in some instances, VLPO sections were DAB-stained brown for c-Fos protein using immunohistochemistry (see previous Immunohistochemistry methodology section) and subsequently counterstained for galanin mRNA using *in situ* hybridization. In brief, sections were acetylated, hybridized overnight using a ^{35}S-labeled cRNA probe synthesized from a plasmid containing the galanin coding sequence,[29] and washed (1 h each in 2× sodium chloride citrate [SSC]/1 mM dithiothreitol (DTT), 50°C; 0.2× SSC/1 mM DTT, 55°C; then 0.2× SCC/1 mM DTT, 60°C). Sections were then treated with RNAase-A (Boehringer-Mannheim, Indianapolis, IN), washed in conditions of increasing stringency (including 30 min in 60°C 0.1× SCC), dehydrated in graded alcohols, and air-dried. Sections were exposed to x-ray film (2 or 3 days; Eastman-Kodak, Rochester, NY), dipped in NTB-2 emulsion (Kodak), exposed for 1 month, developed in D-19 (Kodak), fixed, dehydrated, and cover-slipped.

Cell Counting and Analysis

Using light microscopy, c-Fos positive neurons were identified by dense black nuclear staining, and the DBH-, galanin-, and ADA-positive neurons in the LC, VLPO, and TMN were identified by brown cytoplasmic staining. Locations in the brain were confirmed by staining and reference to the primary literature[14,17,26,30,31] and a rat brain atlas.[24] Four sections through the middle of each structure per animal were counted (blind to treatment) and averaged. Data were analyzed using one-way analysis of variance (ANOVA) and the Bonferroni test and are presented as mean ± SEM. Differences were considered significant at $P < 0.05$.

Discrete Neuronal Lesioning by Ibotenic Acid Microinjection

Using previously established methodology,[26] discrete bilateral VLPO lesions were administered. Briefly, animals were secured in a stereotaxis, and the skull was exposed. After a small hole was drilled in the skull over the VLPO, the dura was incised, a fine glass micropipette (20-μm tip diameter) was stereotaxically lowered into the VLPO (coordinates: −0.6 mm anteroposterior, −8.0 mm dorsoventral, and ± 1.0 mm mediolateral from Bregma), and 15 nl of ibotenic acid (10 nmol) was microinjected by air pressure. The area of neuronal loss induced by the lesion was assessed histologically by staining for c-Fos immunoreactivity (as described by Sherin *et al.*[14] and in previous Immunohistochemistry methodology section) followed by nonspecifically counterstaining with 0.25% thionin (Nissl stain), as previously described.[26] In the region of the lesion, Nissl-stained cells that remained in the VLPO could be easily identified. The absence of c-Fos expression in the VLPO area after dexmedetomidine administration further confirmed lesion success. Lesions were quantified using cell-counting methodology described by Lu *et al.*,[26] which used a counting box placed over the VLPO (framed by the lateral edge of the optic chiasm and extending 300 μm dorsally and 300 μm laterally), and lesions were considered successful if there was > 80% bilateral cell loss.[26] Animals with lesions that missed the VLPO were used as sham-operated controls.

Data Analysis

To assess the effects of VLPO lesions in dexmedetomidine-induced sedation, animals with bilateral VLPO lesions were administered intraperitoneal control NS or dexmedetomidine, 50 μg/kg (a dose sufficient to induce

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LORR for more than 2 h in naïve animals), at approximately 6:45 PM and were connected to the electroencephalogram and electromyogram recording apparatus for 2 h. After 2 h of electroencephalogram and electromyogram recording (approximately 7:00–9:00 PM, a period of maximal wakefulness in naïve animals), animals were transcardially perfused, and brains were sectioned and immunostained (see previous Immunohistochemistry methodology section). The percentages of wakefulness, rapid eye movement (REM) sleep and NREM sleep per hour were plotted from 7:00 to 9:00 PM postlesion in the absence and presence of dexmedetomidine or NS injection and compared with similar recordings made in unlesioned animals. Data were analyzed using one-way ANOVA and the Bonferroni test to determine significant differences in the paired data.

