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Heteromeric Nicotinic Inhibition by Isoflurane Does Not Mediate MAC or Loss of Righting Reflex

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Background: **Neuronal nicotinic acetylcholine receptors (nAChRs) have been implicated in the mechanism of action of isoflurane as they are inhibited at subanesthetic concentrations. Despite clear evidence for nicotinic inhibition at relevant isoflurane concentrations, it is unclear what behavioral result ensues, if any.**

Methods: **The authors have modeled two behaviors common to all general anesthetics, immobility and hypnosis, as minimum alveolar concentration that prevents movement in response to a supramaximal stimulus (MAC) and loss of righting reflex (LORR). They have tested the ability of nicotinic pharmacologic modulators and congenital absence of most heteromeric nAChRs to affect concentration of isoflurane required for these behaviors.**

Results: **Neither mecamylamine, 5 mg/kg, nor chlorisondamine, 10 mg/kg, affected isoflurane MAC. Nicotine caused a small decrease in MAC. None of the above agents had any effect on the concentration of isoflurane required for LORR. Mice genetically engineered to lack the β2 nicotinic gene product were not different in MAC or LORR from controls.**

Conclusions: **Nicotinic antagonists do not cause MAC or LORR. Inhibition of nicotinic acetylcholine receptors by isoflurane is not likely related to its ability to provide immobility and hypnosis in a surgical setting. This is perhaps not surprising as the inhibition of nAChRs *in vitro* is complete at an isoflurane concentration equal to one half of MAC. Nicotinic inhibition may, however, be involved in anesthetic behaviors such as amnesia and analgesia, which occur at lower anesthetic concentrations.**

NEURONAL nicotinic acetylcholine receptors (nAChRs) have been implicated as potential targets of isoflurane because the heteromeric forms of this receptor are inhibited at clinically relevant concentrations.[1,2] The nAChRs are the most potently modulated volatile anesthetic targets identified to date.[3] Additional evidence for the possible involvement of nAChRs in immobility is twofold. First, volatile compounds related to isoflurane have been identified that would be predicted to have anesthetic activity based on their hydrophobicity but do not cause immobility in animals.[4] These compounds are also ineffective at nAChRs.[5] Second, isoflurane contains a chiral carbon, and the stereoisomers can be separated. The positive isomer is approximately 50% more potent than the negative isomer at inhibiting minimum alveolar concentration (MAC) in rats.[6] The same ratio of potency has been observed in molluscan nAChRs.[7] Thus, the neuronal nAChRs fit substantial criteria for playing a role in the mechanism of action of isoflurane. Despite clear evidence that isoflurane modulates nAChRs in a clinically relevant concentration range, a specific behavioral tie between isoflurane modulation of nAChRs (or any other putative target) has not been demonstrated.

Anesthesia is a complex group of behaviors that is readily identified but not simply defined. All general anesthetic agents have in common the propensity to induce immobility and hypnosis. The most commonly studied anesthetic behavior is immobility in response to a noxious stimulus or MAC. Hypnosis is induced by isoflurane at lower concentrations than immobility.[8,9]

To provide evidence for the involvement of nicotinic receptors in immobility and hypnosis induced by isoflurane, we have treated animals with specific nicotinic agonists and antagonists and tested animals with congenital absence of the nicotinic β2 subunit. As isoflurane is an antagonist at heteromeric nAChRs,[1,2] we hypothesized that treatment with other nicotinic antagonists would potentiate the behavioral effects of isoflurane, whereas treatment with the agonist nicotine would reverse them. Further, if isoflurane caused a behavioral response by inhibiting a nAChR that contains the β2 subunit (as most do), the behavior should be absent or reduced in the nicotinic β2 knockout mice.

Methods

With the approval of the University of California-San Francisco animal care committee, 36 male and 20 female 129J mice were studied. In addition, we studied 15 male mice genetically engineered to lack the nicotinic β2 gene product on the background of the c57 Bl/6J strain (β2 knockout) and 15 closely related wild-type cousins.[10] These mice were a gift from Professor Jean Pierre Changeux, M.D., at the Institute Pasteur, Department of Neuroscience, Paris, France. The β2 knockout mice have nearly absent high affinity nicotine binding in the brain. Residual binding in the habenula is thought to be secondary to minor expression of α3 and β4 containing nAChRs.[10,11] Animals were housed five to a cage in the animal care facility for at least 1 week before experimentation. The mice were exposed to 12-h cycles of light and dark and had food and water *ad libitum*. Mice were aged 6–8 weeks at the time of experimentation.

