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Stereoselective Loss of Righting Reflex in Rats by Isoflurane

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Background: **Although it is accepted widely that optically active intravenous general anesthetics produce stereoselective effects in animals, the situation regarding volatile agents is confused. Conventional studies with scarce isoflurane enantiomers have been limited to small numbers of animals and produced conflicting results. By injecting these volatile enantiomers intravenously, however, it is possible to study large numbers of animals and obtain reliable results that can help to identify the molecular targets for isoflurane.**

Methods: **Pure isoflurane enantiomers were administered intravenously to rats after solubilization in a lipid emulsion. The ability of each enantiomer to produce a loss of righting reflex was determined as a function of dose, and quantal dose–response curves were constructed. In addition, sleep times were recorded with each enantiomer. Chiral gas chromatography was used to measure relative enantiomer concentrations in the brains of rats injected with racemic isoflurane.**

Results: **The *S*(+)-enantiomer was 40 ± 8% more potent than the *R*(−)-enantiomer at producing a loss of righting reflex. The *S*(+)-enantiomer induced longer sleep times (by about 50%) than did the *R*(−)-enantiomer. Rats anesthetized by a dose of racemic isoflurane sufficient to achieve a half-maximal effect had essentially identical brain concentrations of the two enantiomers.**

Conclusions: **The *S*(+)-enantiomer of the general anesthetic isoflurane is significantly ($P < 0.001$) more potent than the *R*(−)-enantiomer at causing a loss of righting reflex in rats. This confirms the view that isoflurane acts by binding to chiral sites. The observed degree of stereoselectivity provides a useful guide for ascertaining from *in vitro* experiments which molecular targets are most likely to play major roles in the loss of righting reflex caused by isoflurane. (Key words: Anesthetic mechanisms; consciousness; inhalational anesthetics; Intralipid; stereoisomers.)**

ALTHOUGH there is no doubt that the optical isomers of chemically complex intravenous anesthetics such as etomidate[1,2] and neurosteroids[3] have different potencies in animals, and good evidence that the same is true for ketamine[4,5] and the barbiturates,[6] there has been considerable controversy as to whether this stereoselectivity of anesthetic action extends to any of the volatile agents.[7–9] The earliest attempts to investigate the possible stereoselectivity of volatile anesthetics date to the early 1970s, when small quantities of optically impure mixtures of the halothane enantiomers were synthesized. The limited amount of material then available precluded animal measurements. More than 20 years later, a group at Anaquest (Murray Hill, NJ)[10] succeeded in synthesizing chemically and optically pure isoflurane enantiomers. Using these isoflurane enantiomers we were able to show that the optical isomers exerted stereoselective effects on certain nerve ion channels but were equally soluble in lipid bilayers;[11] this provided strong support for the idea that volatile agents act by binding directly to protein targets.[12] Subsequently, we and other workers have shown that isoflurane acts stereoselectively at the γ-aminobutyric acid type A receptor[13–16] but not at several other targets.[11,17,18] If the extent of stereoselectivity in animal potencies were known, this would provide a very useful guide as to which ion channels are most likely to be relevant to the production of the anesthetic state.[19]

Unfortunately, attempts to determine the general anesthetic potencies of the isoflurane enantiomers have provided contradictory results. After a report[7] that the *S*(+)-enantiomer of isoflurane induced a longer sleep time in mice than the *R*(−)-enantiomer, Lysko and colleagues[8] performed the first determination of the minimum alveolar concentration (MAC) in rats, using a fail-

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ure to respond to a painful stimulus (tail-clamp) as the anesthetic endpoint. Their finding that the *S*(+)-enantiomer was about 50% more potent than the *R*(−)-enantiomer, however, subsequently was contradicted by Eger and colleagues,[9] who were unable to detect any significant difference in MAC.