Results

Systemic Gabazine Attenuates Centrally Administered Dexmedetomidine-induced Anesthesia

Using behavioral measures, we tested the hypothesis that the mechanism of dexmedetomidine-induced sedation (loss of consciousness) converges on the endogenous NREM sleep-promoting pathway by challenging the sedation induced by centrally administered dexmedetomidine with a systemically administered GABA antagonist. Subcutaneous administration of the $GABA_A$ subtype selective antagonist gabazine, 5 mg/kg, induced a rightward shift in the dose required to cause a LORR, an endpoint commonly used as a surrogate measure of loss of consciousness (fig. 2A). Dexmedetomidine alone had an ED_{50} of 0.32 ± 0.11 μg (mean ± SEM), whereas dexmedetomidine in the presence of gabazine pretreatment exhibited a significantly different ($P < 0.05$) ED_{50} of 0.44 ± 0.01 μg. When dexmedetomidine and gabazine were administered systemically, the presence of gabazine significantly ($P < 0.05$) attenuated the sedative response to dexmedetomidine. Subcutaneous gabazine, 5 mg/kg, significantly ($P < 0.05$) increased the latency to (fig. 2B) and decreased the duration of LORR (fig. 2C) induced by subcutaneous dexmedetomidine, 150 μg/kg (both measures indicate reduced anesthetic potency).

Gabazine Administered into the TMN but not LC Attenuates Systemic Dexmedetomidine-induced Anesthesia

In the LC, where discretely administered dexmedetomidine can induce sedation, we found that local administration of the $GABA_A$ antagonist gabazine had no effect on dexmedetomidine-induced sedation (n = 6), whereas systemically administered gabazine significantly ($P <$ 0.05) shifted the dexmedetomidine dose–LORR response curve to the right (fig. 2A). However, gabazine administered more distally into the arousal-promoting TMN (n = 3) attenuated the sedation induced by dexmedetomidine ($P < 0.05$).

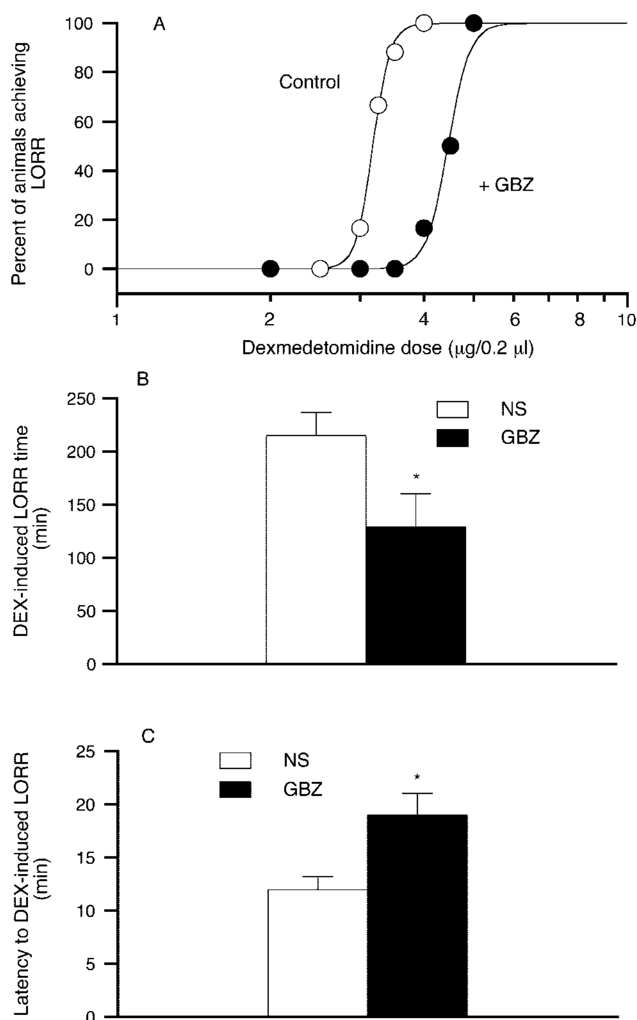

Fig. 2. Behavioral experiments implicate γ-aminobutyric acid receptor type A ($GABA_A$) receptors in dexmedetomidine's sedative mechanism. (*A*) Illustration of systemically administered $GABA_A$ receptor antagonist gabazine (5 mg/kg, subcutaneous) rightward shifts the dexmedetomidine dose–loss of righting reflex (LORR) response curve to centrally administered α_2-adrenoceptor agonist dexmedetomidine (2–6 μg/10 μl, intracerebroventricular; n = 6 per cohort). In addition, systemically administered gabazine (5 mg/kg, subcutaneous; n = 6) attenuates the sedation (*B*) induced by and increases the latency (*C*) of response to systemically administered dexmedetomidine (150 μg/kg, subcutaneous; n = 6). Data are presented as mean ± SEM, and asterisk denotes $P < 0.05$ *versus* NS. DEX = dexmedetomidine; GBZ = gabazine; NS = normal saline.