Control experiments were conducted to demonstrate that central nicotinic blockade persisted over the 3 h

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during which the MAC experiments were conducted. Mice were injected with intraperitoneal mecamylamine, 5 mg/kg. Mecamylamine acts at nanomolar concentrations to inhibit heteromeric nAChRs.[12] After 3 h, mice that had been injected with mecamylamine and control mice were injected with nicotine, 1 mg/kg, subcutaneously. Mice were observed for prostration. There was no evidence of nicotine-induced prostration in mice that had received mecamylamine, whereas mice that received saline were easily identified. This control experiment demonstrates that the nicotinic inhibitory effects of mecamylamine last at least 3 h, the longest period for any experiment.

MAC Testing

Mice were randomized to receive either 1 mg/kg nicotine, 5 mg/kg mecamylamine, or 10 mg/kg chlorisondamine in saline by intraperitoneal injection, or injection of the same volume of saline. Nicotine, the prototypical nicotinic agonist, was used at a dose that is effective in nicotinic pain paradigms.[13] Because of the short half-life of nicotine, animals were injected with nicotine or saline 5 min before testing at each isoflurane concentration. At least 40 min passed between each nicotine injection, the time required for nicotine to be undetectable in plasma.[13,14] The control mice from these experiments did not have significantly different MAC or loss of righting reflex (LORR) values from animals that received a single saline injection, and thus the control animals are pooled.

Mecamylamine, a noncompetitive nicotinic antagonist, was administered at 5 mg/kg, a concentration that results in plasma concentrations of approximately 0.2 μM when measured by gas chromatography-mass spectroscopy in previous experiments. These concentrations result in approximately 50% inhibition of heteromeric nicotinic activation.[12] Chlorisondamine is an irreversible antagonist of nAChRs.[15] Chlorisondamine, 10 mg/kg, completely blocks the locomotor response to nicotine.[15,16]

Minimum alveolar concentration for isoflurane was determined for each mouse by a trained observer blinded to the drug injected or genotype. MAC was determined as previously described.[17] Briefly, animals were placed in individual plastic chambers connected to a circle system containing CO_2 absorber, fan, and oxygen source. The temperature of each mouse was determined rectally and maintained between 36° and 38°C using heating blankets. The MAC of isoflurane was measured as the mean of partial pressures bracketing the animal's response and lack of response to a 1-min tail clamp.[18] If an animal responded to tail clamp, the partial pressure of the inhaled drug was increased in steps of approximately 10% until no response was obtained. The animals were given 30 min to equilibrate after each change of isoflurane partial pressure. The isoflurane partial pressure was measured continuously by gas analyzer and was verified by gas chromatography with each concentration change. MAC was determined for each group as the average MAC for each animal. Male and female animals were tested separately.

Loss of Righting Reflex Testing

Mice were treated with 1 mg/kg nicotine, 5 mg/kg mecamylamine, or 10 mg/kg chlorisondamine in saline by intraperitoneal injection, or with injection of the same volume of saline as described above. Male and female animals were tested separately. A procedure was followed similar to that described by Joo *et al.*[19] Mice were placed in a clear plastic cylinder connected to a circle system containing CO_2 absorber, fan, and oxygen source. The temperature of each mouse was determined rectally and maintained between 36° and 38°C using heating blankets. An investigator blinded to treatment and genotype determined the concentration of isoflurane that resulted in LORR. The LORR was measured as the mean partial pressures of isoflurane bracketing postural response and lack of response to placing the animal in a supine position.

Statistical Analysis

The values for MAC and LORR were calculated as the average of the highest isoflurane concentration tested that resulted in movement and the lowest concentration tested that did not result in movement. The results are expressed as mean ± SE and are compared with an unpaired Student *t* test for significant difference. *P* value less than 0.05 is considered significant.

Results

Minimum Alveolar Concentration

The MAC for isoflurane was 1.47 ± 0.03 for male mice (table 1A) and 1.52 ± 0.07 for female mice. There was no significant difference in MAC between male and female mice. Mice were treated with intraperitoneal mecamylamine, 5 mg/kg, or chlorisondamine, 10 mg/kg, before MAC testing. Neither nicotinic antagonist affected isoflurane MAC in male (table 1A) or female mice. Nicotine, 1 mg/kg, caused a small but statistically significant decrease in MAC in male mice (1.16 ± 0.02 nicotine, Student *t* test, $P < 0.01$). Mice lacking the nicotinic $\beta 2$ subunit had no significant difference in isoflurane MAC when compared with generation matched wild-type control mice (table 1B).

