Anaesthesia with diethyl ether impairs jasmonate signalling in the carnivorous plant Venus flytrap (*Dionaea muscipula*)

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- **Background and Aims** General anaesthetics are compounds that induce loss of responsiveness to environmental stimuli in animals and humans. The primary site of action of general anaesthetics is the nervous system, where anaesthetics inhibit neuronal transmission. Although plants do not have neurons, they generate electrical signals in response to biotic and abiotic stresses. Here, we investigated the effect of the general volatile anaesthetic diethyl ether on the ability to sense potential prey or herbivore attacks in the carnivorous plant Venus flytrap (*Dionaea muscipula*).
- **Methods** We monitored trap movement, electrical signalling, phytohormone accumulation and gene expression in response to the mechanical stimulation of trigger hairs and wounding under diethyl ether treatment.
- **Key Results** Diethyl ether completely inhibited the generation of action potentials and trap closing reactions, which were easily and rapidly restored when the anaesthetic was removed. Diethyl ether also inhibited the later response: jasmonic acid (JA) accumulation and expression of JA-responsive genes (cysteine protease dionain and type I chitinase). However, external application of JA bypassed the inhibited action potentials and restored gene expression under diethyl ether anaesthesia, indicating that downstream reactions from JA are not inhibited.
- **Conclusions** The Venus flytrap cannot sense prey or a herbivore attack under diethyl ether treatment caused by inhibited action potentials, and the JA signalling pathway as a consequence.

Key words: Anaesthesia, anaesthetic, action potential, carnivorous plant, *Dionaea muscipula*, diethyl ether, electrical signal, jasmonic acid, plant movement, Venus flytrap.

INTRODUCTION

The carnivorous plant Venus flytrap (*Dionaea muscipula*) has evolved modified leaves called traps for prey capture (Gibson *et al.*, 2009). The trap consists of two lobes that close rapidly in response to a mechanical stimulus delivered to the trigger hairs protruding from the trap epidermis. Two touches of a trigger hair by an insect prey within 20 s generate two action potentials (APs) which trigger the trap closing reaction (Escalante-Pérez *et al.*, 2011; Volkov, 2019). After rapid closure secures the insect prey, the movement of the entrapped prey in the closed trap results in the generation of further APs (Affolter and Olivo, 1975; Libiaková *et al.*, 2014). The third touch and AP increase cytosolic Ca²⁺ levels in digestive glands. The Ca²⁺ level decays if no further APs are triggered (Escalante-Pérez *et al.*, 2011; Hedrich and Neher, 2018). Prey struggling, repeated mechanical stimulation and the generation of hundreds of APs result in the accumulation of phytohormones from the jasmonate group (Escalante-Pérez *et al.*, 2011; Libiaková *et al.*, 2014; Pavlovič

et al., 2017). Binding of the isoleucine conjugate of jasmonic acid (JA-Ile) to the CORONATINE INSENSITIVE1 (COI1) protein as part of a co-receptor complex mediates the ubiquitin-dependent degradation of JASMONATE ZIM-DOMAIN (JAZ) repressors, resulting in the activation of jasmonate-dependent gene expression (Staswick and Tiryaki, 2004; Chini *et al.*, 2007; Thines *et al.*, 2007; Sheard *et al.*, 2010). Transcriptional activation leads to the synthesis of transport proteins and digestive enzymes that are secreted into the closed trap cavity (Scherzer *et al.*, 2013, 2015, 2017; Libiaková *et al.*, 2014; Böhm *et al.*, 2016a,b). After the prey movement is stopped by exhaustion or death, chemical stimuli from the prey (e.g. chitin, ammonia) further enhance the synthesis of digestive enzymes through jasmonate signalling (Libiaková *et al.*, 2014; Paszota *et al.*, 2014; Bemm *et al.*, 2016; Pavlovič and Mithöfer, 2019).

Several lines of evidence indicate that the generation of electrical signals, jasmonate accumulation and expression of genes encoding digestive enzymes are tightly coupled in the Venus

flytrap. First, repeated mechanical stimulation of trigger hairs is sufficient to induce accumulation of the well-known bioactive compound JA-Ile within the first hour in stimulated traps (Pavlovič *et al.*, 2017). Just two APs are necessary to induce transcription of *JAZ1* within 4 h, and after five APs, *JAZ1* transcripts accumulated to the highest level. More than three APs are necessary to induce significant gene expression of digestive enzymes (e.g. dionain and type I chitinase), and the magnitude of expression is dependent on the number of APs triggered. Gene expression can also be triggered by exogenous application of jasmonic acid (JA), JA-Ile or coronatine without any mechanical stimulus. In contrast, application of the JA perception antagonist coronatine-*O*-methyloxime (COR-MO), which prevents the COI1-JAZ interaction, blocked gene expression despite triggering 60 APs (Böhm *et al.*, 2016a; Bemm *et al.*, 2016).

