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Short communication

Isoflurane-induced surgical tolerance mediated only in part by β3-containing $GABA_A$ receptors

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Abstract

The targets which mediate the actions of the volatile general anaesthetic isoflurane are unknown. Based on pharmacological studies using $GABA_A$ receptor antagonists it has recently been suggested that $GABA_A$ receptors would not mediate the immobilizing action of isoflurane. Using the β3(N265M) knock-in mouse model we found that the mutant mice were less sensitive to the immobilizing action of isoflurane, indicating a role of β3-containing $GABA_A$ receptors in mediating immobility. At high concentrations isoflurane also immobilizes β3(N265M) mice, indicating that other targets also mediate immobility. Thus, our findings support a multisite model for the immobilizing action of isoflurane.

Keywords: $GABA_A$ receptor; General anaesthetic; Knock-in mice; Unconsciousness; Immobility

1. Introduction

Volatile anaesthetics such as isoflurane, halothane and enflurane are frequently used in modern clinical practice to provide hypnosis (unconsciousness) and a loss of responses to noxious-evoked stimuli (immobility). Despite their broad application, their mechanism of action is still only poorly understood (Rudolph and Antkowiak, 2004). Isoflurane strongly modulates the $GABA_A$ receptor in vitro at clinically relevant concentrations, and $GABA_A$ receptors are widely expressed in the central nervous system, including in the spinal cord which largely mediates the immobilizing action of isoflurane (Antognini and Schwartz, 1993; Rampil, 1994). Furthermore, isoflurane appeared to enhance receptor-specific [^{11}C] flumazenil binding, suggesting that it may cause a conformational change of the $GABA_A$ receptor in living human brain (Gyulai et al., 2001). However, these data do not provide any direct evidence concerning the mediation of specific effects of isoflurane, in particular the hypnotic and immobilizing actions by $GABA_A$ receptors.

The potential role of $GABA_A$ receptors in isoflurane action has been assessed by employing $GABA_A$ receptor antagonists. Picrotoxin increased the 50% effective dose (ED_{50}) for the minimal alveolar concentration ca. 60% for isoflurane, ca. 40–50% for ketamine (on top of isoflurane), and ca. 379% for propofol (Sonner et al., 2003). These and similar data (Zhang et al., 2004) were interpreted as demonstrating that while propofol mediates its immobilizing action via $GABA_A$ receptors, isoflurane and ketamine would not do so. However, for ketamine both in vitro and in vivo data are available which indicate that actions of ketamine, whose primary target appears to be the NMDA receptor, are to some degree also mediated by $GABA_A$ receptors (Lin et al., 1992) (Irifune et al., 2000). Thus, the data by Sonner et al. are also compatible with the view that ketamine actions may be partly mediated by $GABA_A$ receptors, raising the question

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whether isoflurane actions may also be partly mediated by $GABA_A$ receptors.

Another approach to identify the clinically relevant targets for general anaesthetics is to generate knock-in mice that carry a point mutation in the potential target receptor which inhibits or abolishes the action of general anaesthetics but leaves the physiological function of the receptor largely intact (Rudolph and Möhler, 2004). For example, in mice carrying the N265M point mutation in the $GABA_A$ receptor β3 subunit the duration of the loss of the righting reflex was significantly reduced and the hindlimb withdrawal reflex was not lost in response to the intravenous anaesthetics propofol and etomidate, indicating that the hypnotic action of these drugs is in part and the immobilizing action of these drugs is mediated essentially completely by $GABA_A$ receptors containing the β3-subunit (Jurd et al., 2003). The response to a neurosteroidal alphaxalone/alphadolone preparation is indistinguishable between β3(N265M) and wild type mice (Jurd et al., 2003), which indicates that the β3(N265M) mice can be hypnotized and immobilized by drugs whose action is not affected by the mutation. Thus, this animal model provides the possibility to test the involvement of β3-containing $GABA_A$ receptors in the action of other general anaesthetics, in particular the volatile anaesthetics. We have demonstrated recently that the immobilizing but not the hypnotic action of halothane and enflurane are impaired in β3(N265M) mice (Jurd et al., 2003). It is known that there are differences in the mode of action of halothane and isoflurane: while halothane reduces noxious-evoked movement at least partly via depression of dorsal horn neurons, isoflurane suppresses movement by an action at more ventral sites in the spinal cord (Jinks et al., 2003). We now studied the hypnotic and immobilizing actions of isoflurane in β3(N265M) and wild type mice.

