Involvement of Tuberomamillary Histaminergic Neurons in Isoflurane Anesthesia

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ABSTRACT

Background: The brain histaminergic system plays a critical role in maintenance of arousal. Previous studies suggest that histaminergic neurotransmission might be a potential mediator of general anesthetic actions. However, it is not clear whether histaminergic tuberomamillary nucleus (TMN) is necessarily involved in the sedative/hypnotic effects of general anesthetics.

Methods: Male Long Evans rats underwent either TMN orexin-saporin/sham lesion or implantation of intracerebroventricular cannula 2 weeks before the experiment. The behavioral endpoint of loss of righting reflex was used to assess the hypnotic property of isoflurane, propofol, pentobarbital, and ketamine in animals. Histaminergic cell loss was assessed by adenosine deaminase expression in the TMN using immunohistochemistry.

Results: Rats with bilateral TMN orexin-saporin lesion induced an average 72% loss of histaminergic cells compared with sham-lesion rats. TMN orexin-saporin lesion or intracerebroventricular administration of triprolidine (an H1 receptor antagonist) decreased the 50% effective concentration for loss of righting reflex value and prolonged emergence time to isoflurane anesthesia. However, TMN orexin-saporin lesion had no significant effect on the anesthetic sensitivity to propofol, pentobarbital, and ketamine.

Conclusions: These findings suggest a role of the TMN histaminergic neurons in modulating isoflurane anesthesia and that the neural circuits for isoflurane-induced hypnosis may differ from those of γ-aminobutyric acid-mediated anesthetics and ketamine.

What We Already Know about This Topic
• The brain histaminergic system plays a critical role in the maintenance of arousal and may be involved as a mediator of general anesthetic actions

What This Article Tells Us That Is New
• The histaminergic tuberomamillary nucleus may play a role in modulating isoflurane anesthesia, and the neural circuits for isoflurane-induced hypnosis may differ from those of γ-aminobutyric acid-mediated anesthetics and ketamine

Although the molecular mechanisms of general anesthesia have been explored extensively during the last decades, how general anesthetics affect neural pathways to cause loss of consciousness remains a mystery. Recent studies indicate that general anesthetics may take effect via suppressing arousal pathways or potentiating sleep pathways.1–5 In the mammalian brain, a small number of cell groups in the brainstem, hypothalamus, and basal forebrain are crucial for the regulation of sleep and wakefulness.6 Among these cell groups, the histaminergic system is one of the most important neuron systems for inducing and maintaining arousal.7–9

Histaminergic neurons that originate from the tuberomamillary nucleus (TMN)10 have been suggested as a possible target site for the sedative effect of some general anesthetics.1,11 Using c-Fos as a marker of neuronal activation, Nelson et al.1 found that systemic administration of the γ-aminobutyric acid–mediated (GABAergic) agents in the rat induced a pattern of change in c-Fos expression similar to that found during nonrapid eye movement sleep, including decreased c-Fos expression in the TMN. When microinjected directly into TMN, γ-aminobutyric acid type A (GABA_A) receptor agonist muscimol produced a dose-dependent sedation, whereas GABA_A receptor antagonist gabazine attenuated the sedative response to systematically administered GABAergic anesthetics. Mice with a specific point mutation in the GABA_A receptor β3 subunit (N265M) showed a decreased anesthetic sensitivity to propofol and pentobarbital,12,13 and the reduced in vivo sensitivity to propofol is mirrored in the TMN neurons, consistent with TMN being a direct target of propofol.1,1

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GABAAergic anesthetics, the inhalational anesthetic halothane was found to inhibit histamine release in a dose-dependent manner in the anterior hypothalamic area, as shown by in vivo microdialysis.14

