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EC NEUROLOGY
Research Article

Halothane Differentially Modifies Serotonin-Induced Depolarizations and Hyperpolarizations of Isolated, Cultured *Lymnaea* Neurons

Asikiya Walcourt[1] and William Winlow[2]*

[1]*Department of Physiology, Howard University College of Medicine, Washington, DC, USA*

[2]*Department of Biology, University of Naples, Federico II, Italy and Institute of Ageing and Chronic Diseases, University of Liverpool, Liverpool, UK*

***Corresponding Author:** William Winlow, Department of Biology, University of Naples, Federico II, Italy and Institute of Ageing and Chronic Diseases, University of Liverpool, Liverpool, UK.

Received: May 22, 2019; **Published:** June 24, 2019

Abstract

In *Lymnaea stagnalis*, serotonin (5-Hydroxytryptamine) (5-HT) evokes depolarizing, hyperpolarizing or biphasic responses in neurons. Here, the actions of varying concentrations of halothane (1 - 4%) on 5-HT-induced hyperpolarizing and depolarizing responses were studied on cultured *Lymnaea* neurons. At low concentrations (1%), halothane first enhanced the depolarizing responses, before producing dose-dependent decrease of such responses. The duration and amplitude of the hyperpolarizing responses first decreased, and then increased in a concentration-dependent manner. We believe that suppression of 5-HT-induced excitatory responses, produced by halothane in *Lymnaea* neurons, is due to a reduction in the sensitivity of the neuronal membrane to exogenous transmitter, whilst enhancing the inhibitory responses.

Keywords: *5-HT; Halothane; Lymnaea; Paroxysmal Depolarization Shifts; Cell Culture; Anaesthetics*

Introduction

In a previous paper, we demonstrated that the electrophysiological characteristics of neurons from the feeding system of the pond snail *Lymnaea stagnalis* (L.), in isolated cell culture, were largely similar to those in the intact brain [1,2]. The synaptic physiology of pairs and groups of cultured *Lymnaea* neurons have previously been studied [1,3]. In experiments using peptidergic synapses of identified *Lymnaea* neurons in culture, clinically relevant concentrations of the general anesthetic halothane (1 - 2%) were shown to cause synaptic depression [4] and to abolish excitatory transmission at lower anesthetic concentrations, than inhibitory transmission [5]. This supports the view that anesthesia is produced by depressant actions at excitatory synapses and potentiation of inhibitory postsynaptic potentials (IPSPS) [6-8]. In this paper we explore the effects of the general anesthetic halothane on isolated cultured neurons in the absence of synaptic inputs from other neuronal network components.

Previous studies have established *Lymnaea stagnalis* (L.) as an appropriate model system for general anesthetic research [9-12]. The work of Spencer., *et al.* [4,5] was performed on peptidergic synapses, but the central nervous system also contains amino acid transmitters such as 5-HT. Therefore, we chose to study the effects of halothane on serotonin-induced responses, using isolated, cultured, identified neurons from the *Lymnaea* feeding system [13]. Previous work using these isolated, cultured *Lymnaea* feeding neurons demonstrated the presence of $5HT_{1a,\ 2\ and\ 3}$ receptors on them [14-16]. The validity of these findings has never been challenged, even though the classification of 5HT receptors has been modified [17]. In the intact brain of *Lymnaea*, the serotonin-containing cerebral giant cells (CGCs) [18,19] and some its follower motoneurons in the buccal ganglia normally respond to serotonin [13]. Preliminary studies using cultured *Lymnaea* neurons have shown 5-HT to produce depolarizing, hyperpolarizing and biphasic responses [14-16]. Hence, we are using these neurons to study the actions of general anesthetics. The CGCs are believed to act as modulators of the ingestive motor pattern [13,20] by providing background excitatory modulation of the feeding central pattern generator (CPG), in the *Lymnaea* feeding system and it has recently been suggested that the excitation is via $5\text{-}HT_{6\text{-}7}$ receptors [21]. Our earlier findings however, showed that both excitation and inhibition

could be evoked via $5HT_2$ and $5HT_3$ receptors [14-16]. Serotonin also has inhibitory actions, exerted via 5-HT_1-type receptors, which is in agreement with our previous findings about $5HT_{1a}$ receptors [14].