Thus, the sequence of signalling events in Venus flytrap resembles the well-known signalling pathway in response to wounding or herbivore attack in non-carnivorous plants (Maffei *et al.*, 2007), supporting the hypothesis that botanical carnivory has evolved from plant-defence mechanisms (Pavlovič and Saganová, 2015; Bemm *et al.*, 2016; Pavlovič and Mithöfer, 2019). The fast electrical signals, generated in local leaves in response to wounding or herbivore attack and propagating to neighbouring leaves, are the first cellular response to biotic and abiotic stresses in plants (Maffei *et al.*, 2007). A breakthrough study in this regard was that by Wildon *et al.* (1992), who revealed the link between electrical signal propagation and biochemical response in tomato plants. Electrical signals are often followed by changes in intracellular Ca^{2+} concentration and generation of reactive oxygen species (e.g. H_2O_2 ; Maffei *et al.*, 2007; Kiep *et al.*, 2015; Nguyen *et al.*, 2018) which often co-propagate together (Gilroy *et al.*, 2014). A direct link between increased cytosolic Ca^{2+} and activation of JA biosynthesis genes by Ca^{2+} /calmodulin-dependent phosphorylation of the JAV1-JAZ8-WRKY5 (JJW) repressor complex was recently provided by Yan *et al.* (2018). As a result, increased levels of jasmonates (JA-Ile in particular) trigger the expression of JA-responsive pathogenesis-related (PR) proteins through a COI1-JAZ-dependent pathway (De Geyter *et al.*, 2012). Recently, we showed that carnivorous plants are not able to distinguish between mechanical stimulation and wounding because these processes share the same signalling pathway with plant defence mechanisms. Both induce electrical signals, jasmonate accumulation and digestive enzyme synthesis, confirming the link among electrical signal propagation, jasmonate accumulation and the expression of digestive enzymes (Krausko *et al.*, 2017; Pavlovič *et al.*, 2017). Moreover, the secreted enzymes predominantly belong to PR-proteins, indicating that carnivorous plants have exploited their hydrolytic properties, further emphasizing the similarity between botanical carnivory and plant defence mechanisms (Hatano and Hamada, 2008, 2012; Schulze *et al.*, 2012).

Recently, we documented that Venus flytraps, sundew traps, *Mimosa* leaves and pea tendrils lost both autonomous and touch-induced movements after exposure to local and general anaesthetics (Yokawa *et al.*, 2018, 2019). General anaesthetics (e.g. diethyl ether) are often defined as compounds that induce a reversible loss of consciousness in humans or loss of righting reflex in animals. Anaesthesia can also be defined as loss of

responsiveness to environmental stimuli. Clinical definitions are extended to include the lack of awareness to painful stimuli, which is sufficient to facilitate surgical applications in clinical and veterinary practice (Franks, 2008). The primary site of action of general anaesthetics in animals and humans is the central nervous system, where these molecules enhance inhibitory neurotransmission or inhibit excitatory neurotransmission (Zhou *et al.*, 2012). Although plants do not have neurons and lack a central nervous system, they are able to generate electrical signals (Fromm and Lautner, 2007; Hedrich *et al.*, 2016). Claude Bernard (1878) concluded that volatile anaesthetics not only act on neurons but also affect physiological processes in all cells (Grémiaux *et al.*, 2014). The electrical signals in plants not only trigger rapid leaf movements in ‘sensitive’ plants, such as *Mimosa pudica* or *D. muscipula*, but also induce physiological processes in non-carnivorous plants (Fromm and Lautner, 2007; Mousavi *et al.*, 2013). Interestingly, our recent study showed that inhibition of rapid trap closure in Venus flytrap by the general anaesthetic diethyl ether is caused by inhibition of electrical signalling. There were no toxic impacts of the anaesthetics used, and the effects were fully and rapidly reversible after their removal (Yokawa *et al.*, 2018).

Although carnivorous plants still do not belong to the model group of plants, the signalling events described above indicate that Venus flytrap is a suitable model for studying inducibility and plant responses to external stimuli under anaesthesia due to its rapid trap movement. Considering the tight coupling between electrical signal propagation and jasmonate signalling in carnivorous plants (Böhm *et al.*, 2016a; Bemm *et al.*, 2016; Krausko *et al.*, 2017), we hypothesize that anaesthesia can impair not only rapid trap movement triggered by APs but also the cascade of jasmonate signalling events leading to activation of the digestive process. Our study showed that the Venus flytrap cannot sense potential insect prey or a herbivore attack under anaesthesia due to blocked jasmonate signalling.