2. Materials and methods

The breeding scheme was as described previously (Jurd et al., 2003). Mice were bred as homozygotes or wild type controls for up to three generations. The sensitivity of β3(N265M) and wild type mice to isoflurane was determined as follows. A chamber consisting of a standard Type III cage and a sealed plexiglass apparatus was equilibrated with isoflurane (0.4–2.7 vol.%, Arovet, Zollikon, Switzerland), enflurane (0.7–4.0 vol.%, Abbott, Baar, Switzerland) or halothane (0.55–2.5 vol.%, Arovet, Zollikon, Switzerland) with fresh air flow at a rate of 7.5 l/min. The concentrations of anaesthetic were determined at the bottom of the cage by an infrared gas analyzer (Capnomac Ultima, Datex-Ohmeda, Tewksbury, MA, USA). The chamber was heated with a heating pad from below. A group of up to eight mice (female mutant and wild type animals on the 129/Sv–129/SvJ background) was placed into the chamber and allowed to equilibrate to the respective concentration of anaesthetics for 20 min. Four independent groups were examined with isoflurane, three with enflurane and two with halothane. An observer blinded to the genotype of the animals then examined the presence of the righting reflex and the hindlimb withdrawal reflex in a quantal fashion. Mice were handled through airtight access ports. Mice were allowed to recover for at least 24 h before being tested again. Data were fitted to a non-linear logistic equation, yielding half-effect concentrations (EC_{50}), slopes and estimates of their

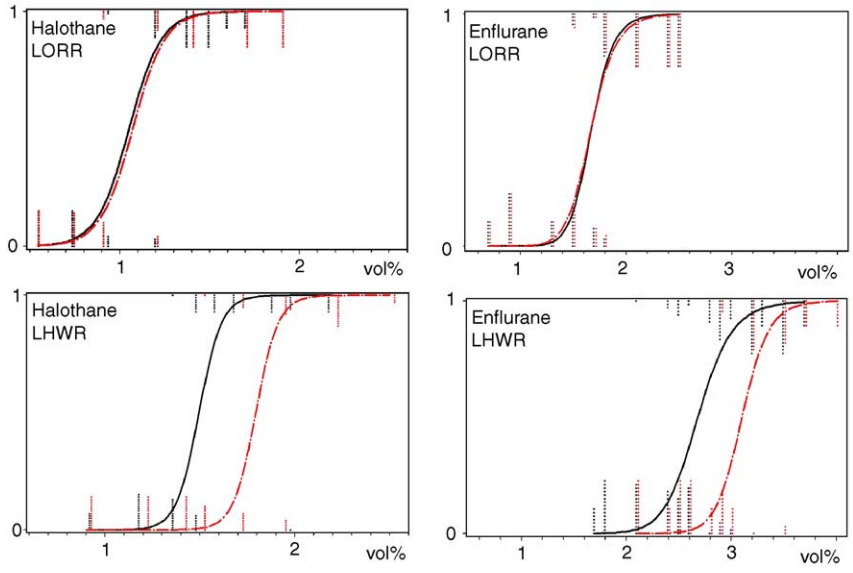

Fig. 1. Dose–response data for the hypnotic (top panels) and immobilizing (bottom panels) actions of halothane (left panels) and enflurane (right panels) in β3(N265M) and wild type mice. Mice were exposed to halothane and enflurane. The presence (value 0) or the absence (value 1) of the righting reflex and the hindlimb withdrawal reflex were determined. Each point represents a single measurement. Black: wild type mice, gray or red: β3(N265M) mice. The solid (black) and interrupted (gray or red) lines represent the dose–response curves computed using the method of Waud (see Materials and methods) for wild type mice and β3(N265M) mice, respectively. The numbers on the *x* axis denote the vol.% of the corresponding anaesthetic agent. LORR = loss of righting reflex; LHWR = loss of hindlimb withdrawal reflex. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

respective standard errors, followed by goodness of fit and equal slope tests (Waud, 1972). The data on halothane and enflurane are included for comparison and represent the same experiment from which EC_{50} and slope values were published previously (Jurd et al., 2003). All values are given as mean±S.E.M. All experiments were approved by the veterinary office of the Kanton Zürich.