To our knowledge there have been no attempts to determine whether or not isoflurane shows stereoselectivity in its ability to cause a loss of righting reflex in mammals, an anesthetic endpoint that is achieved at concentrations lower than MAC and might be expected to show a greater degree of stereoselectivity (if any exists).[20] In order to circumvent the fact that there are only relatively small quantities of the pure enantiomers available (all previous studies were limited to very small numbers of animals), we have exploited the fact that a loss of righting reflex in rats[21] and mice[22] can be induced by the intravenous administration of small quantities (about 10 and 1 μl, respectively) of volatile general anesthetics solubilized in the lipid emulsion Intralipid (Intralipid 20%, Pharmacia Laboratories, Milton Keynes, United Kingdom). Using this method of administration we have been able to use a sufficiently large number of animals so that reliable quantal dose–response curves for the isoflurane enantiomers can be established.

Materials and Methods

This study conforms to the United Kingdom Animals (Scientific Procedures) Act of 1986. Experiments were performed on 147 male Sprague–Dawley rats (B&K Universal, Hull, United Kingdom) weighing approximately 300 g. Rats were given free access to food and water and maintained on a 12-h light–dark cycle.

Preparation of Isoflurane Enantiomer Solutions

The isoflurane enantiomers we used were chemically synthesized[10] and provided by Huang and his colleagues (Anaquest). We established using gas chromatography that the *R*(−)- and *S*(+)-enantiomers were 99.6% and 99.5% chemically pure, respectively, and had optical purities of 99.4% and 100.0%, respectively. Racemic isoflurane was obtained from Abbott Laboratories (Queenborough, Kent, United Kingdom) and found to be 99.1% chemically pure and to consist of an equimolar mixture of the two enantiomers. Solutions of racemic isoflurane or the pure enantiomers were prepared as follows: A quantity (approximately 4 ml) of Intralipid emulsion (Intralipid 20%) first was weighed into a 5-ml glass gas-tight syringe (SGE International, Ringwood, Victoria, Australia). Then a quantity (ranging from 40–250 mg) of either racemic isoflurane or one of the pure enantiomers was added to the syringe, which then was capped tightly and weighed. The syringe then was rotated gently for 15 min in order to solubilize the isoflurane in the Intralipid. It was easy to establish by visual inspection that even the largest quantities of isoflurane used readily dissolved. We confirmed gravimetrically that there was negligible loss (<2% over 4 h) of the volatile anesthetic from these capped syringes.

Determination of Loss of Righting Reflex and Sleep Times

The loss of righting reflex was determined in rats over a wide range of anesthetic doses. For each dose a syringe was mounted on a computer-controlled syringe pump (Harvard Apparatus, South Natick, MA), which was set to deliver 400 μl of solution (accurate to ±1 μl) over 20 s through a short length of polytetrafluorethylene (PTFE) tubing into a tail vein (through a 24-gauge, 19-mm Neoflon cannula, BOC Ohmeda, Helsingborg, Sweden). We determined using gas chromatography that there was no significant loss of isoflurane either into or through the PTFE tubing. A specially designed stainless steel fitting ensured that the tube from the pump could be connected to an implanted cannula leaving a small "dead volume" (< 15 μl). Immediately after the injection, the animal was placed on its back carefully and scored as *anesthetized* if it failed to completely right itself within 30 s from the start of the injection. Any animal that succeeded in righting itself within this time was scored as *awake*. The procedure was recorded on videotape and sleep times subsequently were measured. Sleep time was defined as the time between the start of the injection and the time at which the animal managed to right itself. With the exception of 10 animals used at the lowest concentrations of racemic isoflurane, animals were used once only. For the experiments with the *R*(−)-enantiomer the average animal weight was 311 ± 12 g; for the *S*(+)-enantiomer it was 301 ± 20 g; and for the racemate it was 307 ± 12 g (mean ± SD). Experiments were carried out at an ambient temperature of 22 ± 1°C.

Quantal dose–response data were fitted according to the method of Waud[23] to a logistic equation of the form

$$P=\frac{100A^b}{A^b+(ED_{50})^b} \quad (1)$$

in which *P* is the percentage of the population anesthetized, *A* is the anesthetic dose, *b* is a parameter that

represents the steepness of the dose–response curve, and ED_{50} is the anesthetic dose for a half-maximal effect.