MATERIALS AND METHODS

Plant material and culture conditions

The Venus flytrap (*D. muscipula* Ellis.) is native to the subtropical wetlands of North and South Carolina on the East Coast of the USA. Experimental plants were grown under standard glasshouse conditions at the Department of Biophysics of Palacký University in Olomouc (Czech Republic) and the Department of Plant Physiology of Comenius University in Bratislava (Slovakia). As substrate we used well-drained peat moss in plastic pots placed in a tray filled with distilled water to a depth of 1–2 cm. Daily temperatures fluctuated between 20 and 35 °C; relative air humidity ranged from 50 % to 100 %; and the maximum daily irradiance reached 1500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR).

Experimental set-up

The Venus flytrap plants were incubated in 15 % diethyl ether for 2 h in a polypropylene bag. The approximate volume of diethyl ether was calculated using ideal gas constant (standard

state of gas as $22.4 \text{ L}^{-1} \text{ mol}$). To obtain 15 % vapour in 1 litre of air, a liquid phase of 700 μL diethyl ether (74.12 g mol^{-1} , 0.71 g cm^{-3}) was allowed to evaporate inside the sealed polypropylene bag. This was sufficient to anaesthetize the plants, as we found in our previous study, and the plants did not react to mechanical stimulation by rapid trap closure (Yokawa *et al.*, 2018). Thereafter, one group of plants served as a non-stimulated control, and the second group was mechanically stimulated or wounded. For this, a small opening in the polypropylene bag was made. For mechanostimulation, the trigger hairs were mechanically stimulated twice within a short period of time and then once every 3 min for 2 h (40 times together) with the tip of a pipette (which had been melted by heat and then hardened at room temperature to avoid a wound response by the sharp tip, see Pavlovič *et al.*, 2017). In the second experiment, a trap was pierced/wounded with a needle twice within a short period of time and then 40 times every 3 min. In one of the experiments, extracellular measurements of electrical signals were performed on a separate group of plants during stimulation (see below). After 2 h of stimulation, the plants were removed from the bag to allow the plants to recover from anaesthesia. Immediately thereafter, the traps from other groups of plants were sampled for phytohormone analysis. Ten hours later, the traps from the third group of plants were sampled for quantitative PCR (qPCR). At the same time, the non-stimulated traps under diethyl ether for 4 h were also harvested. Control plants, bagged non-stimulated plants or identically stimulated plants but in the absence of diethyl ether were also harvested for phytohormone analysis and qPCR at the same time points (Supplementary Data Fig. S1A). For each method, different groups of plants were used because wounding during sampling could activate the jasmonate signalling pathway.

Extracellular measurements of electrical signals

Each Venus flytrap incubated in diethyl ether for 2–4 h in a polypropylene bag with attached electrodes inside was mechanically stimulated or wounded as described above. Mechanical stimulation or wounding was performed through a small opening in the bag. For recovery, the bag was cut off, and the trigger hair was touched repeatedly every 100 s. Control traps without anaesthetics were also measured. APs were measured on the trap surface inside a Faraday cage with non-polarizable Ag/AgCl surface electrodes (Scanlab Systems, Prague, Czech Republic) fixed with a plastic clip and moistened with a drop of conductive EV gel (Hellada, Prague, Czech Republic) as described previously (Ilík *et al.*, 2010; Pavlovič *et al.*, 2017).

Quantification of phytohormone tissue level

Two hours after initiation of mechanical stimulation and wounding under anaesthesia (4 h etherized), trap tissue samples were collected. Control traps without any stimuli under anaesthesia, as well as stimulated (positive control) and non-stimulated (negative control) control traps without anaesthesia were also harvested. The traps were cut off with scissors and immediately (within 10 s) frozen in liquid nitrogen and stored

at -80°C until analysis. Ten minutes after diethyl ether removal the remaining traps on plants were mechanically stimulated to ensure that plants were only anaesthetized and not dead (the traps had to close). Quantification of JA, JA-Ile, JA-valine (Ja-Val), *cis*-12-oxo-phytodienoic acid (*cis*-OPDA), 9,10-dihydrojasmonic acid (9,10-DHJA), abscisic acid (ABA), salicylic acid (SA) and indole-3-acetic acid (IAA) was performed according to the method described by Floková *et al.* (2014). Two independent technical measurements were performed on four or five biological replicates.

Real-time qPCR

To study the induction of gene expression in the trap tissue, two corresponding genes of well-characterized proteins from digestive fluid were chosen: the cysteine protease dionain (Schulze *et al.*, 2012; Risør *et al.* 2016) and chitinase I (Paszota *et al.*, 2014). To determine the effect of anaesthesia on gene expression, we had to find the time point where the induction of gene expression is high. Therefore, we first collected 100 mg of trap tissue from plants after 0, 2, 6, 12, 24 and 48 h from initiation of mechanical stimulation, wounding or external application of 2 mM JA under a normal atmosphere (air).

