Isoflurane exerts antinociceptive and hypnotic properties at all ages in Fischer rats

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Background. Some anaesthetic agents exhibit an age-dependent analgesic effect, for example nitrous oxide, which is ineffective in newborn rats. We investigated whether a similar time dependency existed for the responses to the volatile anaesthetic isoflurane.

Methods. The analgesic and hypnotic properties of isoflurane at various ages was assessed using four cohorts of Fischer rats aged approximately 7, 16, and 28 days and adults (11–12 weeks old). Intraplantar administration of formalin mimicked inflammatory pain, and its effects were assessed using immunohistochemical (c-Fos staining) and behavioural paradigms. The hypnotic properties of isoflurane were assessed using loss of righting reflex.

Results. Formalin administration produced a typical nociceptive response observed both behaviourally and immunohistochemically in all age groups; these nociceptive responses were significantly attenuated by isoflurane 0.5% at each age (P<0.05). Interestingly 7-day-old animals showed a significantly more potent hypnotic response than older animals (P<0.01): with adult rats being most resistant to isoflurane induced hypnosis (P<0.05).

Conclusion. In contrast to nitrous oxide, isoflurane is an effective antinociceptive agent in neonatal rats. If the data can be extrapolated to clinical scenarios these results suggest that isoflurane may be analgesic in newborns as well as adult humans. In addition, isoflurane is a potent hypnotic, especially in the very young, which is in contrast to the neonate’s relative resistance to anaesthesia as assessed by minimum alveolar concentration.

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Effective analgesia in neonates is critical as unopposed pain states may precipitate significant long-term psychological and physiological sequelae including hyperalgesia.1–4 The assumption that analgesic agents used in the adult population will be equally efficacious in the paediatric population has recently been challenged.5 We found that nitrous oxide was an ineffective antinociceptive agent in young Fischer rats. The mechanism may relate to differences in pain processing systems between mature and immature animals; notably, neonates lack functional descending inhibitory neurons (DIN)6,7 connecting spinal and supraspinal sites. Nitrous oxide requires functional DIN to exert antinociceptive actions and thus is ineffective in the neonate.5 Therefore, the use of analgesic agents, which require functional DIN to be effective in the young may be contraindicated.

Isoflurane is known to mediate nociception through both DIN-dependent and DIN-independent mechanisms.8 In addition, isoflurane is known to modulate nociceptive pathways in a complex manner via both pronociceptive and antinociceptive actions. However, at the level of the spinal cord isoflurane is thought to exert an antinociceptive effect.8 We have shown previously antinociceptive efficacy using an in vitro paradigm of the neonatal rat spinal cord,9 suggesting that isoflurane may be effective in vivo when functional DIN are absent (e.g. the neonate). Furthermore, in a model of spinal cord transection, where DIN are rendered non-functional, isoflurane inhibited wide dynamic neuron sensory transmission.10 We therefore sought to clarify the antinociceptive action of isoflurane in vivo at various ages in the formalin test using both behavioural and immunohistochemical markers of nociception.
Isoflurane is a potent hypnotic agent in both the adult and neonate; however, the neonate is relatively resistant to the anaesthetic effects of volatile anaesthetic agents assessed by MAC, an operational descriptor. We assessed the hypnotic effects of this agent at four age groups in order to quantify the confounding effects on the behavioural response to nociceptive stimuli and to examine the age dependency of isoflurane induced hypnosis.

Methods

General procedures and animals
The study protocol was approved by the Home Office (UK). Fischer rats used for the entire study (B&K Universal, Grimston Aldbrough Hull, UK) were provided ad libitum with food and water, and artificial lighting between 06.00 and 18.00. The date of birth for the animals was defined as 0-day-old. Experiments were performed on rat pups of 7, 16, and 28 days old and on adult rats (11–12 weeks old), which are equivalent to neonate, toddler, adolescent, and adult.