Determination of Brain Concentrations of Isoflurane

In a few cases (n = 4), we determined the relative concentrations of the two isoflurane enantiomers in the brains of rats that had been anesthetized by an ED_{50} dose of racemic isoflurane. At about the time that it was anticipated the animal would recover consciousness (about 30 s), it was killed by cervical dislocation and the brain rapidly removed (in about 60 s). The brain then was homogenized in heptane (by vortexing and sonication) and centrifuged (at about 2,000*g*), and the relative concentrations of the *R*(−)- and *S*(+)-enantiomers determined using chiral chromatography.

Gas Chromatography

Samples of isoflurane, usually diluted in heptane (total volume 0.5–2.0 μl) were injected into the gas chromatograph (split–splitless injector, electron capture detector; model 8600, Perkin Elmer, Beaconsfield, Bucks, United Kingdom). We used an 80-m long Chiraldex G-TA capillary column (Advanced Separation Technologies, Whippany, NJ) having an internal diameter of 0.25 mm, which allowed the two enantiomers to be well resolved (see Results). The parameters were a split ratio of 100:1, injector temperature of 250°C, detector temperature of 350°C, and oven temperature of 60°C (isothermal).

Statistical Analysis

Values throughout this article are given as the mean ± SEM or SD, as appropriate. Statistical significance was assessed using the Student *t* test.

Results

Dose–Response Curve for Racemic Isoflurane

In preliminary experiments we established that Intralipid emulsions containing more than about 2% isoflurane by volume were able to induce a rapid loss of righting reflex (within a few seconds). We also established that injections of 400 μl Intralipid alone (n = 5) caused no obvious behavioral effects in the animals. Using 400 μl as the standard total bolus volume, we then determined a dose–response curve for the loss of righting reflex induced by racemic isoflurane over a dose range of 2.9–15.9 μl isoflurane. We used 43 animals in total with an average weight of 307 ± 12 g (mean ± SD).

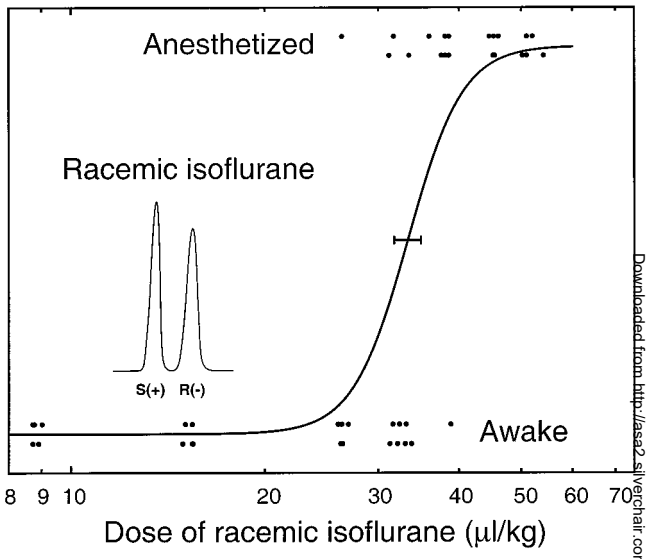

Fig. 1. Dose–response data for racemic isoflurane causing a loss of righting reflex in rats. Each point (filled symbol) represents a single animal that was scored either as anesthetized or awake at the dose marked on the abscissa (in several cases the points partially overlap; 43 animals were used in total). For clarity of presentation, the data points have been staggered alternately in the vertical direction. The line represents the dose–response curve computed using the method of Waud (see Materials and Methods and equation 1) with the following values: ED_{50} = 33.3 ± 1.6 μl/kg (mean ± SEM; see error bar), slope parameter *b* = 10.2 ± 3.6. (*Inset*) A gas chromatogram trace for the racemic isoflurane. Analysis showed the material was 99.1% isoflurane, of which 50% was the *S*(+)-enantiomer and 50% was the *R*(−)-enantiomer.