With the exception of the ionotropic $5HT_3$ receptors, it should be noted that, all the other 5HT receptor families are composed of G-protein coupled metabotropic receptors [17]. Previously, modifications of synaptic transmission of the CGCs by halothane in *Lymnaea* have been investigated, but only excitatory synapses were studied in detail [22]. Here, the interactions between halothane and excitatory and inhibitory serotonin responses are investigated on cultured CGCs or their follower buccal cells in isolation.

Materials and Methods

Preparation of neurons

Experiments were carried out on identified neurons from either the buccal ganglia or the CGCs of the cerebral ganglia cultured in isolation in defined medium for 1 - 2 days as previously described [14]. Intracellular recordings were made from these neurons at 20°C in either HEPES saline for control experiments or using different anaesthetic concentrations prepared in HEPES saline. The perfusion circuit was designed to minimise anaesthetic loss and is described elsewhere [9]. The vaporised halothane concentrations (%v/v) are related to the halothane concentrations (mM) obtained from our recording baths under standard flow conditions at 20^0C, which contained 0.43 to 0.47 mM halothane [23,24]. These values relate well to the clinical concentrations found in mammalian blood [25]. Using Neurolog DC amplifiers all cells were held at a membrane potential of -60mV. Anaesthetic-induced changes (if any) in the resting membrane potential were usually small (a few mV) and were compensated for by intracellular injections of DC-current to return the neurons to their original resting potentials. Experiments were carried out between mid-May and late-July.

Experimental procedures

The effects of anaesthetics were assessed as follows. (1) After stable recording conditions were achieved, 10^{-3}M 5-HT was pressure ejected onto the neuron surface via a puffer pipette attached to a WPI PV 800 picopump set at 5psi and for 500ms pressure pulses. The puffer pipette allowed for controlled application of 5-HT during electrophysiological recordings. (2) After consistent and repeatable (3-4) responses to 5-HT were obtained on a neuron, (3) the perfusion of 1% halothane was commenced and stopped either when paroxysmal depolarization shifts (PDSs) occurred, or when the cell went quiescent. (4) Three or more responses to reapplication of 5-HT obtained in the presence of anaesthetic were averaged for quantitative comparisons with controls. Steps (3) and (4) were then repeated for each anaesthetic concentration. (5) The preparation was washed in fresh HEPES saline until complete recovery of the cells from anaesthetic occurred. In some experiments however, up to two hours of continuous washout was required for complete recovery, although in a few cases, cells were lost before complete recovery could be achieved.

Drugs

The volatile anaesthetic agent used was halothane (redistilled from Fluothane^R I.C.I. Ltd). This was dissolved directly into HEPES saline according to the methods of Girdlestone., *et al.* [10] and stored in sealed glass containers. The concentrations of halothane used were 1, 2, and 4%. No further gassing of the solution was performed during experimentation. 10^{-3}M serotonin (creatinine sulphate complex, Sigma) was dissolved in HEPES saline and freshly prepared before experiments.

Statistical analysis

The results are expressed as means ± standard error of mean (SEM) obtained from different experiments with the same anaesthetic concentrations. Statistical analyses were performed using the Student's t test.

Results

The effects of halothane applications (1 - 4%), were investigated in 14 neurons (5 CGCs and 9 buccal motoneurons) cultured in isolation. In all cases, application of 1% halothane in saline produced either immediate quiescence, or quiescence following a series of paroxysmal depolarization shifts (PDSs), as was previously reported in other groups of neurons [11,12]. For the more detailed analysis of excitatory and inhibitory responses found on buccal 4 cells and CGCs no differences could be found between these different cells and they have considered together.