We previously reported that application of histamine into the basal forebrain facilitated emergence from isoflurane anesthesia, whereas histamine H1 receptor blockade delayed such emergence.15 Although this finding suggests that histaminergic neurotransmission is a potential modulator of isoflurane anesthesia, it is not clear whether TMN histaminergic neurons are necessarily involved in the sedative/hypnotic effects of isoflurane. To further investigate the involvement of the histaminergic neurons in modulating the hypnotic effects of isoflurane, we performed a selective lesion of the histaminergic neurons in the posterior hypothalamus of Long Evans rats and studied the effects of the general anesthetic isoflurane. The involvement of brain histamine system in isoflurane anesthesia was also investigated by pharmacologic disruption of histamine signaling at the receptor level by intracerebroventricular administration of histamine receptor antagonists. We used the loss of righting reflex (LORR) as our primary measure for sedation because it reflects one important facet of the sedative state in humans—unconsciousness.4 Because the TMN has been implicated previously in the actions of GABAAergic anesthetics, including propofol and pentobarbital, but not the N-methyl-D-aspartate receptor antagonist ketamine,2 the effect of TMN lesion on propofol, pentobarbital, and ketamine also was investigated. We hypothesized that histamine depletion would change the anesthetic response to isoflurane, propofol, and pentobarbital but not ketamine.

Materials and Methods

Animals
All procedures were approved by the Animal Care Committee of the University of Western Ontario (London, Ontario, Canada) and conducted according to the guidelines of the Canadian Council for Animal Care. Experiments were performed on 53 adult male Long Evans rats (235–280 g; Charles River Canada, St. Constance, Quebec, Canada), with 40 rats used in the TMN lesion study and 13 rats used in the pharmacologic study. All animals were given water and regular rat chow ad libitum and housed under climate-controlled conditions with a 12-h light-dark cycle, with lights on at 7:00 AM. The temperature in the room was maintained at 21°–23°C.

TMN Lesion
TMN neurons were bilaterally lesioned using orexin-2 conjugated to saporin (orexin-saporin) that binds to the orexin-2 receptors on the TMN neurons, as was done in previous studies.16–18 Briefly, animals were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal) and placed in a stereotaxic frame. After a small hole was drilled in the skull over the TMN, the dura was incised, a 30-gauge stainless-steel cannula was stereotaxically lowered into the TMN (coordinates in millimeters: P4.2, L ± 1.0, and 9.6 ventral to the skull surface),19 and 250 nl orexin-saporin (250 ng/μl; Advanced Targeting Systems, San Diego, CA) was infused over 5 min using a Hamilton syringe (Hamilton Company, Reno, NV) driven by an infusion pump. After the infusion, the cannula was left in place for 10 min and then withdrawn slowly. In the sham lesion, rats were subjected to the same surgical procedure except that saline solution (250 nl) was infused into the TMN. The rats were then left in their home cages for a recovery period of 2 weeks. In an effort to minimize the number of animals used in research, each rat was tested for two or three general anesthetics in a random order and separated by 1 week.

Implantation of Lateral Ventricle Cannula
Animals used for pharmacologic study received unilateral implantation of a 26-gauge guide cannula 2 weeks before experimental investigation. The guide cannulas were implanted with the animals receiving sodium pentobarbital (60 mg/kg, intraperitoneal) and placed in a stereotaxic frame. The guide cannula was placed above the left lateral ventricle (coordinates in millimeters: P 0.8, L 1.4, and 3.0 ventral to the skull surface)20 and fixed to the skull with dental acrylic. Drug infusion was made via a 30-gauge cannula connected to a Hamilton syringe. The 30-gauge cannula was inserted into the guide cannula and protruded 1 mm beyond its ventral tip.

Anesthetic Response to Isoflurane in Orexin-saporin and Sham-lesion Rats
The behavioral endpoint of LORR was used to investigate the hypnotic properties of isoflurane in animals, following previously described methods with slight modifications.2,26 To determine the 50% isoflurane (Forane; Baxter Corporation, Mississauga, Ontario, Canada) concentration for LORR, animals were individually placed in a small acrylic glass chamber connected to a vaporizer and oxygen source. Isoflurane was administered to each rat with an increment concentration of 0.125% from 0.625% to 1.375% atmosphere with 1 l/min of 100% oxygen. The concentration of isoflurane was verified by an infrared gas analyzer (RGM5250; Ohmeda, Louisville, CO). Each concentration of anesthetic was maintained for a minimum equilibration period of 15 min, after which the chamber was rotated to place the rat on its back. A rat was considered to have LORR if it did not turn onto all four limbs within 30 s and that result was confirmed in two subsequent trials.