c-Fos Expression Pattern during Dexmedetomidine-induced Anesthesia Mirrors NREM Sleep

We next used immunohistochemical techniques to assess whether dexmedetomidine acts on a NREM sleep-promoting pathway (fig. 1). Activation of a cellular immediate early gene *c-fos*, and subsequent accumulation of expressed c-Fos protein, is often used as evidence of neuronal activity.[32,33] Although the link between c-Fos

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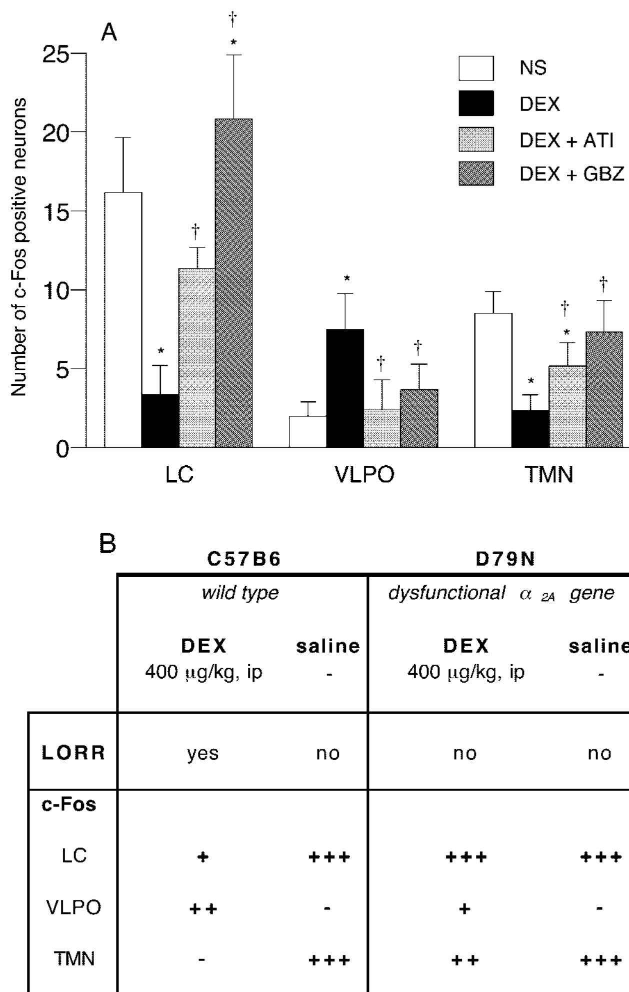
expression and neuronal activation is indirect, c-Fos levels have been shown to change in different parts of the brain during spontaneous sleep–wake episodes,[14,30,31,34] and these changes are taken as surrogate markers for neuronal activity. Dexmedetomidine-induced sedation resulted in a pattern of c-Fos expression in the LC, VLPO, and TMN that was qualitatively and quantitatively similar to that seen previously during endogenous normal NREM sleep,[30,31] *i.e.*, decreased c-Fos expression in the LC, increased in the VLPO, and decreased in the TMN (fig. 3A). We further examined the effect of dexmedetomidine on c-Fos expression after administration of the α_2-adrenoceptor antagonist atipamezole or the $GABA_A$ receptor antagonist gabazine. Fifteen-minute pretreatment with intraperitoneal atipamezole, 2 mg/kg (a dose that blocks dexmedetomidine-induced sedation), inhibited the dexmedetomidine-induced changes in c-Fos expression in the LC, VLPO, and TMN described previously. Pretreatment with intraperitoneal gabazine, 5 mg/kg, blocked dexmedetomidine-induced c-Fos changes in the TMN but not in the LC or VLPO (fig. 3A).