Loss of Righting Reflex

The concentration of isoflurane that effected a LORR was 0.56 ± 0.10% for male mice (table 2A) and 0.59 ± 0.18% for female mice. There was no significant difference in response between the genders. When mice were treated with the nicotinic antagonists mecamylamine and chlorisondamine or the agonist nicotine, there was

Table 1. Isoflurane Supramaximal Stimulus

	MAC Male	SE	N	MAC Female	SE	N	*P* Value
Pharmacologic agents							
Saline*	1.47	0.03	30	1.52	0.07	12	—
Mecamylamine, 5 mg/kg*	1.44	0.05	20	1.58	0.04	11	NS
Chlorisondamine, 10 mg/kg*	1.42	0.04	10	NA	NA	NA	NS
Nicotine, 1 mg/kg*	1.16	0.02	10	NA	NA	NA	< 0.001
Genetic modulation							
Nicotinic $\beta 2$ knockout†	1.37	0.14	9	NA	NA	NA	NS
Generation-matched wild type†	1.38	0.11	7	NA	NA	NA	—

After receiving saline, 10 mg/kg chlorisondamine, 5 mg/kg intraperitoneal mecamylamine, or 1 mg/kg nicotine injection, male and female 129J mice were tested for isoflurane supramaximal stimulus (MAC). There was no significant difference in the MAC concentration of isoflurane with any antagonist. Nicotine treatment caused a small decrease in MAC. Isoflurane MAC was also tested in the nicotinic $\beta 2$ knockout mice and generation-matched wild-type controls. Data are mean ± SE.

* 129J strain genetic background. † C57B1/6J strain genetic background.

NS = not significant; NA = not attempted.

no effect on the concentration of isoflurane that caused a LORR (table 2A). There was no difference in the concentration of isoflurane that resulted in LORR between mice with a congenital absence of the nicotinic $\beta 2$ subunit and generation matched control mice (table 2B).

Discussion

Two nicotinic antagonists, mecamylamine and chlorisondamine, that readily cross the blood-brain barrier and influence other nicotinic-mediated behaviors do not cause immobility or hypnosis at the doses studied. Neither do these drugs decrease the dose of isoflurane required for MAC and LORR. Plasma levels of mecamylamine, 5 mg/kg given intraperitoneally (approximately 200 nM), were equivalent to those that resulted in approximately 50% inhibition of current from heterologously expressed $\alpha 4\beta 2$ nAChRs.[12] The plasma concentration of chlorisondamine is difficult to interpret because it has acute and long-lasting effects as a result of intracellular accumulation and, thus, was not measured. However, 10 mg/kg is at the high end of doses studied in other behavioral research in mice.[15,16] We believe that adequate doses of the inhibitors were used to cause significant neuronal nicotinic inhibition, thus the lack of effect of these agents on the dose of isoflurane required for MAC and LORR is indicative of a lack of involvement of nicotinic inhibition in these behaviors.

Nicotine, the prototypical nicotinic agonist, at a concentration that can produce analgesia, caused a small decrease in MAC. Isoflurane is a nicotinic antagonist. If nicotine were acting to counteract isoflurane-induced immobility, we would expect an increase in MAC. The small decrease in MAC caused by nicotine may be the result of an analgesic effect.

By concluding that nicotinic inhibition by isoflurane does not mediate immobility or hypnosis, we do not mean to say that nicotinic inhibition does not play a role in the action of isoflurane as an anesthetic. All general anesthetic agents act as amnestic agents, and many have nociceptive and autonomic effects as well. These anesthetic behaviors usually occur at concentrations lower than those that result in MAC and LORR. It is possible that nicotinic inhibition by isoflurane and other anesthetic drugs plays a role in these anesthetic actions.

For many years, the nicotinic receptors have been considered putative targets for the anesthetic actions of isoflurane. We present evidence that the high affinity receptors for nicotine likely do not play a role in MAC or LORR. Anesthesia cannot be thought of as a single entity

Table 2. Isoflurane Loss of Righting Reflex

	LORR Male	SE	N	LORR Female	SE	N	*P* Value
Pharmacologic agents							
Saline*	0.56	0.10	8	0.59	0.18	8	—
Mecamylamine, 5 mg/kg*	0.59	0.02	5	NA	NA	NA	NS
Chlorisondamine, 10 mg/kg*	0.56	0.10	8	0.57	0.12	8	NS
Nicotine, 1 mg/kg*	0.54	0.04	8	0.61	0.05	8	NS
Genetic modulation							
$\beta 2$ Knockout†	0.68	0.3	6	NA	NA	NA	NS
Generation-matched wild type†	0.63	0.2	6	NA	NA	NA	—

After receiving saline, 10 mg/kg chlorisondamine, 5 mg/kg mecamylamine, or 1 mg/kg intraperitoneal nicotine, male and female mice were tested for the concentration of isoflurane that resulted in loss of righting reflex (LORR). There was no significant difference in the concentration of isoflurane that caused LORR with any nicotinic modulator. Data are mean ± SE.

* 129J strain genetic background. † C57B1/6J strain genetic background.

NA = not attempted; NS = not significant.

with several behavioral aspects. Rather, anesthesia is a group of behavioral responses induced through actions on multiple targets, some perhaps unknown.

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