It was suggested that anesthesia induction and emergence may use different neural substrates,2 so the time to LORR and time for recovery also were investigated. To measure the time required to loss of righting, animals were individually placed in a small acrylic glass chamber with 1.375% isoflurane vaporized in 1 l/min oxygen. The concentrations of
Histamine Receptor Blockade and Righting Reflex

Studies
Histaminergic receptors consist of three main types: H1 and H2 receptors mainly excite the postsynaptic membrane, and H3 receptors suppress the presynaptic release of histamine and other neurotransmitters.21 To determine whether disruption of histamine signaling modulates isoflurane response, H1 or H2 receptor antagonist was administered intracerebroventricularly, to block histamine receptor-mediated effects in the brain. Animals received, in random order, intracerebroventricular injection of saline (1 μl), the selective H1 receptor antagonist triprolidine (25 μg/1 μl, Sigma-Aldrich Co., Oakville, Ontario, Canada), or the selective H2 receptor antagonist cimetidine (25 μg/1 μl, Sigma-Aldrich Co.) before isoflurane was administered. Stepwise increases in isoflurane concentration were performed every 15 min to determine the dose response for LORR, as described in Anesthetic Response to Isoflurane in Orexin-saporin and Sham-lesion Rats. In a separate experiment, all rats were subjected to isoflurane, 1.375%, immediately after receiving an intracerebroventricular injection of saline (1 μl), triprolidine (5–25 μg/1 μl), or cimetidine (5–25 μg/1 μl). The times to loss and return of righting reflex were measured. The doses of triprolidine and cimetidine were chosen based on their ability to block histamine-induced responses as determined by in vivo and in vitro studies.15,22–24 Each rat was subjected to drug or saline application, in random order, separated by at least 4 days.15

Anesthetic Response to Propofol, Pentobarbital, and Ketamine in Orexin-saporin/Sham-lesion Rats
Anesthetic responses to propofol, pentobarbital, and ketamine were also studied using a cumulative- or single-dose protocol, derived from previously described methods with slight modifications based on our pilot study.25,26 For cumulative doses, a rat was repeatedly injected with intraperitoneal propofol or ketamine, first at 40 mg/kg and then in nine increments of 20 mg/kg, at 10-min intervals, for a total of 220 mg/kg. Cumulative doses of intraperitoneal sodium pentobarbital started with 15 mg/kg, and eight increments of 5 mg/kg were given, at 10-min intervals, for a total of 55 mg/kg. The dose-LORR relationship of each anesthetic was established, and the ED_{50} was estimated from the dose–response equation described in Statistical Analysis. In the single-dose study, a single intraperitoneal dose corresponding to the ED_{50} of the dose–response curve was injected (i.e., 200 mg/kg propofol, 40 mg/kg pentobarbital, or 200 mg/kg ketamine), and the durations of times of onset and recovery of LORR were measured. After administration of an anesthetic, a rat was gently positioned on its back every 15 s to test for righting. LORR was considered to occur at the time when a rat lay in the supine position for 30 s without turning onto four limbs and was confirmed by two subsequent trials. A rat was then left in the supine position until it recovered the righting reflex on its own, which is considered as return of the righting reflex.

Immunohistochemistry and Histology
At the end of experiments, animals with orexin-saporin/sham lesion were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneal) and transcardially perfused with saline (0.9%), followed by paraformaldehyde (4%) at pH 7.4. Brains were postfixed in paraformaldehyde, 4%, overnight at 4°C and then dehydrated in 0.1 M phosphate-buffered saline containing sucrose, 18%, at 4°C. Blocks containing the hypothalamus were cut on a freezing microtome into 40-μm coronal sections. TMN neurons expressed adenosine deaminase (ADA), which was detected as described previously using an avidin-biotin complex immunohistochemistry method.18 Briefly, the tissue sections were incubated with the primary antibody against ADA (1:500; Millipore Bioscience Research Reagents, Temecula, CA) for 2 days at 4°C, followed by incubation with biotin-conjugated secondary antibody (1:200; Millipore Bioscience Research Reagents) at room temperature for 1 h. The sections were then placed in the peroxidase-avidin-biotin complex for 1 h (1:100; Vectastain; Vector Laboratories, Burlington, Ontario, Canada), and the reaction product was visualized by diaminobenzidine.