The actions of halothane on serotonin evoked depolarizations

Six preparations (3 CGCs and 3 buccal 4-cells) were used to examine the effects of halothane over a concentration-range of 1 - 4%, on the depolarizing responses produced by 5-HT. The amplitude of the excitatory effects of halothane were first enhanced (in 1%), and then depressed in a concentration-related manner, between in 2 and 4% (Figure 1). Similar responses have previously been demonstrated in the intact brain of *Lymnaea stagnalis* [14]. However, the duration of the responses clearly decreased in a dose-dependent manner (Figure 1). In control experiments, application of 5-HT produced depolarization (16.75 ± 0.37 mV) and excitation in silent neurons, the duration (74.63 ± 1.05 sec) of which far out-lasted the application period of 500ms. In the presence of 1% halothane, action potentials did not occur, but the amplitude of the depolarizing response to 5-HT application was large and substantially enhanced (35.50 ± 2.77 mV) at the first application. The mean duration of these responses was 51.78 ± 1.03 sec. It then gradually diminished with successive applications (Figure 1B). In 2% halothane, the amplitude of the 5-HT response was significantly reduced (20.17 ± 1.41 mV) as compared with that produced in 1% halothane ($P << 0.05$), while the duration of these responses also reduced (49.11 ± 2.23 sec). In 4% halothane, the 5-HT-evoked responses were further reduced in amplitude (10.08 ± 0.47 mV) as compared with the control ($P << 0.05$), and the duration (27.56 ± 0.73 sec) ($P << 0.01$) as compared with that in 1% halothane. On washing out halothane, the normal response to 5-HT application recovered within 27 minutes (18.67 ± 2.45 mV) amplitude and (62.22 ± 1.54 sec) duration. These data are summarised in figure 2.

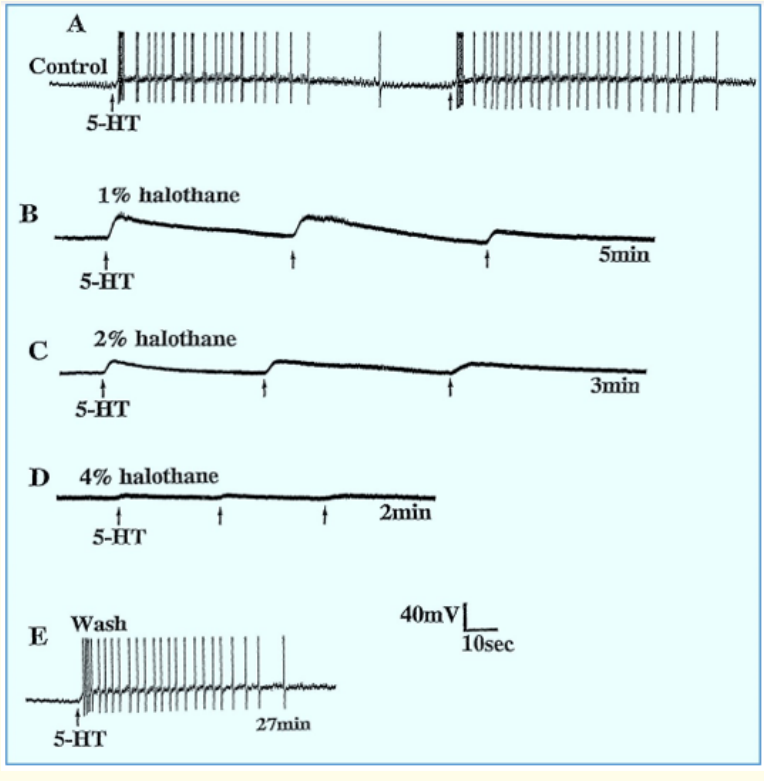

Figure 1: *Effects of halothane (1 - 4%) application on depolarizing responses induced by 5-HT on a CGC. (A) 5-HT evoked depolarization and excitation at the points of application (arrows) in a previously silent CGC. The responses far outlasted application. (B) After 5 minutes of bath perfusion of 1% halothane, spike generation was blocked, but the first application of 5-HT produced a depolarizing response which gradually reduced with subsequent applications. (C) In 2% halothane, the depolarizing responses were further reduced and (D) 4% halothane virtually abolished them. (E) Full recovery occurred after 27 minutes of washing in Hepes saline. In A and E, the action potential peaks were clipped by the pen recorder (Em = -60mV).*