The dose–response curve, calculated using the method of Waud (see Materials and Methods),[23] is shown in figure 1. The dose–response curve was very steep, with a slope parameter *b* of 10.2 ± 3.6 and an ED_{50} of 33.3 ± 1.6 μl/kg (mean ± SEM). For this dose–response curve the injected dose was calculated in terms of microliters of isoflurane per kilogram of body weight. Essentially identical values were obtained if the doses were calculated using the average animal weight rather than the individual animal weights (not shown).

Experiments with the Isoflurane Enantiomers

Having arrived at a standard protocol, we next determined dose–response curves for each of the two isoflurane enantiomers. The curves we obtained are shown in figure 2, and the ED_{50} values and slope parameters are given in table 1. Once again, essentially identical values were obtained if the doses were calculated using the average, rather than the individual, animal weights. It was clear from these dose–response curves that the *S*(+)-enantiomer was significantly ($P < 0.001$) more po-

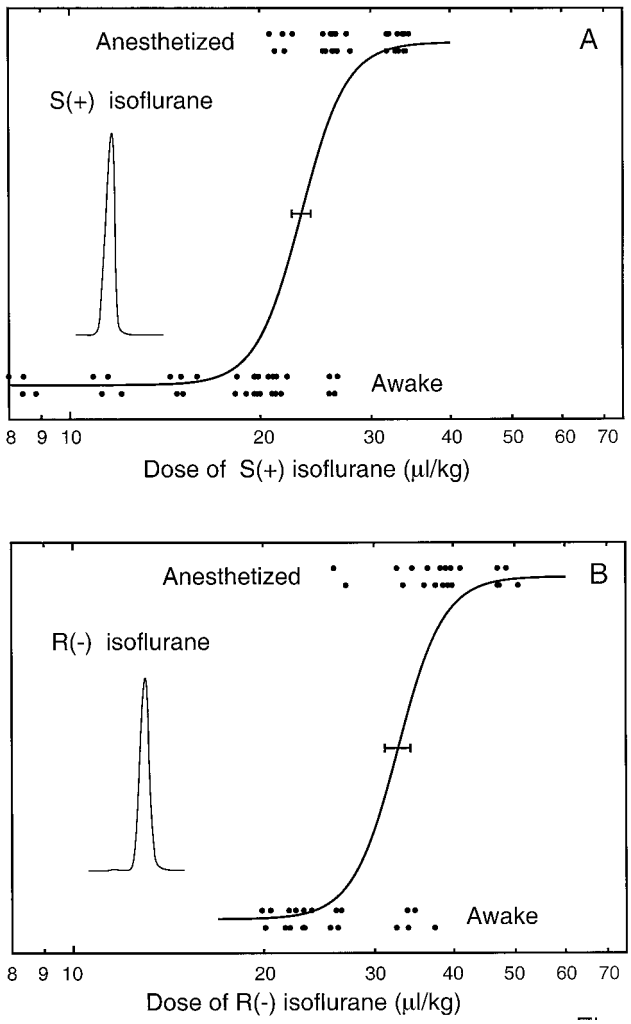

Fig. 2. Dose–response data for the enantiomers of isoflurane causing a loss of righting reflex in rats. (*A*) *S*(+)-isoflurane. The dose–response curve had values of ED_{50} = 23.3 ± 0.8 μl/kg and slope parameter *h* = 12.0 ± 3.2; 60 animals were used (in several cases the data points overlap). (*Inset*) Gas chromatogram trace for *S*(+)-isoflurane. Analysis showed the material to be 99.5% isoflurane, of which 100.0% was the *S*(+)-enantiomer and 0.0% was the *R*(−)-enantiomer. (*B*) *R*(−)-isoflurane. The dose–response curve had values of ED_{50} = 32.7 ± 1.5 μl/kg and slope parameter *h* = 11.9 ± 3.8; 40 animals were used. (*Inset*) Gas chromatogram trace for *R*(−)-isoflurane. Analysis showed the material to be 99.6% isoflurane, of which 99.4% was the *R*(−)-enantiomer and 0.6% was the *S*(+)-enantiomer.