Based on this experiment, we chose the 12-h time point for mechanostimulation and wounding under anaesthesia. First, the plants were enclosed in polypropylene bags with diethyl ether for 2 h and then repeatedly mechanically stimulated for 2 h or wounded as described above. After 4 h of anaesthesia, the plants were removed from the bag. Ten hours later, 100 mg of trap tissue sample was harvested (Supplementary Data Fig. S1A). Control plants without anaesthesia in the air were also mechanically stimulated or wounded (positive controls) or were without any stimulation (in the air and under diethyl ether, negative controls).

To determine the effect of JA under diethyl ether treatment, the plants were again enclosed in polypropylene bags with diethyl ether for 2 h. We applied 2 mM JA to the trap surface (volume dependent on the size of the trap), and the second group of plants had no JA application but was still under a diethyl ether atmosphere. The same was done in the control air-only plants. After 7 h, trap samples were collected for qPCR analyses (Supplementary Data Fig. S1B). The sampling time was shorter than in the previous experiment because prolonged exposure of plants to diethyl ether caused damage to trap tissue. This is probably caused by non-polar aprotic solvent properties of diethyl ether and its interactions with lipid membranes if exposed for prolonged periods.

To exclude the lethal impact of diethyl ether on plants, the recovery of gene expression was also investigated. After 2 h in diethyl ether, the plants were removed from the bag and kept for 2 h in air. The plants were then mechanically stimulated or wounded as described above, and after 10 h, trap samples were collected for qPCR analyses.

RNA extraction and gene expression were performed as described previously (Saganová *et al.*, 2018) using the primers detailed in Supplementary Data Table S1. All samples for PCR experiments were analysed in four biological and three technical replicates.

Western blotting

To detect and quantify cysteine protease (dionain) and type I chitinase, polyclonal antibodies against these proteins were raised in rabbits by Agrisera (Vännäs, Sweden) and Genscript (Piscataway, NJ, USA) as described previously (Pavlovič *et al.*, 2017). Digestive fluid was collected 48 h after the beginning of mechanical stimulation and wounding only from plants in the air (no digestive fluid was secreted under diethyl ether). SDS-PAGE, silver staining and Western-blot were done as described previously (Pavlovič *et al.*, 2017).

Statistical analyses

All data are from biological replicates, and each biological sample was analysed in two or three technical replicates. Before statistical analyses, the data were tested for homogeneity of variance (Brown–Forsythe test). If homogeneity was fulfilled, one-way analysis of variance (ANOVA) with Tukey's post-hoc test was used (Origin 8.5.1, Northampton, MA, USA). If homogeneity was not fulfilled, multiple comparison by Welch's test with Bonferroni-corrected alpha was used (Microsoft Excel).

RESULTS

Anaesthesia inhibits electrical signalling and trap closing reactions

The trap of the Venus flytrap plant generates typical APs in response to mechanical stimulation of trigger hairs or to wounding. Two APs resulted in rapid trap closure within 1 s (Fig. 1A, Supplementary Data Movies S1 and S4). The shape, duration and amplitude of APs triggered by mechanostimulation and wounding were the same (Supplementary Data Fig. S2). However, after 2 h under diethyl ether anaesthesia, the trap lost the closing response and the ability to generate APs in response to both stimuli (Fig. 1B, Supplementary Data Movies S2 and S5). One hundred seconds after removal of diethyl ether, APs with a reduced amplitude and increased half-width were detected (Fig. 2A), but they were not able to trigger trap closure. In some traps, the first AP was detected after 200 s during recovery. The amplitude and spike half-width of the recorded APs gradually recovered (recorded every 100 s, Fig. 2B, C). When

the amplitude of APs was lower and the spike half-width longer during recovery, more touches, and thus more APs, were necessary to induce rapid trap closure. The closing response of the trap was fully restored within 10–15 min, and again, only two touches were sufficient for trap closure after recovery (Supplementary Data Movies S3 and S6).

Anaesthesia inhibits the accumulation of jasmonates

In a previous study, we found that tissue levels of jasmonate in Venus flytrap were highest within the first 2 h of stimulation (Pavlovič *et al.*, 2017). Therefore, we chose this time point for phytohormone analysis under anaesthesia. We found a clear activation of the JA signalling pathway for both mechanostimulation and wounding, consistent with our previous study (Pavlovič *et al.*, 2017). There was a more than 300-fold increase in the JA tissue level for both types of stimulation in air (Fig. 3A, I). The bioactive compound JA-Ile increased 23- and 13-fold in response to mechanostimulation and wounding, respectively (Fig. 3B, J). The content of other oxylipins (JA-Val, *cis*-OPDA, 9,10-DHJA) did not change significantly (Fig. 3C–E, K–M). Under anaesthesia, diethyl ether completely inhibited jasmonate accumulation in response to mechanostimulation (Fig. 3A, B). In response to wounding, there was only a slight but significant increase of jasmonates. The JA level increased seven-fold, and the bioactive JA-Ile level increased two-fold (Fig. 3I, J). This increase probably did not reach the threshold level for activation of the JA signalling pathway, as indicated by the qPCR data (see below). The JA-Val content decreased in wounded plants under ether atmosphere in comparison to air (Fig. 3K). A similar, but not significant, pattern was detected also for *cis*-OPDA (Fig. 3L). The levels of other plant hormones (ABA, IAA) did not change significantly (Fig. 3F, H, N, P). There was a trend towards a two-fold non-significant increase in SA level in response to both stimuli, irrespective of treatment (air vs. diethyl ether; Fig. 3G, O).