α_{2A}-Adrenoceptor Mutation Blocks Dexmedetomidine Anesthesia and c-Fos Changes in Sleep Circuitry

We examined dexmedetomidine-induced c-Fos expression in transgenic mice lacking functional α_{2A}-adrenoceptors to assess the mechanisms of the dexmedetomidine-induced sedation. Gene targeting was used to introduce a point mutation into the α_{2A}-adrenoceptor subtype in the C57B6 strain mouse genome, rendering it dysfunctional in the eponymous D79N mouse. The α_{2A} subtype is known to be responsible for the sedative component of dexmedetomidine's anesthetic action,[7,35] whereas α_{2B} and α_{2C} subtypes are uninvolved in sedation but may play a role in analgesia.[36] In D79N animals, dexmedetomidine cannot induce LORR at any dose. In C57B6 mice, 2 h of dexmedetomidine-induced sedation at the beginning of the dark cycle (rodent waking cycle, 7:00–9:00 PM) resulted in a c-Fos pattern that was qualitatively similar to that seen in rats (see the preceding Results section), *i.e.*, reduced c-Fos expression in the LC and TMN and elevated c-Fos in the VLPO relative to NS-administered (awake) animals. However, none of these changes were observed in D79N mice administered the same dose of dexmedetomidine (400 μg/kg); in this case, the pattern of c-Fos expression did not differ from that induced by NS (fig. 3B).



	C57B6 wild type DEX 400 µg/kg, ip	C57B6 wild type saline -	D79N dysfunctional α 2A gene DEX 400 µg/kg, ip	D79N dysfunctional α 2A gene saline -
LORR	yes	no	no	no
c-Fos				
LC	+	+++	+++	+++
VLPO	++	-	+	-
TMN	-	+++	++	+++

Fig. 3. Dexmedetomidine sedation induces c-Fos expression pattern of nonrapid eye movement (NREM) sleep. (*A*) Sedation induced by systemically administered α_2-adrenoceptor agonist dexmedetomidine (500 μg/kg, intraperitoneal) at night (rodent waking period) results in a pattern of c-Fos expression (a surrogate marker of neuronal activation) in the locus coeruleus (LC), ventrolateral preoptic nucleus (VLPO), and tuberomammillary nucleus (TMN) similar to that seen previously during NREM sleep, *i.e.*, a decrease in c-Fos expression in the LC and TMN and an increase in the VLPO. α_2-Adrenoceptor antagonist atipamezole (ATI; 2 mg/kg, intraperitoneal) reverses these changes in all three areas, and the γ-aminobutyric acid receptor type A ($GABA_A$) receptor antagonist gabazine (5 mg/kg, intraperitoneal) reverses them in the VLPO and TMN but further increases c-Fos expression in the LC relative to wakefulness. Asterisks denote $P < 0.05$ *versus* NS, crosses denote $P < 0.05$ *versus* dexmedetomidine, and data are shown as mean ± SEM. (*B*) The changes in c-Fos expression during sedation induced by α_2-adrenoceptor agonist dexmedetomidine (400 μg/kg, intraperitoneal) were not observed in transgenic D79N mice lacking a functional α_{2A}-adrenoceptor subtype, indicating that these changes are causally linked to the induction of sedation. DEX = dexmedetomidine; GBZ = gabazine; LORR = loss of righting reflex; NS = normal saline.

VLPO Lesioning Attenuates Dexmedetomidine-induced Anesthesia

To confirm that the changes in c-Fos expression observed in the endogenous NREM sleep pathway (in the LC, VLPO, and TMN) and during dexmedetomidine-induced sedation are causally related to the sedative state and to assess the sequential relationship of the changes in neuronal activity in these nuclei, we tested dexmedetomidine's sedative efficacy after discretely destroying the VLPO using behavioral (sedation measured by electroencephalogram) and immunohistochemical indices. Bilateral VLPO lesions were administered by microinjec-