Nociception experiments
Three cohorts were used at each age group: saline group, oxygen 100% with formalin i.p.i.; isoflurane 0.5% in oxygen and formalin i.p.i.; isoflurane+ formalin group, isoflurane 0.5% in oxygen and formalin i.p.i. The gases were delivered at a flow rate of 2 litre min^{-1}. The concentration of isoflurane was measured using infrared gas spectroscopy (Model 5250 RGM, Ohmeda). Twenty minutes after oxygen or isoflurane administration, formalin 5% (or saline) was injected subcutaneously into the plantar surface of the animal’s left hind paw. The volumes of formalin or saline injected were adjusted for each age group as reported previously and were as follows: 10 μl for 7 days old; 15 μl for 16 days old; 20 μl for 28 days old; 50 μl for adults.

Nociceptive intensity scoring
Immediately after injection of formalin, behaviour was recorded for 60 min with a video camera (MegaPixel, Digital Handycam, Sony) positioned approximately 50 cm beneath the floor of the chamber to allow an unobstructed view of the paws (visible via a video monitor) and to facilitate recording of animal behaviour. The chamber and holding area for pups waiting to be tested were maintained at room temperature throughout the experiment.

Nociceptive behaviour was assessed in the 7-day-old pups (n=3–4) for the presence (‘1’) or absence (‘0’) of flexion, shaking, and whole body jerking per epoch of time and calculated as nociceptive score=T/300, where T is the duration (s) of nociceptive behaviour exhibited during consecutive 300 s post-injection epochs.

Loss of righting reflex
At each age we investigated the sedative effect of isoflurane using the loss of righting reflex (LORR) endpoint defined as the inability of animals to right themselves when positioned in a supine position. The percentage of animals with LORR at concentrations of isoflurane 0.1% and above (using increments of 0.1%) was used to establish dose–response curves (n=8–10). After adjusting the vaporizer to achieve a new concentration, a 35-min equilibration period was allowed to elapse before testing.

Immunohistochemical staining and quantitative counting of c-Fos
Ninety minutes after the formalin injection, animals were deeply anesthetized with pentobarbital (100 mg kg^{-1}, i.p.) and perfused with paraformaldehyde 4% (n=4 per age group). The whole spinal cord was removed. The lumbar enlargement was sectioned transversely at 30 μm and was then stained for c-Fos as described previously. Briefly, sections were incubated for 30 min in H₂O₂ 0.3% in methanol and thereafter washed three times in 0.1 M phosphate-buffered saline (PBS). Following this, the sections were incubated for 1 h in a ‘blocking solution’ consisting of donkey serum 3% and Triton-X 3% in PBS (PBT) and subsequently incubated overnight at 4°C in 1:5000 goat anti-c-Fos antibody (sc-52-G, Santa Cruz Biotechnology, Santa Cruz, CA) in PBT with donkey serum 1%. The sections were then rinsed three times with PBT and incubated with 1:200 donkey anti-goat IgG (Vector laboratories, Burlingame, CA) in PBT with donkey serum 1% for 1 h. The sections were washed again with PBT and incubated with avidin–biotin–peroxidase complex (Vector Laboratories) in PBT for 1 h. The sections were rinsed three times with PBS and stained with 3,3'-diaminobenzidine (DAB) with nickel ammonium sulphate to which hydrogen peroxide was added (DAB kit, Vector Laboratories). When the staining was complete, the sections were rinsed in PBS followed by distilled water and mounted, dehydrated with ethanol 100%, cleaned with 100% xylene and covered with cover slips.

Photomicrographs of three sections per animal were scored ipsilaterally for c-Fos-positive neurons by an observer who was blinded to the experimental treatment. Sections expressing maximal levels of c-Fos were selected for scoring. For the purpose of localizing the c-Fos-positive cells to functional regions of the spinal cord, each section was...
divided into A/B (laminae I–II or the superficial area), C (laminae II–IV or nucleus proprius area), D (laminae V–VI or the neck area, and E (laminae VII–X or the ventral area).\textsuperscript{15}

**Data analysis**

Nociceptive intensity scoring against time in each animal was plotted and the area under curve (over a 60 min time period) (AUC) from each animal was calculated. Mean c-Fos-positive neurons for three representative sections in each region, as described above, was the aggregate score for each animal. The results of nociceptive intensity or c-Fos-positive neurons are reported as means (SEM). Statistical analysis was performed by one-way analysis of variance, followed by Newman–Keuls test. A $P$ value <0.05 was regarded as statistically significant.