Transcription of JA-responsive genes is inhibited under anaesthesia

First, we analysed the time dependence of the mRNA levels of two selected JA-responsive genes in Venus flytrap: the cysteine

FIG. 1. Electrical signalling in the Venus flytrap (*Dionaea muscipula*) under anaesthesia with diethyl ether. (A) Two touches of trigger hairs (arrows) or wounds generate two action potentials and rapid trap closure. (B) Action potentials are not generated in response to two touches (arrows) when exposed to diethyl ether.

FIG. 2. Recovery of electrical signalling after removing of diethyl ether in the Venus flytrap (*Dionaea muscipula*). (A) Recovery of action potentials (APs) in response to mechanical stimulation and wounding. Representative APs from four independent experiments are depicted. (B) Recovery of AP amplitude. (C) Recovery of AP spike half-width. Black bars – mechanostimulation, red bars – wounding. Data are means $\pm$ s.e., $n = 4$. There were no significant differences between APs generated in response to wounding and mechanical stimulation.

protease *dionain* and *chitinase I* (Böhm et al., 2016a; Bemm et al., 2016). The kinetics of the upregulation of mRNA levels for both genes were similar. The highest mRNA level was found between 12 and 24 h after the first AP was triggered for both types of stimulation. At 48 h, the mRNA levels declined. The kinetics of mRNA levels were different for the external application of JA; the mRNA levels of both genes gradually increased over 48 h (Supplementary Data Fig. S3A, B). The protein

product of these genes was detected in digestive fluid after 48 h (Supplementary Data Fig. S4). Based on these results, we chose the earliest possible time point, where the upregulation of gene expression was evident, to investigate the effect of anaesthesia. In this experiment, plants were exposed to diethyl ether for 2 h, and the traps were mechanostimulated or wounded for the next 2 h. The diethyl ether was removed, and the traps were sampled 10 h later (12 h after the first mechanostimulus, Supplementary

FIG. 3. Accumulation of phytohormones in trap tissue of the Venus flytrap (*Dionaea muscipula*) 2 h after mechanostimulation and wounding in the air and under anaesthesia with diethyl ether. (A–H) Mechanostimulation, (I–P) wounding. (A, I) Jasmonic acid (JA), (B, J) isoleucine conjugate of jasmonic acid (JA-Ile), (C, K) valine conjugate of jasmonic acid (JA-Val), (D, L) *cis*-12-oxo-phytodienoic acid (*cis*-OPDA), (E, M) 9,10-dihydrojasmonic acid (9,10-DHJA), (F, N) abscisic acid (ABA), (G, O) salicylic acid (SA), (H, P) indole-3-acetic acid (IAA). C, control; M, mechanostimulation; W, wounding. Values are means $\pm$ s.d. from four biological replicates, $n = 4$ –5. Different lower-case letters denote statistically significant differences at $P < 0.05$.

Data Fig. S1A). [Figure 4](#) clearly shows that the mRNA levels of both investigated genes (*dionain*, *chitinase 1*) were not increased under anaesthesia and were comparable with the non-stimulated control in air or diethyl ether. Two hours after removing diethyl ether, the Venus flytrap was again able to upregulate gene expression in response to mechanostimulation and wounding (Supplementary Data Fig. S5).

External application of JA bypassed electrical signalling and restored gene expression under anaesthesia

We used the following experiment to determine whether we can bypass the inhibition of electrical signalling by direct application of JA and thus restore gene expression under anaesthesia. The plants were exposed to diethyl ether for 2 h. Then, a few drops of 2 mM JA were applied to the trap surface, and the plants were kept for 7 h under anaesthesia, which was the

longest possible time to avoid tissue damage. The traps were then sampled for qPCR (Supplementary Data Fig. S1B). [Figure 5B](#) shows that JA clearly restored the expression of *chitinase 1* under diethyl ether. By contrast, *Dionaea* showed a rather weak non-significant response ([Fig. 5A](#)); however, at the 6 h time point, the increase was not statistically significant in the experiment depicted in Supplementary Data Fig. S3A. This result is consistent with the finding that *chitinase 1* expression increased earlier (somewhere between 2 and 6 h) than *dionain* (between 6 and 12 h, Supplementary Data Fig. S3) in response to mechanical stimulation, wounding and JA application.