3. Results

The hypnotic and immobilizing responses to isoflurane, and for comparative purposes halothane and enflurane (see also Jurd et al., 2003) were assessed in β3(N265M) mice in which the $GABA_A$ receptors containing the β3 subunit are rendered insensitive to a variety of general anaesthetics and in wild type mice. For halothane and enflurane, the dose–response curves for the loss of the righting reflex were indistinguishable between the two genotypes (halothane: EC_{50} wild type=1.06±0.04 vol.%, EC_{50} mutant=1.07±0.04 vol.%, $P>0.05$ ($n=16$); enflurane: EC_{50} wild type=1.67±0.04 vol.%, EC_{50} mutant=1.67±0.04 vol.%, $P>0.05$ ($n=24$)), indicating that the hypnotic action of these agents is not mediated by β3-containing $GABA_A$ receptors (Fig. 1). However, the dose–response curves for the loss of the hindlimb withdrawal reflex were significantly different between genotypes (halothane: EC_{50} wild type=1.49±0.03 vol.%, EC_{50} mutant=1.80±0.04 vol.%, $P<0.01$ ($n=16$); enflurane: EC_{50} wild type=2.68±0.04 vol.%, EC_{50} mutant=3.1±0.04 vol.%, $P<0.01$ ($n=24$)), indicating that the immobilizing action of enflurane and halothane are mediated in part by β3-containing $GABA_A$ receptors. Similar results for halothane and enflurane have previously also been reported using β3 knockout mice (Quinlan et al., 1998).

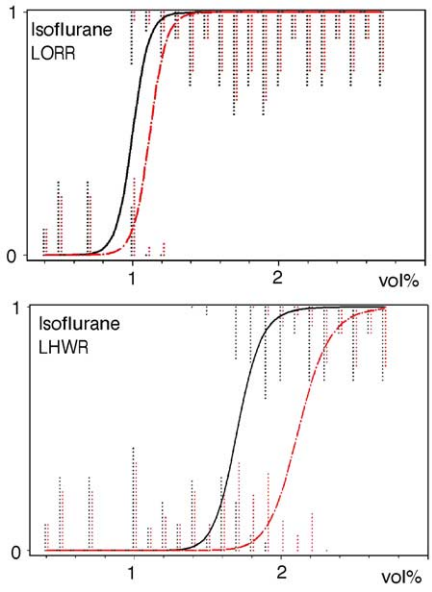

Fig. 2. Dose–response data for the hypnotic (top) and immobilizing (bottom) actions of isoflurane in β3(N265M) and wild type mice. Mice were exposed to isoflurane. The presence or absence of the righting reflex (top panel) and the hindlimb withdrawal reflex (bottom panel) were determined. For explanation of symbols see legend to Fig. 1.

When testing isoflurane, we found that the EC_{50} for the loss of the righting reflex was higher in mutant mice compared to wild type mice (EC_{50} mutant=1.12±0.05 vol.%, slope=17.15±4.76; EC_{50} wild type=1.00±0.05 vol.%, slope=18.31±3.68; $P<0.05$), indicating that the β3(N265M) mice are slightly less sensitive to the hypnotic effect of isoflurane, in contrast to enflurane and halothane (Fig. 2). The isoflurane-induced loss of the hindlimb withdrawal reflex was shifted to higher concentrations in the β3(N265M) mice (EC_{50} wild type=1.70±0.02 vol.%, slope=20.80±3.07; wild type mice: EC_{50} mutant=2.11±0.03 vol.%, slope=20.19±2.74; $P<0.05$). Thus, this test indicates that the immobilizing action of isoflurane requires significantly higher doses of isoflurane in the β3(N265M) compared to wild type mice (Fig. 2). This suggests that the immobilizing action of isoflurane is in part mediated by $GABA_A$ receptors containing the β3 subunit. Although at high concentrations of isoflurane all β3(N265M) mice get immobilized, it is noteworthy that at the concentration at which 50% of the wild type mice are immobilized, as measured by the loss of the hindlimb withdrawal reflex (1.70±0.02 vol.%), hardly any of the β3(N265M) mice have lost this reflex. In summary, these results demonstrate a reduced sensitivity of the β3(N265M) mice not only for the immobilizing action of halothane and enflurane, but also for isoflurane.