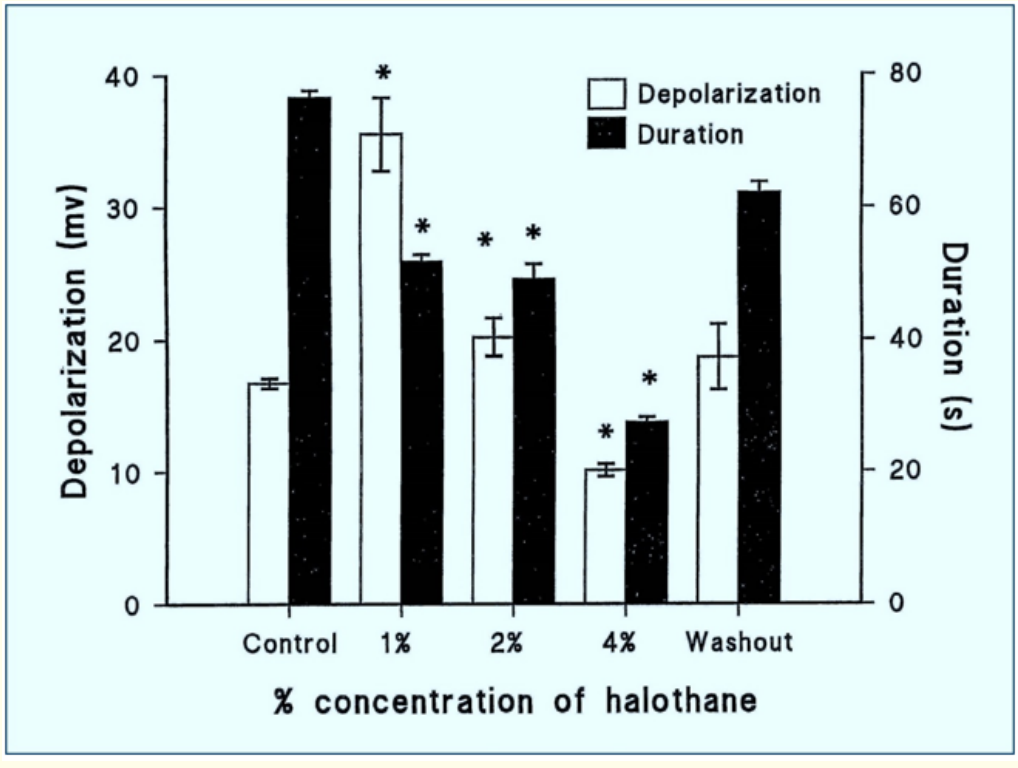

Figure 2: *Summary histogram of the effects of 1-4% halothane on both the amplitude (open bars) and duration (shaded bars) of the depolarizing responses produced by 10^{-3}M 5-HT on CGCs and buccal-4 cells (n = 6). In controls, pressure ejection of 5-HT produced depolarization (16.75 ± 0.37 mV), the duration of which was 74.63 ± 1.05 sec. 1% halothane increased the amplitude to 35.50 ± 2.77 mV, but reduced the duration of this response to 51.78 ± 1.03 sec (P << 0.01). The addition of 2% halothane however, reduced both the amplitude and duration of the 5-HT-induced depolarizing response to 20.17 ± 1.41 mV and 49.11 ± 2.23 sec respectively (P << 0.01). In 4% halothane, this depolarizing response was significantly reduced to 10.08 ± 0.47mV (amplitude) (P << 0.05), and 27.56 ± 0.73 sec (duration) (P << 0.01) (n = 6). After washout of the preparation, the 5-HT-induced depolarizing responses returned to normal (18.67 ± 2.45 mV) and the duration of these responses were 62.22 ± 1.54 sec.*