DISCUSSION

In this study, we showed that during diethyl ether treatment, Venus flytrap plants could not sense their environment, and after ‘waking up’, did not ‘remember’ what occurred. This was shown by inhibition of APs and trap movement, an inability

FIG. 4. Gene expression in the air and under anaesthesia with diethyl ether in the Venus flytrap (*Dionaea muscipula*). The traps were kept in the air or under diethyl ether for 2 h and then mechanostimulated 40 times or pierced/wounded by needle for the next 2 h. The diethyl ether was then removed and trap tissue was sampled for qPCR after 10 h. Relative expression of *dionain* after (A) mechanostimulation and (B) wounding. Relative expression of *chitinase* after (C) mechanostimulation and (D) wounding. Gene expression for the non-stimulated control in the air was set as 1. Data show mean expression $\pm$ s.e. from four biological replicates ($n = 4$). Different lower-case letters denote statistically significant differences at $P < 0.05$.

to accumulate jasmonates and no induction of genes encoding digestive enzymes and thus no physiological response. Our observations resemble those in animals and humans where general anaesthesia suppresses central nervous system activity. The volatile anaesthetic halothane (halogenated derivative of ether) produced a concentration-dependent depression of AP amplitude accompanied by an increased spike half-width with complete inhibition at 3 vol.% in mammalian nociceptors (MacIver and Tanelian, 1990). Electrical signalling in the Venus flytrap was fully recovered in the range of minutes; a similar recovery period was recorded in mammal neurons (MacIver and Tanelian, 1990). During this period, more than two touches were necessary to induce rapid trap closure, supporting the summation of smaller subthreshold charges of APs necessary for trap closing reactions, consistent with electrical memory in the Venus flytrap (Volkov et al., 2008, 2009). Several previous studies have indicated that 'shy' plants (*M. pudica*) are also sensitive to anaesthesia. The leaf closing reaction after mechanical stimulus was inhibited by exposure to diethyl ether, halothane and lidocaine but not ketamine (Milne and Beamish, 1999; De Luccia, 2012; Yokawa et al., 2018). Although electrical signals were not investigated in these studies, it is tempting to assume that anaesthesia also inhibited electrical signals, because electrical signalling and rapid trap movements are tightly coupled (Fromm and Lautner, 2007).

Two hypotheses regarding the mechanisms of anaesthetic action in animals and humans have been proposed: the lipid (membrane) theory and protein (receptor) theory (Franks, 2008; Rinaldi, 2014), with several modifications (Lerner et al., 1997; Tang and Xu, 2002). Meyer (1899) and Overton (1901) concluded that solubilization of a lipophilic general anaesthetic in the lipid bilayer of the neuron causes its malfunction and anaesthetic effect. On the other hand, Franks and Lieb (1984) demonstrated that the relationship reported by Meyer (1899) and Overton (1901) could be reproduced using a soluble protein. Until now, many proteins have been shown to contribute to general anaesthesia. Among them are γ -aminobutyric acid type A receptor (GABA_A), glutamate gated *N*-methyl-D-aspartate (NMDA) receptors, and potassium and sodium channels (Mihic et al., 1997; Orser et al., 2002; Weir, 2006; Zhou et al., 2012; Herold and Hemmings, 2012.). Diethyl ether was shown to interact with GABA_A, NMDA receptors and the potassium channel TREK-1 in animals (Martin et al., 1995; Patel et al., 1999; Krasowski and Harrison, 2000; Zhou et al., 2012). However, the exact nature of general anaesthetic-protein interactions remains a mystery. Anaesthetics may bind to the preformed cavities on proteins by fitting into structurally compatible pockets (key-lock mechanism), causing structural perturbation to the protein channel. Volatile general anaesthetics may have not changed the structure of the membrane channel

FIG. 5. Gene expression in response to exogenous application of 2 mM jasmonic acid (JA) after 7 h in the air or under diethyl ether in the Venus flytrap (*Dionaea muscipula*). The plants were kept in the air or under diethyl ether for 2 h and then a few drops of JA were applied on the trap surface. The plants were kept in the same conditions for the next 7 h and the traps were then sampled for qPCR. (A) Relative expression of *dionain*. (B) Relative expression of *chitinase I*. Data show mean expression $\pm$ s.e. from four biological replicates ($n = 4$). Different lower-case letters denote statistically significant differences at $P < 0.05$.

by a key-lock mechanism but by changing its dynamics by becoming an integral part of amphipathic domains where they can either disrupt the association of the channel with its surroundings or facilitate the formation of structured water clusters within the protein (Tang and Xu, 2002). Another proposed explanation is a combination of the lipid and protein hypotheses: anaesthetics alter the cell membrane properties and may distort the channel protein to block channel function (Lerner et al., 1997; Andersen and Koeppel, 2007).