The number of ADA-immunoreactive cells in TMN was counted, by an observer blind to the treatment group, in three standardized coronal sections and averaged to yield a single mean for each rat. The three sections were selected based on the morphology of the structures, in particular the shape of the third ventricle, and corresponded approximately to P3.8, P4.16, and P4.3 of the rat brain atlas of Paxinos and Watson.19 Cell survival was estimated by the ratio of the number of ADA-immunoreactive cells in orexin-saporin-lesion rats compared with that in sham-lesion rats. Cannula placements in the lateral ventricle were verified histologically in 60-μm frozen sections of the brain stained with thionin.

Statistical Analysis
Data were expressed as mean ± SEM and 95% CI if indicated. GraphPad Prism software version 4.0 (GraphPad Prism, Inc., San Diego, CA) was used for statistical evaluation. Differences in the expression of ADA-positive cells be...
Tuberomamillary nucleus (TMN) orexin-saporin–lesion rats had decreased requirement for isoflurane to induce the loss of righting reflex and longer emergence time from isoflurane anesthesia. The dose–response curve for TMN orexin-saporin–lesion rats (blue lines) and sham-lesion rats (red lines) shows the percentage of animals that lost their righting reflex with stepwise increases in isoflurane concentration. The EC_{50} is smaller for orexin-saporin–lesion rats (crosses) than for sham rats (open circles) (orexin-saporin: n = 10, sham: n = 11, P = 0.0003) (A). The TMN orexin-saporin lesion failed to change time of onset of isoflurane anesthesia (orexin-saporin: n = 17, sham: n = 18, P > 0.05) but significantly prolonged emergence time (orexin-saporin: n = 17, sham: n = 18, P = 0.01) (B). Ore-Sap = orexin-saporin.

**TMN Lesion Enhanced Anesthetic Sensitivity to Isoflurane**

A recent study suggests that the neural substrates governing transitions into and out of the anesthetized state may not be identical. Therefore, we first examined whether the anesthetic actions of isoflurane are altered by TMN histaminergic lesion by using an increasing isoflurane protocol. Exposure to isoflurane produced anesthetic effects or hypnosis in a dose-dependent manner in both groups of rats. However, the orexin-saporin–lesion rats demonstrated increased sensitivity to isoflurane compared with sham-lesion rats. The concentration at which one half of the rats showed an LORR was 0.95 ± 0.04% (95% CI: 0.89–1.00%) in sham lesion (n = 11) and 0.82 ± 0.01% (95% CI: 0.81–0.84%) in orexin-saporin–lesion (n = 10) rats (P = 0.0003, fig. 2A). Next, we examined whether the onset and emergence of isoflurane anesthesia or hypnosis was changed in orexin-saporin–lesion rats. We found that orexin-saporin–lesion and sham rats showed similar onset time to LORR when exposed to isoflurane, 1.375% (orexin-saporin lesion: 6.90 ± 0.29 min, n = 17; sham: 7.39 ± 0.32 min, n = 18, P > 0.05). However, the emergence time (time to recover righting reflex in room air after 30 min in isoflurane, 1.375%) was significantly prolonged in the orexin-saporin–lesion rats (6.67 ± 0.58 min, 95% CI: 5.43–7.90 min, n = 17) compared with the sham-lesion rats (4.49 ± 0.59 min, 95% CI: 3.26–5.73 min, n = 18, P = 0.01, fig. 2B).
administration of triprolidine (5–25 μg/1 μl) with saline injection (crosses) failed to alter the isoflurane-induced LORR dose–response curve, induction time, or the emergence time compared with saline-treated animals (P > 0.05, n = 6 for both groups, fig. 3C–D).