tent than the *R*(−)-enantiomer. We investigated this further by measuring the sleep times over the range of doses for which the dose–response data for the two enantiomers overlapped (between 20 and 35 μl/kg). In order to obtain statistically meaningful results, we pooled the data within three contiguous dose ranges (20–25 μl/kg, 25–30 μl/kg, and 30–35 μl/kg) and expressed the results as mean sleep times. These data are plotted in fig. 3 and tabulated in table 2. Consistent with the observed differences between the ED_{50} values (fig. 1, table 1), the *S*(+)-enantiomer consistently induced a significantly longer sleep time ($P < 0.05$ or $P < 0.01$, unpaired Student *t* test) than the *R*(−)-enantiomer for each of the dose ranges studied.

Table 1. Dose–Response Parameters for Loss of Righting Reflex

Isoflurane Enantiomer	ED_{50} (μl/kg)	Slope Parameter (*h*)	No. of Animals
R(−)	32.7 ± 1.5	11.9 ± 3.8	40
S(+)	23.3 ± 0.8	12.0 ± 3.2	60
Racemate	33.3 ± 1.6	10.2 ± 3.6	43

Errors are SEM.

Relative Concentrations of Isoflurane Enantiomers in the Brain

We injected a few animals with an ED_{50} dose of racemic isoflurane, and just before we anticipated that they would regain consciousness we sacrificed the animals and determined the relative brain concentrations of the *R*(−)- and *S*(+)-enantiomers using chiral gas chromatography (see Materials and Methods). Figure 4 shows a

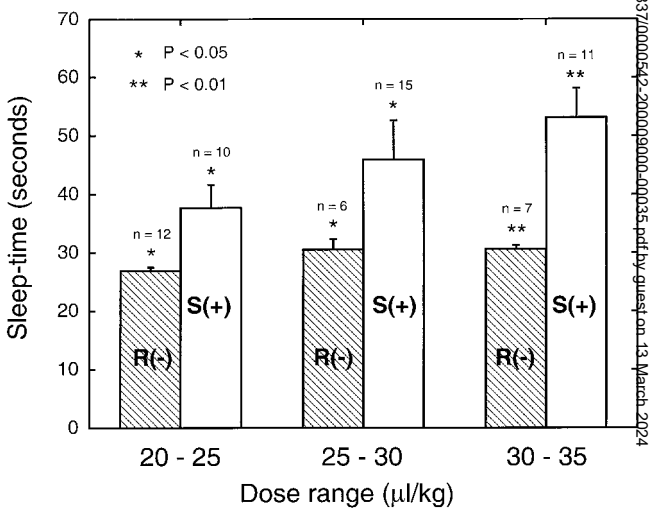

Fig. 3. Sleep times recorded from the start of the intravenous injection. Mean sleep times were calculated for three dose ranges (20–25 μl/kg, 25–30 μl/kg, and 30–35 μl/kg). For each dose range and for each optical isomer, between 6 and 15 animals were used (see values above each histogram bar). The error bars shown are standard errors in the mean. For each dose range there was a significant difference between the sleep times induced by the *S*(+)- and *R*(−)-enantiomers (unpaired Student *t* test).

Table 2. Sleep-time Data for the Isoflurane Enantiomers

Dose Range (μl/kg)	*S*(+) Sleep-time (s)	No. of Animals	*R*(−) Sleep-time (s)	No. of Animals
20–25	37.7 ± 3.9	12	26.9 ± 0.6	10
25–30	45.9 ± 6.7	15	30.5 ± 1.8	6
30–35	53.1 ± 5.0	11	30.6 ± 0.7	7

Errors are SEM.

representative gas chromatogram trace. These experiments showed that the ratio of the concentrations of the *S*(+)-enantiomer to the *R*(−)-enantiomer was not significantly different from unity (1.019 ± 0.011, mean ± SEM; n = 4 brains; $P > 0.15$, paired Student *t* test).