The actions of halothane on serotonin-evoked hyperpolarizations

5-HT evoked pure hyperpolarizing responses in 5 neurons (2 CGCs and 3 buccal 4-cells). An example is shown with a 4-cell in figure 3A (mean duration: 23.40 ± 1.63 sec; mean amplitude: 10.00 ± 3.50 mV). In the presence of 1% halothane, PDS was initiated within 50sec in this cell, as is characterized by the long-lasting depolarization plateau seen on a faster time base (Figure 3B). After, 2 minutes in 1% halothane, the duration of the hyperpolarizing responses produced by 5-HT was reduced to 13.36 ± 0.93 sec; and the mean amplitude was 10.05 ± 3.00 mV (Figure 3C) and in 2% halothane, to 15.37 ± 0.66 sec, while the mean amplitude became 6.52 ± 2.45 mV (Figure 3D). In 4% halothane however, the 5-HT responses became much more prolonged and deeper (Figures 3E and 3F). 1 minute after bath perfusion of 4% halothane, the duration of the hyperpolarizing response caused by 5-HT was 25.61 ± 1.29 sec, and the mean amplitude was 20.87 ± 1.32 mV (Figure 3E). After 3 minutes, these responses lasted significantly longer than in controls (Figure 3F) (duration = 62.34 ± 2.87sec, and amplitude = 25.47 ± 4.36 mV), (P < 0.001). After 45 minutes of washout in fresh Hepes saline, normal firing was being restored (Figure 3G). These data are summarised in figure 4.

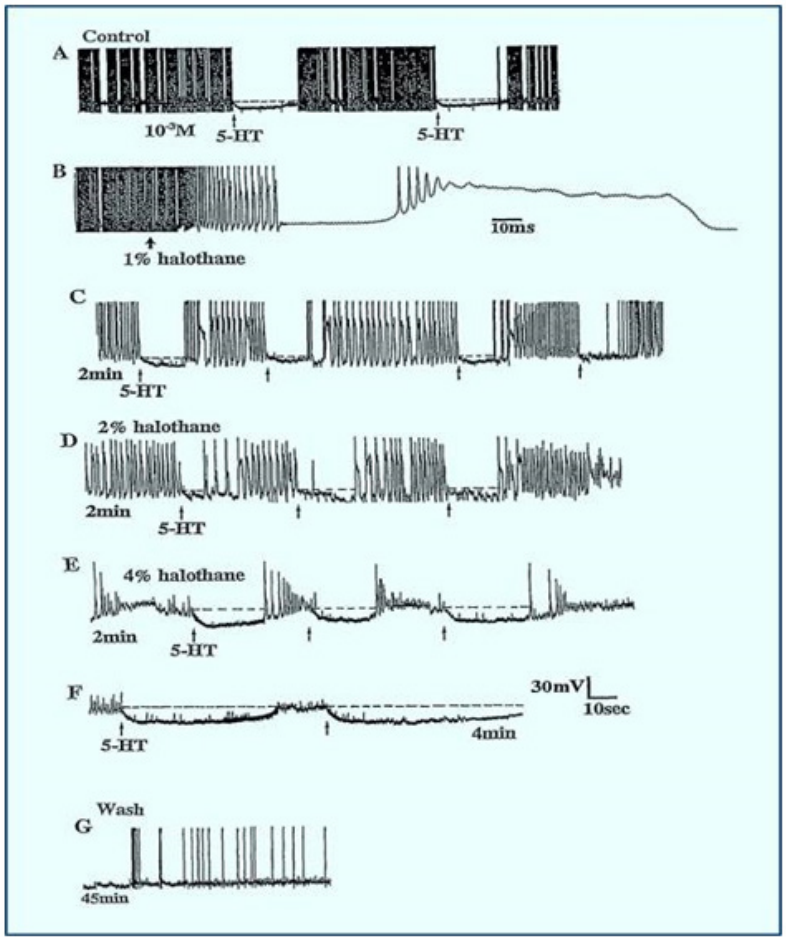

Figure 3: *Enhancement of the hyperpolarizing responses of 5-HT by halothane (1 - 4%) in a buccal 4 cell. (A) Application of 5-HT (arrows) evoked hyperpolarization and inhibition. (B) In 1% halothane, PDS was rapidly initiated as is evidenced by the plateau on a faster time base. (C) 2 minutes later, 5-HT still produced hyperpolarization and spike inhibition. (D) In 2% halothane, cell hyperpolarization and spike inhibition was still maintained. (E) After 1 minute in 4% halothane, application of 5-HT evoked much more prolonged and deeper hyperpolarizing responses than in 1 and 2% halothane. (F) 3 minutes later, 5-HT evoked hyperpolarizing responses of much longer duration. (F) 45 minutes after washing in Hepes saline, the cell started to recover from the anaesthetic.*