**TMN Lesion Did Not Change Anesthetic Sensitivity to Propofol, Pentobarbital, or Ketamine**

The TMN lesion did not affect the sedative response to propofol, pentobarbital, or ketamine. TMN-lesion and sham-control rats had similar ED50 (propofol: 68.27 ± 1.09 mg/kg vs. 77.16 ± 1.09 mg/kg; pentobarbital: 26.15 ± 1.04 mg/kg vs. 25.46 ± 1.04 mg/kg; ketamine: 150.10 ± 1.06 mg/kg vs. 136.90 ± 1.07 mg/kg, n = 12 for orexin-saporin lesion and n = 8 for sham, respectively; P > 0.05, fig. 4A–C).

When a single dose of propofol (200 mg/kg), pentobarbital (40 mg/kg), or ketamine (200 mg/kg) was administered, there was no significant difference between orexin-saporin–lesion and sham-lesion rats in the time to LORR or time to recover the righting reflex (P > 0.05, n = 10 for orexin-saporin–lesion and n = 7 for sham-lesion rats, respectively, fig. 4D–F).

**Discussion**

We found that histaminergic neuronal depletion in the TMN significantly increased the susceptibility to isoflurane anesthesia. When cumulative concentrations of isoflurane were introduced, TMN orexin-saporin–lesion rats, compared with sham-lesion rats, lost their righting reflex with significantly lower concentrations of isoflurane. In addition, when a constant concentration of isoflurane (1.375%) was administered, the time to righting reflex recovery once anesthetic administration had been terminated was prolonged in orexin-saporin–lesion rats compared with sham-lesion rats. Moreover, central administration of triprolidine, an H1 receptor antagonist, produced a leftward shift in dose–response curve and a dose-dependent delay in emergence from isoflurane anesthesia. Therefore, results from both TMN lesion and histaminergic blockade suggest that neural histamine is involved in the mechanism of isoflurane anesthesia.

The increased sensitivity to isoflurane anesthesia and delayed emergence from isoflurane may be associated with the role of TMN in mediating arousal. TMN histaminergic neurons were silent during sleep, fired moderately during quiet awake periods, and typically fired maximally during active waking with “motor activities” or with “a high level of vigilance.” TMN outputs to the hypothalamus, basal forebrain, and cortex may help to promote arousal and cortical activation. Thus, histamine administration in the basal forebrain facilitated, whereas H1 receptor antagonist delayed, the emergence from isoflurane anesthesia. TMN likely exerts its effect in concert with other neuronal groups that control the sleep–wake cycle, including orexinergic neurons in the lateral hypothalamus, noradrenergic neurons in the locus coeruleus, and serotonergic neurons in the dorsal
By lesioning TMN neurons or blocking the H1 receptor, the arousal effects of TMN neurons are diminished, although the animals did not show marked changes in wakefulness. It is possible that loss of histaminergic activity may cause selective deficits in cognitive or arousal functions, instead of global changes in levels of wakefulness, thus leading to increased sensitivity and duration of isoflurane anesthesia. Isoflurane has been reported to reduce c-Fos expression of orexinergic neurons, which mediate their arousal effects through activation of H1 receptors. Isoflurane also decreased acetylcholine release in the somatosensory cortex.

The time to the onset of LORR was not affected by either TMN lesion or acute administration of histamine receptor antagonists. It may be because LORR onset is a dynamic effect that depends on isoflurane’s effects on brain targets other than TMN. This contrasts with a leftward shift of the LORR isoflurane dose–response curve, determined under steady-state isoflurane conditions in rats with TMN lesion (fig. 2A) or with H1 receptor blockade (fig. 3A). The current results are different from the results seen in mice with genetically ablated orexin neurons or orexin-1 receptor blockade; those mice showed no change in sensitivity to isoflurane but a delayed emergence time (compared with control animals) associated with isoflurane. Although general anesthetics and sleep may share some similarities in the mechanism of loss of consciousness, TMN lesions did not significantly affect the total amount of sleep. Histaminergic neurons do not appear to regulate spontaneous wakefulness and sleep, although they modulate the sensitivity and duration of isoflurane anesthesia.