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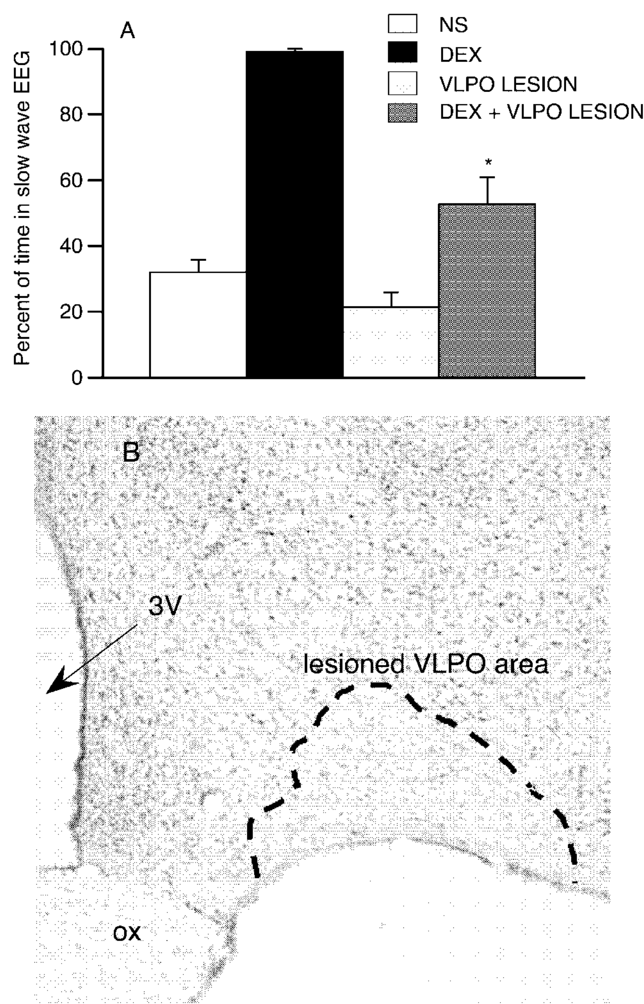

Fig. 4. Bilateral ventrolateral preoptic nucleus (VLPO) lesions attenuate dexmedetomidine-induced sedation. (*A*) Discrete bilateral lesions in the VLPO attenuate the ability of systemically administered α_2-adrenoceptor agonist dexmedetomidine (50 μg/kg, intraperitoneal) to induce sedation between 7:00 and 9:00 PM, a period of rodent maximal wakefulness. (*B*) Depiction of an example of the area lesioned in a Nissl- and c-Fos–stained (DAB visualized) section. DEX = dexmedetomidine; EEG = electroencephalogram; NS = normal saline; ox, = optic chiasm; 3V = third ventricle.

tion of the nonspecific excitotoxin ibotenic acid, which does not destroy fibers of passage.

Bilateral VLPO lesions reduced the percentage of time that animals spent in an electroencephalogram-assessed slow wave sleep state during the 2-h period (7:00–9:00 PM, the beginning of the dark cycle, a period of maximal wakefulness) after dexmedetomidine administration (fig. 4). Further, we found that VLPO lesions blocked dexmedetomidine-induced decreases in c-Fos expression at the level of the TMN but not at the LC. The level of NREM sleep in lesioned and unlesioned animals was consistent with the findings of Lu *et al.*,[26] who reported that VLPO lesions decrease natural sleep in rats during the same 2-h period of the day, further indicating that the lesions performed in this study successfully destroyed VLPO neurons.

Discussion

The aim of this study was to determine whether the mechanism of sedation induced by the α_2-adrenoceptor agonist dexmedetomidine converges on an endogenous NREM sleep-promoting pathway. We found that pretreatment with the $GABA_A$ receptor agonist gabazine decreases the sedative potency of dexmedetomidine, suggesting that $GABA_A$ receptors may be involved downstream of the LC, the key site for initiation of dexmedetomidine sedation. We further observed that the c-Fos changes induced by 90 min of dexmedetomidine sedation were qualitatively similar to those induced during NREM sleep (a decrease at the LC, an increase at the VLPO, and a decrease at the TMN). It is likely that these changes are being initiated at α_2-adrenoceptors because they can be prevented by the α_2-adrenoceptor antagonist atipamezole at a dose sufficient to block dexmedetomidine-induced sedation. Further, dexmedetomidine did not induce the same changes in neuronal activity in mice genetically modified to have mutated α_{2A}-adrenoceptor subtype receptors (in which dexmedetomidine cannot induce sedation).