Discussion

The aim of the experiments presented here was to observe the actions of halothane on 5-HT-induced phenomena on identified cultured *Lymnaea* neurons in isolation. Thus, the synaptic effects of presynaptic cells were entirely excluded, simplifying the interpretation of the present results. The data clearly indicate that halothane first enhanced the amplitude of induced depolarizations at low doses (1%), and subsequently depressed them in a dose-dependent manner at higher concentrations (Figure 2), while prolonging the durations and amplitudes of the inhibitory responses produced by 5-HT (Figure 4).

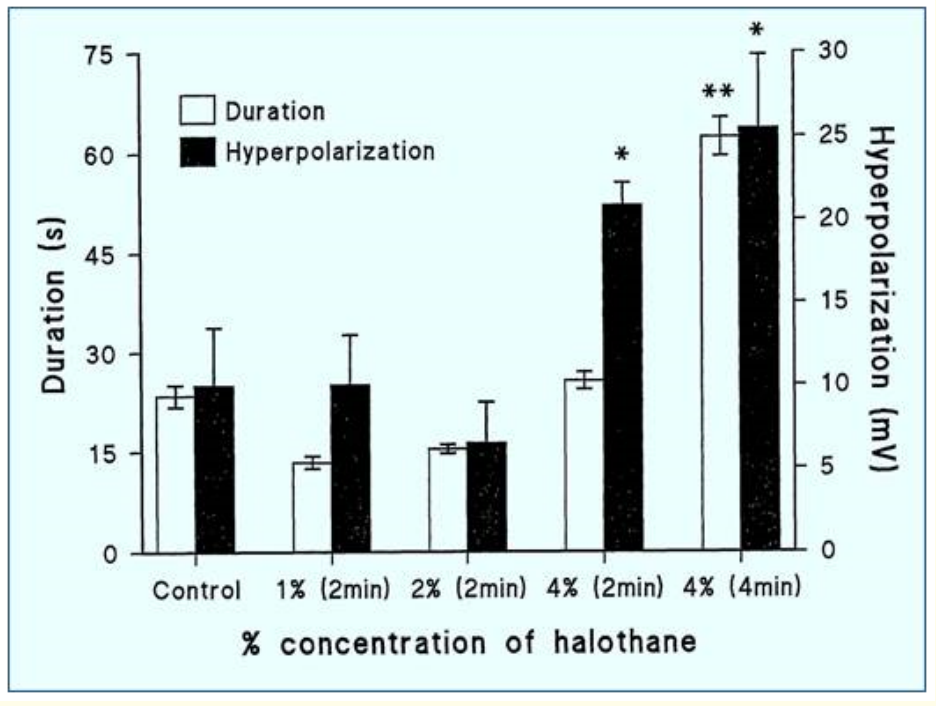

Figure 4: *Halothane enhances both the durations (open bars) and amplitudes (shaded bars) of the hyperpolarizing responses evoked by 5-HT on CGCs and buccal-4 cells in a concentration-dependent manner. In control experiments, 10^{-3}M 5-HT produced hyperpolarizing responses of 23.40 ± 1.63 sec duration and 10.00 ± 3.50 mV amplitude (n = 5). In 1% halothane, although the duration of hyperpolarization reduced to 13.36 ± 0.93 sec in 2 minutes, the amplitude did not change much (10.05 ± 3.00 mV). In 2% halothane, both parameters were reduced to 15.37 ± 0.66sec (duration) and 6.52 ± 2.45 mV (amplitude). Addition of 4% halothane significantly increased the amplitude of the 5-HT-induced responses after 2 minutes, without much affecting the duration of this response within 2 minutes. After 4 minutes in this anaesthetic concentration, both the duration and amplitude of the responses were significantly enhanced (P << 0.01). All cells showed complete or partial recovery from this treatment after washing.*