We found that the TMN neuron lesion failed to affect anesthetic response to propofol, pentobarbital, or ketamine; no significant effect was found in the LORR dose response, the onset of LORR, or the return of righting when animals were given a single anesthetic dose. The lack of TMN lesion effect on ketamine anesthesia is expected, but the lack of TMN lesion effect on propofol and pentobarbital anesthesia does not support our hypothesis that was partly based on administration of a GABAA receptor agonist, muscimol, into the TMN. This is likely the effect of muscimol inactivation differing from that of the TMN lesion. In the current study, the orexin-saporin lesion depleted 72% of the TMN neurons with ADA immunoreactivity, which may include both histaminergic and nonhistaminergic neurons; adjacent hypothalamic neurons with orexin-2 receptors would also be lesioned. In addition, the TMN lesion induced cell death, which occurred over a period of several days, allowing compensatory effects that would not emerge after acute inactivation of the TMN by muscimol. Muscimol, at the dose administered, also would affect neurons with GABAA receptors in the TMN and adjacent structures in the hypothalamus, perhaps including the supramammillary area.

Fig. 4. Tuberomamillary nucleus (TMN) orexin-saporin lesion did not change anesthetic sensitivity to propofol, pentobarbital, or ketamine. The dose–response curve for TMN orexin-saporin–lesion rats (blue lines) and sham-lesion rats (red lines) showing the percentage of animals that lost their righting reflex with intraperitoneally administered propofol (40–220 mg/kg) (A), pentobarbital (15–55 mg/kg) (B), and ketamine (40–220 mg/kg) (C). There were no statistical differences in the ED50 between orexin-saporin–lesion (crosses) and sham-lesion (open circles) rats (orexin-saporin: n = 12, sham: n = 8, P > 0.05). Effect of a single intraperitoneal injection of propofol (200 mg/kg) (D), pentobarbital (40 mg/kg) (E), and ketamine (200 mg/kg) (F) on time to loss of righting reflex and time to return of righting reflex. There were no statistical differences between the orexin-saporin–lesion and sham-lesion rats (orexin-saporin: n = 10, sham: n = 7, P > 0.05). Ore-Sap = orexin-saporin.
fusion of muscimol with time also may affect distant structures. It should be noted that histaminergic neurons may not be critical for GABAergic anesthetics to induce loss of consciousness because the discrete application of anesthetics into the TMN caused only sedation, not LORR.3 The different anesthetic responses in TMN-lesion animals observed in this study suggest that isoflurane anesthesia may involve neural circuits different from those responsible for propofol, pentobarbital and ketamine anesthesia. The topographical distribution of different types of receptors or ion channels likely contributes to the regional selective nature of these general anesthetics. At the molecular level, isoflurane may not act as strongly on GABA_A receptors as propofol and pentobarbital. Correlating in vivo anesthetic effects with ex vivo receptor density data supports a GABAergic mechanism of action for propofol but not for isoflurane.37 The action of isoflurane may involve a more complex mechanism and several receptor systems, including neuronal nicotinic acetylcholine receptors, voltage-gated potassium channels, and two-pore domain potassium channels.38–40 How these molecular effects relate to the LORR induced by isoflurane anesthetics remains unclear.

Our study has several important limitations. First, it is possible that delayed emergence from anesthesia was secondary to the distribution or elimination of the drug. Clarifying this issue would require the measurement of brain isoflurane concentration at induction and emergence of anesthesia. Second, we observed that isoflurane response was enhanced by TMN lesion and H1 receptor blockade. It remains unknown whether the brain histamine system plays a generalized role in mediating loss of consciousness induced by inhalational anesthetics. Future studies using other anesthetics, including sevoflurane, desflurane, and etomidate, are needed for a full understanding of the dose of isoflurane anesthesia remains unclear.

In summary, the current results extend our previous findings that TMN histaminergic activity is involved in modulating isoflurane anesthesia. These results also suggest that the neural circuits for isoflurane-induced anesthesia may differ from those of GABAergic anesthetics and ketamine.

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