In questioning whether the activation of the VLPO during dexmedetomidine-induced sedation has a causal role, we found that slow wave electroencephalogram activity after dexmedetomidine administration (indicative of NREM sleep) was significantly attenuated by discrete bilateral VLPO lesions. This finding led us to conclude that the activation of GABAergic and galaninergic neurons in the VLPO is necessary for the sedative response. The patterns of c-Fos expression induced by dexmedetomidine in VLPO-lesioned animals demonstrate the relationship of one nucleus to another. The upstream dexmedetomidine-induced decrease in c-Fos expression in the LC was unchanged in VLPO-lesioned animals, but the downstream dexmedetomidine-induced decrease in c-Fos expression in the TMN was not observed in the absence of a functional VLPO. We further elucidated the hierarchical nature of these changes by demonstrating that discrete administration of gabazine into the TMN blocks dexmedetomidine-induced changes in the TMN but not at the level of the LC or VLPO.

It is known that discrete VLPO lesions prevent normal sleep behavior and render animals insomniac.[26] Recent advances[15,17,29,37,38] suggest that the VLPO is a site that uses GABA to inhibit arousal pathways. VLPO neurons are sleep-active (show c-Fos expression during sleep) and project to all of the ascending monoaminergic, cholinergic, and peptidergic arousal-promoting sites in the brain: the histaminergic TMN, noradrenergic LC, serotonergic dorsal raphe (DR), cholinergic pedunculopontine tegmental nucleus (PPTg), cholinergic laterodorsal tegmental nucleus (LDTg), and the orexinergic perifornical area (PeF).[17] The VLPO neurons are identified by three distinct characteristics. First, they contain the colocal-

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ized inhibitory neurotransmitters GABA and galanin in rats (nearly 100% colocalization, whereas many surrounding neurons contain GABA only).[17] Second, projections densely innervating the TMN originate from a dense cluster of VLPO neurons just lateral to the optic chiasm and a diffuse population of cells extending dorsally and medially from this cluster. Third, VLPO neurons are uniquely sleep-active (*i.e.*, express c-Fos during sleep and show increased firing rates during sleep).[14,38]

The inhibition of the TMN by GABA and galanin, believed to be released by VLPO neurons,[20,37] is hypothesized to play a key role in causing sleep. GABA and galanin are observed in the TMN,[39–42] where they have an inhibitory effect on TMN release of histamine in the cortex and subcortical regions, and are implicated in loss of wakefulness. Yang and Hatton[37] recorded from the TMN while stimulating the VLPO region and found $GABA_A$-mediated IPSPs in the TMN. $GABA_A$ receptor agonist injections here cause sleepiness, and $GABA_A$ receptor antagonists cause wakefulness.[23,43]

Several caveats arising from our experiments are worth discussing. First, we have used rat and mouse models in this study, which raises issues of translatability between species. Transgenic experiments were introduced only as corroborating data and were conducted in mice rather than rats because rats with dysfunctional α_{2A}-adrenoceptor genes (mutations) are not yet available. Mice and rats react similarly to dexmedetomidine, although mice require a higher minimum dose to induce sedation, and it is believed that both species[29] use similar sleep-promoting neuronal circuitry. Second, we have used two endpoints as measures of anesthetic-induced sedation, namely, behavioral LORR and telemetric electroencephalogram and electromyogram assessments. LORR assesses immobility (a feature of loss of consciousness) and equates to loss of consciousness in humans, whereas electroencephalogram and electromyogram measure slow wave electroencephalogram activity, which accompanies sedation. Third, although we used a small cohort size in the VLPO lesion experiments, our results were robust enough to show statistical significance ($P < 0.05$). Finally, we focused this study on the TMN as representative of arousal-promoting nuclei innervated by the VLPO but do not suggest that the TMN is the only wake promoter whose activity is modulated by dexmedetomidine. Rather, we suggest that further experiments are likely to reveal that other loci in the ascending arousal system are also indirectly inhibited by dexmedetomidine.

This is a novel demonstration of a neuronal pathway causally connected with the hypnotic response to a general anesthetic. The potential implications of using general anesthetics (or hypnotic drugs) that act *via* similar mechanisms as natural sleep to induce loss of consciousness are profound. A hypnotic agent that could produce the same reparative changes as natural sleep (*i.e.*, hormone and immune function changes) may speed recovery time in an intensive care setting and counteract the effects of sleep deprivation, a common problem in intensive care units and for surgical patients during recovery. We suggest that α_2-adrenoceptor agonist sedatives activate sleep pathways that work on nuclei upstream of the VLPO at the level of the LC, and thus may produce more restorative sleep than that induced by downstream modulation of the same pathway by GABAergic agents.

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