LORR concentration response data were fitted according to the method of Waud\textsuperscript{16} to a logistic equation of the form:

$$P = \frac{100C^n}{C^n + (ED_{50})}$$

where $P$ is the percentage of the population anaesthetized, $C$ is the drug concentration, $n$ is the slope parameter, and $ED_{50}$ is the drug dose for half a maximal effect.

**Results**

**Behavioural nociceptive response**

Time course of the nociceptive response of each cohort in each age category is presented in Figure 1. Isoflurane significantly decreased the AUC relative to oxygen after formalin i.p.i. (Table 1).

During the pre-injection period, all animals were awake and active. Following injection with formalin in the presence of oxygen 100%, the 7-day-old animals exhibited intense nociceptive behaviour. Administration of isoflurane at 0.5% attenuated this nociceptive response.

Similarly formalin i.p.i. in the 16-day-old, 28-day-old, and adult groups led to intense nociceptive behaviour. For each cohort there was a significant decrease in nociceptive behaviour with administration of isoflurane at 0.5%.

**Immunohistochemical nociceptive response**

Formalin-induced c-Fos expression at the lumbar level of the spinal cord ipsilateral to the site of injection increased at all age groups in the outer laminae of the dorsal horn.

<table>
<thead>
<tr>
<th></th>
<th>Air+formalin</th>
<th>Iso+formalin 0.5%</th>
<th>Air+saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>7-day-old</td>
<td>10 (0.72)</td>
<td>0.56 (0.59)*</td>
<td>0.17 (0.08)*</td>
</tr>
<tr>
<td>16-day-old</td>
<td>66 (3.1)</td>
<td>2.8 (0.9)*</td>
<td>0.2 (0.1)*</td>
</tr>
<tr>
<td>28-day-old</td>
<td>79 (9.1)</td>
<td>9.2 (7.8)*</td>
<td>0.4 (0.35)*</td>
</tr>
<tr>
<td>Adult</td>
<td>117 (10)</td>
<td>10 (1.3)*</td>
<td>2.2 (1.0)*</td>
</tr>
</tbody>
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* $P<0.01$, $^*P<0.001$ relative to air+formalin group at the corresponding age group.

**Fig 1** Nociceptive scoring time curves (after formalin injection) from (a) 7-, (b) 16- and (c) 28-day-old, and (d) adult Fischer rats treated with formalin or saline by intraplantar injection (i.p.i.) in the presence of oxygen or oxygen/0.5% isoflurane [mean (SEM), n=3–4]. On the y-axis nociceptive intensity with lower values indicating less nociceptive behaviour.
Exposure to isoflurane at 0.5% significantly suppressed c-Fos expression in this region at all ages indicating suppression of nociception (Fig. 3).

Isoflurane administration reduced c-Fos expression in lamina A/B (I–II) in response to formalin by 60% \((P<0.001)\) in 7-day-old rats, 40% \((P<0.05)\) in 16-day-old rats, 74% \((P<0.001)\) in 28 day-old rats, and 34% \((P<0.05)\) in adult rats (Fig. 3).