Surprisingly, similar proteins that are suspected as possible targets of the volatile anaesthetic diethyl ether in animals and humans have also been discovered in plants, where they are also responsible for electrical signalling. First, glutamate receptor-like proteins (GLRs) in plants are the most closely related proteins to NMDA channels in mammals. They even share similar extensive sequence identity and secondary structure (Lam et al., 1998; Weiland et al., 2016). GLR3.3 and GLR3.6 are Ca^{2+} channels that mediate the propagation of wound-induced electrical and Ca^{2+} signals in *Arabidopsis* from damaged to

undamaged leaves (Mousavi et al., 2013; Salvador-Recatalà et al., 2014; Hedrich et al., 2016; Toyota et al., 2018). Moreover, glutamate, which acts as an excitatory neurotransmitter in the vertebrate central nervous system, accumulates in response to wounding in *Arabidopsis*, and GLRs act as sensors that convert the wound signal into an electrical signal that propagates to distant organs where defence responses are induced (Toyota et al., 2018). Salvador-Recatalà et al. (2014) and Hedrich et al. (2016) extended these studies and found that APs triggered by cold water and wounding are not inhibited in local leaves of *glr3.3* and *glr3.6* double mutants. Therefore, the elicitation and propagation of APs is independent of GLR3.3 and GLR3.6 in plants, and they are only important for channelling the signal to neighbouring systemic leaves. However, systemic electrical signals were not found in the Venus flytrap (Pavlovič et al., 2017). These findings are consistent with the results of De Luccia, (2012), who found that ketamine, which mediates anaesthesia by blockade of the NMDA receptor in animals, had no effect on the trap closing reaction in the Venus flytrap and closing leaflets in *M. pudica*.

In addition to Ca^{2+} influx, which is important for the initial depolarization during AP generation, efflux of Cl^- accelerates depolarization with the subsequent K^+ efflux/influx needed for repolarization (Felle and Zimmermann, 2007). The anion QUAC1-type channels and AKT2/3 and SKOR/GORK-type K^+ channels have been proposed to be involved in AP generation in plants (van Bel et al., 2014; Hedrich et al., 2016). Indeed, AKT2 modulates tissue excitability and GORK shapes APs in *Arabidopsis* (Cuin et al., 2018). Diethyl ether activated another potassium channel, TREK-1, causing hyperpolarization of the membrane in mammals and inhibiting excitability (Patel et al., 1999; Peyronnet et al., 2014). The most closely related proteins in plants are TPK potassium channels, which are known to be involved in mechanosensing and controlling membrane potential (Becker et al., 2004). The third receptor that was suspected to be a target of diethyl ether anaesthesia in animals is the GABA_A receptor. GABA is the main inhibitory neurotransmitter in the central nervous system of vertebrates and exerts its inhibitory effect by activating Cl^- currents through the GABA_A receptor, hyperpolarizing the membrane and inhibiting excitability. GABA_A receptor function is allosterically enhanced by diethyl ether and its halogenated derivatives (Krasowski and Harrison, 2000). Decades ago, GABA was shown to rapidly accumulate in plant tissue in response to different biotic and abiotic stresses, but its receptor was unknown. Recently, the first GABA receptor was found in plants and identified as aluminium-activated malate transporter (ALMT, Ramesh et al., 2015; Žárský, 2015). Activation of ALMT results in depolarization of the membrane, and inversely, GABA inhibition results in hyperpolarization of the membrane potentials, generating a state of low excitability (Žárský, 2015). Although the outcome is surprisingly similar to the effect of GABA on animal neurons, there is no sequence homology to the GABA_A receptor except for the small region responsible for the GABA interaction (Ramesh et al., 2015). This finding decreases the probability that diethyl ether may have the same effect on two unrelated proteins, even if it is only a positive allosteric modulator. As we lack exact data that would allow us to identify the molecular bases underlying the initiation and propagation of

APs in plants, it is impossible to identify the protein target of an anaesthetic on electrical signals in plants. Either other molecules represent targets of diethyl ether in plants or the membrane theories proposed by Meyer (1899), Overton (1901) and Lerner *et al.* (1997) are relevant in the case of plants as we supposed also in our previous study (Yokawa *et al.*, 2018).