Halothane, alphaxalone and pentobarbitone have been shown to enhance ɣ-aminobutyric acid (GABA)-mediated inhibition in the guinea-pig olfactory cortex [26,27]. This is considered by several authors to be the principal mechanism of action of anaesthetics, given the importance of GABA as an inhibitory transmitter in vertebrates and whose effects have also been demonstrated on *Lymnaea* neurons [28]. However, actions on other transmitter systems and receptors may also be significant and in the experiments reported here, e.g. the enhanced hyperpolarizations induced by 5HT were greatly enhanced with 4% halothane. Halothane has previously been shown to evoke hyperpolarization of neurons as a result of increased potassium conductance [29] in response to FMRFamide [30] in *Lymnaea* neurons. Similar results have also been reported by Spencer., *et al.* [4,5], but the ionic basis of the enhanced hyperpolarizations reported here remains a matter of conjecture.

Halothane and other anaesthetics have also been shown to produce potent depression at some excitatory synapses [4,5] in *Lymnaea.* The suppression in the amplitude of EPSPs observed by these authors in their experiments may be explained by a combination of pre- and postsynaptic depressant actions of the anaesthetic, or by a decrease in the number of quanta of released transmitter, since *in situ* preparations were used in their experiments. However, the use of single synaptically isolated neurons in culture, such as were used in the present experiments suggests that halothane might have depressed excitatory responses either by reducing the sensitivity of the neurons to applied 5-HT, or by interfering with ion channels, or a combination of both mechanisms may also have occurred. However, at low concentrations (1% halothane),

the 5-HT-evoked depolarizing response was shown to be first enhanced in amplitude, before subsequent depression at higher concentrations. The duration of these responses was also shown to be reduced in a dose-dependent manner. Similar enhancement in the depolarizing responses to applied met-enkephalin in the presence of halothane has also been reported in *Lymnaea* neurons [31]. Hyperpolarizing responses produced by FMRFamide were enhanced at low doses [4,5] suggesting that different transmitter systems respond differently to anaesthetics. In the whole animal preparation, McCrohan., *et al.* [32], demonstrated that halothane produced an increase in the locomotor and feeding motor activity during the progress of anaesthesia, which was believed to be equivalent to the excitatory stage of anaesthesia as described in man [33]. Furthermore, in the system we have tested here, halothane, at low concentrations (1 - 2%), was also shown to first reduce both the amplitudes and durations of the 5-HT-induced hyperpolarizing responses, before producing an increase in both parameters at higher anaesthetic concentrations (Figures 3 and 4). Similar results have also been reported in rat pyramidal cell somata, where the iontophoretic application or pressure ejection of GABA was shown to cause hyperpolarization, which was reduced by 55% in the presence of low concentration of halothane (0.5 mM) [34]. Furthermore, using rat mesencephalic reticular (MRF) neurons Shimoji., *et al.* [35] also reported that the inhalational anaesthetics, isoflurane and halothane blocked inhibitory responses during the lighter anaesthetic stages, while augmenting actions on the inhibitory responses during deep anaesthesia. This suggests that in the intact preparation, anaesthetics may produce an enhancement of depolarizing responses by first causing a reduction in the duration and amplitude of hyperpolarizing responses at low doses, (Figure 1B), before release from inhibition to express exaggerated inhibition at higher concentrations. Thus, at higher concentrations of anaesthetics, there appear to be reductions in depolarizing responses and enhancement of hyperpolarizing responses, at least as far as serotonin-based systems are concerned.

Serotonin is involved in virtually all behaviours in mammals and any reduction in 5HT release can result in elimination of locomotor activity [36,37]. However, extracellular serotonin concentrations decline after administration of the volatile anesthetic isoflurane and maintenance doses of halothane [36]. Isoflurane also causes inhibition of medullary raphé serotonergic neurons in a dose dependent manner suggesting a possible mechanism for anesthesia [38]. However, the $5HT_3$ ligand gated receptors are thought to play only a minor role in producing immobility in the presence of volatile anesthetics [39] and the major inhibitory $GABA_A$ receptors now appear to be important anesthetic targets [40].