Discussion

There have been several studies over the years that have investigated the anesthetic and physiologic effects of the intravenous administration of volatile general anesthetics, usually[21,22,24–26] but not always[27,28] solubilized in a lipid emulsion. The most comprehensive data are available for halothane, which appears to have a very similar pharmacologic profile whether administered by injection or by inhalation.[21,25,26] Virtually no information is available for the intravenous administration of isoflurane, although a recent study[22] reported an ED_{50} of 0.7 μl isoflurane, equivalent to 17.5 μl/kg, for inducing a loss of righting reflex in mice. Because of species and protocol differences, a direct comparison cannot be made; however, our value of 33.3 ± 1.6 μl/kg for the ED_{50} of racemic isoflurane (fig. 1) is at least comparable.

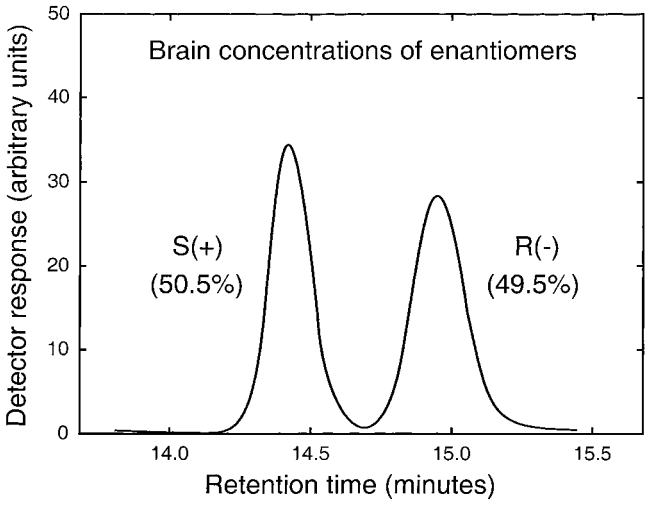

Fig. 4. A representative gas chromatogram trace showing the relative proportions of *S*(+)-isoflurane and *R*(−)-isoflurane in the rat brain after an intravenous ED_{50} dose of racemic isoflurane. The ratio of the *S*(+)-enantiomer concentration to the *R*(−)-enantiomer concentration was 1.019 ± 0.011 (mean ± SEM; n = 4 brains) which is not significantly different from unity ($P > 0.15$, paired Student *t* test).

The central aim behind the experiments reported here was to determine whether or not the two enantiomers of isoflurane had different anesthetic potencies in animals. The data presented in figures 2 and 3 show that, unequivocally, they do, at least for loss of righting reflex and length of sleep time. The dose–response curves for the two enantiomers shown in figure 2 have slope parameters that do not differ significantly (table 1), but the *R*(−)-enantiomer has an ED_{50} value that is 40% larger than that of the *S*(+)-enantiomer. The significantly ($P < 0.001$) greater potency of the *S*(+)- over the *R*(−)-enantiomer at causing a loss of righting reflex also is reflected in the sleep-time data (fig. 3, table 2). These sleep-time data show that, at a given dose, the *S*(+)-enantiomer caused the animals to sleep for 40 to 74% longer than did the *R*(−)-enantiomer. A surprising result was that the potency of the racemic mixture at causing a loss of righting reflex was not significantly different from that of the less potent enantiomer (table 1). The same behavior, however, has been seen with the enantiomers of isoflurane[29] and halothane[30] causing immobilization in *Caenorhabditis elegans*, as well as with the enantiomers of thiopental causing a loss of righting reflex in mice.[31] Although many simple molecular models would predict that the potency of the racemate would lie somewhere between those of the individual enantiomers, there are more complex models that can account for these observations. On the other hand, it is also possible that the explanation lies at the whole-animal level, and more work is needed to address this question.