4. Discussion

The mechanism of action of the commonly used volatile anaesthetic isoflurane is controversial. In vitro studies have shown that it strongly potentiates the activity of $GABA_A$ receptors, glycine receptors, serotonine receptors and kainate receptors, while it strongly inhibits the activity of neuronal nicotinic acetylcholine receptors and AMPA receptors. Little inhibition is observed at GABA ρ1 receptors, muscle nicotinic acetylcholine receptors and NMDA receptors (Krasowski and Harrison, 1999). The observations that β3-containing and β2-containing $GABA_A$ receptors are mediating the hypnotic activity of etomidate (Jurd et al., 2003) (Reynolds et al., 2003) and β3-containing $GABA_A$ receptors are mediating the immobilizing action of etomidate and propofol (Jurd et al., 2003) in conjunction with the finding that isoflurane strongly potentiates the activity of $GABA_A$ receptors would suggest that isoflurane might exert its clinical actions by activating $GABA_A$ receptors. This view has however recently been challenged based on the action of $GABA_A$ receptor agonists in rats (Sonner et al., 2003; Zhang et al., 2004). We therefore investigated whether in β3(N265M) mice the sensitivity to isoflurane is altered or not.

In contrast to the intravenous anaesthetics propofol and etomidate, whose hypnotic actions are strongly impaired in β3(N265M) mice, the sensitivity of the β3(N265M) mice to the hypnotic response of the volatile anaesthetics halothane and enflurane was unchanged. Thus, although halothane and enflurane activate presumably the same β2- and β3-containing $GABA_A$ receptors as propofol and etomidate, at least the β3-containing $GABA_A$ receptors do not play a

significant role in mediating halothane's and enflurane's hypnotic action, in contrast to propofol and etomidate. Thus, it can be concluded that most likely targets other than β3-containing $GABA_A$ receptors mediate the hypnotic action of halothane and enflurane.

In the current study, we observed a small but significant increase of the EC_{50} for isoflurane in the β3(N265M) mice, indicating a reduced sensitivity of the β3(N265M) mice to the hypnotic action of isoflurane. Further studies are required to explore the potential involvement of β3-containing $GABA_A$ receptors in this response in more detail.

The immobilizing action of etomidate and propofol appears to be mediated by β3-containing $GABA_A$ receptors, since the immobilizing action of these drugs is absent in β3(N265M) mice (Jurd et al., 2003). This finding is indirectly supported by the observation that the immobilizing action of etomidate is still present in β2(N265S) mice (Reynolds et al., 2003). Our present results show that the immobility EC_{50} values for isoflurane, halothane and enflurane are increased in β3(N265M) mice. At defined concentrations of volatile anaesthetics, the response is significantly different between β3(N265M) and wild type mice. E.g., at the isoflurane EC_{50} value determined in wild type mice for the loss of the hindlimb withdrawal reflex, β3(N265M) mice essentially do not loose this reflex. In contrast to propofol and etomidate, complete immobility can still be achieved in β3(N265M) mice at higher concentrations of isoflurane, halothane and enflurane. Thus, β3-containing $GABA_A$ receptors appear to be involved in mediating the immobilizing action of isoflurane, halothane and enflurane, in particular at concentrations of these drugs which are just about sufficient to mediate immobility and thus clinically significant. Primarily at higher concentrations, these drugs–in sharp contrast to propofol and etomidate–also exert immobility via other targets. Our results are in line with another report (Liao et al., 2005) reporting that the immobilizing minimal alveolar concentration value for isoflurane, determined by tail clamping after exposing the same animals to increasing concentrations of isoflurane, is increased by 17% in β3(N265M) mice ($1.93 \pm 0.12\%$ versus $1.65 \pm 0.16\%$, mean ± S.D., $n = 14$; $P < 0.001$). The minor differences in absolute values between the two studies are likely due to variations in methodology, and our experiments described in this paper provide data for three commonly used volatile anaesthetics using the same methodology.

While we cannot formally exclude the possibility that in β3(N265M) mice the sensitivity of targets other than β3-containing $GABA_A$ receptors to the anaesthetic agents examined here might be changed e.g. by as yet undetected compensatory mechanisms, an argument speaking against this possibility is the finding that the neurosteroidal anaesthetic mix alphaxalone/alphadolone, whose action is not affected by the β3(N265M) point mutation in vitro (Siegwart et al., 2002) has an indistinguishable action on wild type and mutant mice (Jurd et al., 2003). Our results thus support a multiple target hypothesis for isoflurane, halothane and enflurane with respect to immobility, with the β3-containing $GABA_A$ receptors playing a visible but nevertheless limited role especially at relatively low concentrations of these drugs.

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