**Discussion**

Isoflurane at 0.5% possesses antinociceptive efficacy at all developmental stages tested in Fischer rats. Extrapolation of these findings suggests that isoflurane 0.5% may be analgesic in humans from neonatal ages onwards. In contrast to neonatal resistance to isoflurane anaesthesia as measured by MAC, isoflurane is a potent hypnotic in the very young. This information not only dissociates MAC and hypnosis in the very young, but highlights the complex interaction between analgesia, immobility, and hypnosis that contributes to MAC assessment. Furthermore, increasing age appears to correlate with increasing resistance to the hypnotic effects of isoflurane as assessed by loss of righting reflex. As the hypnotic effect of isoflurane may confound the behavioural assessment of nociception, we complemented behavioural assessment with immunohistochemical c-Fos assays of antinociceptive efficacy. However, there are still some limitations using c-Fos for this kind of study although it could be considered as a classic marker of pain circuitry. In terms of the anatomical pattern of c-Fos expression in the spinal cord following noxious stimuli, there is a difference between immature and adult rats where it is located more in superficial dorsal region of the spinal cord in fetus but in all laminae of the dorsal horn in adults and this was confirmed by the present study (Figs 2 and 3). It has also been suggested that the sensitivity of c-Fos expression following noxious stimuli in rats is age and stimulus-intensity dependent. Another limitation is that it would have been ideal to include a isoflurane+saline group for better comparison. However, this has no implications for the antinociceptive efficacy of isoflurane and hence this group was omitted originally in the experimental protocol. Nevertheless, these limitations would not affect our conclusion, as we did not compare the antinociceptive efficacy of isoflurane directly between age groups. The comparisons were made with aged-matched controls only.

The data are qualitatively different from those that we reported recently with nitrous oxide in which no antinociceptive effect (neither behaviourally nor immunohistochemically) was noted in animals younger than 23 days old. In contrast, we have found xenon to be effective at all ages in this paradigm, possibly because of the fact that xenon acts at the level of the spinal cord. Isoflurane is known to possess direct action at the level of the spinal cord and this is reflected in vivo in 7-day-old rats where DIN are not functional, yet c-Fos expression, stimulated by formalin, is attenuated. The mechanism whereby isoflurane
**Fig 3** Number [mean (SD), n=4] of c-Fos positive cells at the lumbar level in response to plantar formalin injection from the four age-groups of animals receiving either oxygen (black bars) or oxygen/isoflurane 0.5% (dotted bars) or in response to saline injection and oxygen (white bars). *P<0.05; **P<0.01 relative to oxygen+formalin group. The x-axis represents the respective laminae (ipsilateral to formalin injection) within which the c-Fos positive cells were distributed. For 7-day-old pups the laminae are A/B, C, D, and E, respectively, as equivalent to the five laminae in older animals (laminae I–II, laminae II–IV, laminae V–VI, and laminae VII–X).
rats have significantly lower ED₅₀ than 16-day-old, 28-day-old, and adult rats (P<0.01). Adult rats have significantly higher ED₅₀ than younger rats (P<0.05).

7-day-old data against the 9-day-old MAC data² for neonate, is 2.34:0.25 while the adult it is 1.12:0.65 (comparison of our adult rat data against that of Orliaguet and colleagues²). This indicates that the neonate is more susceptible to the hypnotic effects of isoflurane than the adult and while we have started to describe mechanistic features of these effects in the adult³ we are yet to elucidate mechanisms of hypnosis in the very young. Furthermore, it is also possible that other aspects of the anaesthetic state are less sensitive in the neonate such as immobility and pain processing mechanisms. This interesting facet of anaesthetic agents deserves further exploration.

Anesthesia in the neonate has recently been associated with long-term detrimental side effects.⁵⁹ Whether this is a correlate of anaesthesia, individual anaesthetic agents or a combination of anaesthetic agents is currently unknown. However, isoflurane at 0.75% and above induced neurodegeneration in rats when administered alone⁶⁰ and therefore isoflurane should not be regarded as entirely safe in the neonate if the results of this animal study can be extrapolated to humans. It is clear that the provision of satisfactory analgesia and anaesthesia must be balanced against the potential to cause harm, and thus a well-defined anaesthetic profile of an agent at each age must be established.

In summary, isoflurane at 0.5% is an effective antinociceptive agent at developmental stages equivalent to the human neonate and older. Thus unlike nitrous oxide, which has no antinociceptive effect in this model,⁵ isoflurane is a potent antinociceptive agent. In addition, isoflurane is a potent hypnotic agent at each age tested. However the neonate, while insensitive to isoflurane anaesthesia as assessed by MAC, is extremely sensitive to the hypnotic effect of isoflurane. Further investigation of the MAC-fraction of isoflurane in the newborn is warranted.

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Isoflurane, antinociceptive and hypnotic effect and age

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