5-HT responses have been classed into different 7 receptor families [41] not only based on the actions of selective agonist and antagonist drugs [42], but also based on the types of responses they mediate and molecular biological studies of their structure. Thus, the $5\text{-}HT_3$ receptors were shown to be ligand-gated ion channels [17], the activation of which caused fast depolarizing responses of 5-HT, due to an increase in Ca^{2+} conductance in mammalian neurons [43,44]. Although we have not yet determined which categories of 5HT receptor are most affected by general anaesthetics, we have observed that the depolarizing responses produced by the first application of 5-HT were enhanced in 1% halothane (Figure 1), but this gradually diminished in amplitude with time and with subsequent applications. It is possible that these 5-HT responses became desensitized with repeated applications. Such desensitization or tachyphylaxis has been described as a general characteristic of the action of 5-HT in many preparations: cortical neurons [45]; guinea-pig *taeniae coli* preparations [46], or molluscan hearts [47]). In one experiment however, the excitatory responses evoked by 5-HT reversed to hyperpolarizations which grew in amplitude in the presence of 4% halothane. Similar responses to 5-HT have previously been observed in cortical neurons [45] and also in *Aplysia* L7/L10 synapse in response to Ach [48]. This can be explained in terms of the heterogeneity within the $5\text{-}HT_3$ receptor subtype which has been reported elsewhere [49,50]. One receptor subtype which is probably more susceptible to anaesthetic action may first be blocked, thus exposing the other which is probably less susceptible to anaesthetic action. However, it is difficult to explain such responses in the context of the present experiments. Indeed, it has also been demonstrated that ethanol and ketamine act to potentiate the electrical responses elicited by $5\text{-}HT_3$ receptor activation in rabbit nodose ganglion neurons [51]), but their mechanism(s) of action are not known. It is possible in the present experiments, that 1% halothane acted in the same manner as ketamine to potentiate the probable $5\text{-}HT_3$ responses in these neurons.

It is of interest that the isolated motor neurons described here can all support PDS and that this is generated in the presence of halothane (Figure 3). Similar findings have been discussed elsewhere [52], but it should also be noted that PDS could be generated in the CGCs and the buccal 1, 2 and 4 cells in the intact brain when synaptically isolated from each other by zero calcium/ high magnesium saline in the presence of 1% halothane [53]. PDS may be due to suppression of calcium activated potassium currents due to blockade of voltage gated potassium

currents which have the effect of unmasking persistent sodium currents [54]. However, general anesthetics usually raise intracellular calcium concentration [12,55,56] which would then activate potassium currents, which means that we need to seek another explanation for generation of PDS.

Conclusions

It can be concluded, that the suppression of transmitter-induced excitatory responses produced by halothane in *Lymnaea* neurons is due to a reduction in the sensitivity of the neuronal membrane to applied transmitter and to the enhancement of inhibitory responses. Both of these effects appear to act together to produce the final block in synaptic transmission. Since 5-HT receptors seem to play a part in anaesthetic interaction with 5-HT responses, it will be interesting in future to explore the role of the individual receptor subtypes and how they interact with halothane in producing synaptic efficacy or blockage. Significantly, since in other *Lymnaea* neurons, enhancement, instead of the suppression of depolarizing responses to applied neuropeptides in the presence of increasing halothane concentrations occurs, it is clear that different transmitter systems respond differentially to general anaesthetics. Finally, PDS was initiated in the neurons in this study, but the ionic mechanisms underlying it still need clarification.

Acknowledgements

We are grateful for support for AW, who was a Commonwealth Academic Scholar. Thanks are also due to Messrs. D. Johanson, D. Harrison, and to the late J. Wray for technical assistance and advice. This work was funded by the Association of Commonwealth Universities, Ohmeda (Keighley), and Nuffield Foundation.

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