There is an intriguing parallel to the effects of anaesthetics on animals and humans. Anaesthesia induces loss of responsiveness to environmental stimuli as well as loss of pain perception during surgical operation. Pain sensing in humans results from the action of prostaglandins on peripheral sensory neurons (nociceptors) and on central sites within the spinal cord and the brain (Funk *et al.*, 2001; Ricciotti and FitzGerald, 2011). Tissue injury triggers cyclooxygenase-2 (COX-2) in peripheral tissue to convert arachidonic acid to prostaglandin E₂ (PGE₂), resulting in stimulation/sensitizing of the nociceptor in peripheral nerves to send a signal for pain to the central nervous system. The oxylipin pathway leading to prostaglandin synthesis in animals is mimicked in plants by a similar pathway that leads to the synthesis of jasmonates (Pan *et al.*, 1998). We do not claim that plants feel pain, but accumulate structurally similar molecules as warning signals. We believe that the suppression of jasmonate accumulation under anaesthesia is mediated by the inhibition of electrical signalling, which is tightly coupled to the JA response in non-carnivorous plants (Mousavi *et al.*, 2013; Toyota *et al.*, 2018) and carnivorous plants (Böhm *et al.*, 2016a; Bemm *et al.*, 2016). However, the mechanism of action differs strongly between animals and plants. Whereas the production of oxylipins in plants is mainly downstream from electrical signalling, in animals, it is upstream (prostaglandins sensitize nociceptors for pain). Thus, under anaesthesia, the warning signal (prostaglandins) in animals can be synthesized but is not sensed; in plants, the warning signal (JA) is not synthesized at all. Because of this, exogenous application of JA under anaesthesia can bypass inhibited electrical signalling in plants and trigger the response (Fig. 5). Although wounding still slightly increased JA and JA-Ile accumulation under anaesthesia in this study (Fig. 3I, J), this increase probably did not reach the threshold level for activation of gene expression. This slight increase after wounding but not mechanostimulation can be explained by disruption of cell walls and release of cellular contents and signalling molecules which may activate JA biosynthesis directly or indirectly (Ellis *et al.*, 2002; Mielke and Gasperini, 2019). A further increase of JA level is mediated by Ca²⁺ which derepresses JA biosynthesis genes, and accumulated JA-Ile may then transcriptionally activate expression of JA biosynthesis genes by a positive feedback mechanism (Yan *et al.*, 2018; Wang *et al.*, 2019). Ca²⁺ is involved in the generation of APs in Venus flytrap, and its increased level in the cytoplasm was detected after the third AP (Hodick and Sievers, 1988; Krol, 2006; Escalante-Pérez *et al.*, 2011). Therefore due to the inhibited APs, Ca²⁺ does not enter the cytoplasm, and this stage of JA accumulation is probably blocked under anaesthesia.

In conclusion, we have shown that the carnivorous plant Venus flytrap cannot sense its environment during anaesthesia with diethyl ether. This situation resembles the effect of general anaesthesia on animals and humans, resulting in a total lack of sensation. After removing the anaesthesia, the recovery of sensitivity is very fast. We have shown that one of the many possible

targets of anaesthetics are electrical signals (APs), which affect the later reactions, such as generation of warning signals (JA) and transcription of JA-responsive genes. Because jasmonates are important stress hormones that redirect gene expression from growth to defence (Pauwels *et al.*, 2009; Attaran *et al.*, 2014), the use of anaesthesia during vegetative propagation and plant manipulation in horticultural practice may be plausible, although more experimental studies with non-carnivorous plants are needed. The fact that anaesthesia inhibits electrical signal propagation not only in animals but also in plants, and that in both it affects their sensibility indicates a remarkable similarity between animals and plants.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Figure S1: Timeline of experimental set-up for diethyl ether treatment. Figure S2: Comparison of action potentials triggered on the same plant by mechanostimulation and 200 s later by wounding. Figure S3: Time course of gene expression in response to mechanostimulation, wounding and external application of jasmonic acid over 48 h in the Venus flytrap. Figure S4: Protein profile and immunodetection of cysteine protease and VF-1 chitinase in the digestive fluid of the Venus flytrap after 48 h. Figure S5: Recovery of gene expression after anaesthesia in the Venus flytrap. Table S1: Primer sequences and their properties for the Venus flytrap. Movie S1: Mechanical stimulation of trigger hairs twice results in rapid trap closure in the Venus flytrap. Movie S2: The trap remains open after repeated mechanical stimulation of trigger hairs under anaesthesia with diethyl ether in the Venus flytrap. Movie S3: 700 s after removing of diethyl ether the trap reaction to mechanostimulation is restored in the Venus flytrap. Movie S4: Wounding the trap by needle triggers rapid trap closure in the Venus flytrap. Movie S5: The trap remains open after wounding under anaesthesia with diethyl ether in the Venus flytrap. Movie S6: 700 s after removal of diethyl ether the trap reaction to wounding is restored in the Venus flytrap.

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A.P. and F.B. designed the study, A.P. measured electrical signals, M.L. and B.B. performed qPCR, J.J., I.P. and O.N. performed phytohormone analysis, and A.P. wrote the manuscript and provided materials and financial support. All authors discussed the results and contributed to the manuscript. The authors declare no conflicts of interest.

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