The degree of stereoselectivity we have observed is in line with that which might have been predicted on the basis of Pfeiffer's observations.[20] Pfeiffer noted an inverse correlation between the degree of stereoselectivity for a particular drug and its average human dose: the more potent the drug, the greater the ratio of potencies between the two enantiomers. The potency ratio of 1.4

that we have found for the isoflurane enantiomers would be expected, according to Pfeiffer's rule,[20] for a drug with an average human dose of the order of 1 g. This is comparable to the dose (3.3 g isoflurane) that one might estimate if our value of 33 μl/kg for the rat is applied to a human (body weight of about 70 kg).

It is possible that the degree of stereoselectivity we have observed is affected to some extent by differing pharmacokinetics of the two enantiomers; differences have been reported in the manner in which the isoflurane enantiomers interact with serum albumin,[32,33] and it is conceivable that these or other factors could result in a differential delivery to (or loss from) the brain. Ideally this would be tested by anesthetizing animals with the individual enantiomers and measuring the concentrations in the brain. We found, however, that the variability between animals (associated with the loss of isoflurane during brain removal and its subsequent extraction from the tissue) was large compared with the precision needed. The best we could do was to determine if there had been any depletion of one enantiomer more than the other in the brains of animals that had been anesthetized by a racemic mixture of isoflurane. We found that the concentrations of the two enantiomers in the brain did not differ significantly ($P > 0.15$). It therefore seems unlikely that the extent of the stereoselectivity we have observed was affected appreciably by differential binding or diffusion.

Our findings are difficult to compare with those of previous studies because of differences in animal species, numbers of animals, and the anesthetic endpoint used. Nonetheless, our observation that the *S*(+)-enantiomer is significantly more potent than the *R*(−)-enantiomer at causing both a loss of righting reflex and a loss of consciousness is consistent with some earlier results.[7,8] Lysko *et al.*[8] measured MACs and found that the *S*(+)-isomer was about 50% more potent than the *R*(−)-isomer at preventing a response to a painful stimulus in rats; Harris *et al.*[7] found that the *S*(+)-isomer induced 20–60% longer sleep times in mice. On the other hand, Eger *et al.*[9] concluded that the MAC values for the two optical isomers did not differ significantly in rats. Our results cannot resolve this controversy as to whether MAC does[8] or does not[9] differ for the isoflurane enantiomers, and further work is needed in order to resolve this issue. Nonetheless, our results do show that if loss of righting reflex is used as the anesthetic endpoint, stereoselective effects are observed. One should bear in mind in considering the implications of our results that different molecular targets might underlie different anesthetic endpoints. Because the sites underlying the MAC endpoint appear to lie in the spinal cord,[34–36] but the righting reflex involves both spinal and supraspinal regions, different anatomic targets are certainly possible.

The fact that the mirror-image isomers of isoflurane can have different animal potencies has some important implications for anesthetic mechanisms. First, the observation that the isoflurane enantiomers act differently in animals yet are equally soluble in water (necessarily)[1] and lipid bilayers (despite their chiral nature)[37] reinforces the notion that general anesthetics bind directly to their protein targets.[12] More importantly, it establishes the stereoselectivity of isoflurane anesthesia in animals as a useful guide to which of the many possible molecular targets are pharmacologically relevant,[19] at least as far as those that underlie the loss of righting reflex are concerned. It can be argued that those putative targets that show opposite or no stereoselective effects *in vitro* are unlikely to play a dominant role in isoflurane-induced loss of righting reflex, but those that show a degree of stereoselectivity comparable to that found in animals are more likely to be relevant. From the available evidence on ion channels, for example, these considerations would favor roles for γ-aminobutyric acid type A receptors[13–16] and baseline potassium channels[11,38,39] but argue against roles for voltage-gated L-type calcium channels[18] and A-type potassium channels.[11] Finally, the degree of stereoselectivity that we have found for the loss of righting reflex in rats (a factor of 1.4) is not that much smaller than the *maximum* stereoselectivity found for molecular targets in *in vitro* systems (about a factor of 2). It follows from this that there is unlikely to be a large number of genetically unrelated targets underlying isoflurane-induced loss of righting reflex, because if this were the case it would be very difficult to account for the extent of the stereoselectivity observed in animals.

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