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Research in Context

Anesthetics Stop Diverse Plant Organ Movements, Affect Endocytic Vesicle Recycling, ROS Homeostasis, and Block Action Potentials in Venus Flytraps

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• **Background and Aims** Anesthesia for medical purposes was introduced only in the 19th century. However, the physiological mode of anesthetic drug actions on the nervous system remains unclear. One of the deep mysteries is how these different compounds, with no structural similarities and even chemically inert elements such as the noble gas xenon, act as anesthetic agents inducing loss of consciousness. Our main goal is to find out if anesthetics affect the same or similar processes in plants, as it is known from animals and humans.

• **Methods** A single-lens reflex camera was used to follow organ movements in plants before, during, and after recoveries from their exposures to diverse anesthetics. Confocal microscopy was used to analyze endocytic vesicle trafficking. Electrical signals were recorded using a surface AgCl electrode.

• **Key Results** Mimosa leaves, pea tendrils, Venus flytraps and sundew traps – all lost both their autonomous and touch-induced movements after their exposures to anesthetics. In Venus flytrap, this was shown to be due to the loss of action potentials under diethyl ether anesthesia. The same concentration of diethyl ether immobilizes pea tendrils. Anesthetics impede also seed germination and chlorophyll accumulation in cress seedlings. Endocytic vesicle recycling and reactive oxygen species (ROS) balance, as observed in intact Arabidopsis root apex cells, was also affected by all anesthetics tested in our study.

• **Conclusions** Plants are sensitive to several anesthetics with no structural similarities. Similarly as in animals and humans, anesthetics used at proper concentrations block action potentials and immobilize organs via effects on action potentials, endocytic vesicle recycling, and ROS homeostasis. Plants emerge as ideal model objects to study general questions related to anesthesia, as well as to serve as a suitable test system for human anesthesia.

Introduction

The use of ether for medical purposes, such as anesthesia, was introduced and first described in 1818 by Michael Faraday, famous for his work on electromagnetic fields (Bergman, 1992). In 1846, its utility was first demonstrated during a surgical procedure to remove a tumor from the neck of a patient that had inhaled ether vapor, painlessly. Before that, “surgery” and “pain” were synonymous (Rinaldi, 2014). Many different chemicals have been found to induce anesthesia. These include diethyl ether, chloroform, halothane, isoflurane and xenon. In the current pharmaceutical market, an enormous amount of anesthetic drugs are being produced industrially. However, despite the fact that many anesthetics have been used over a 150-year period, the exact mode of anesthetic drug action on the animal nervous system is still controversial. One of the deep mysteries is how these different compounds with no structural similarities, even chemically inert elements such as xenon (a noble gas), behave as anesthetic agents inducing loss of consciousness (Cullen and Gross, 1951; Lawrence *et al.*, 1946; Turin *et al.*, 2014; Sonner and Cantor, 2013; Sonner, 2008; Pauling, 1961). In the early history of anesthesia research, the theory of the Meyer-Overton correlation was proposed (Meyer, 1899; Overton, 1901). It explains that the magnitude of anesthesia of different compounds correlates well with their lipid-solubility. Nowadays, this ‘general’ theory has been abandoned and researchers are attempting to find out specific receptors or neurons responding to anesthetics (Franks, 2008). Remarkably, anesthesia extends to plants. Claude Bernard demonstrated that the sensitive plant, *Mimosa pudica* L., was unresponsive in closing leaves upon touch under diethyl ether. He arrived at a conclusion: plants and animals must share a common biological essence that is disrupted by anesthetics (Bernard, 1878; Perouansky, 2012;

Grémiaux *et al.*, 2014). After the work by Claude Bernard, many plant physiologists reported similar effects of anesthetics on plants (Bancroft and Rutzler, 1931; Bübbing, 1934; Perouansky, 2012; De Luccia, 2012; Grémiaux *et al.*, 2014). However, it is still not known if effects of anesthetics in plants are related to plant action potentials and which cellular processes are affected by these compounds in plant cells.

Relevance of anesthetics for plants is obvious also from the fact plants generate their own endogenous anesthetics such as ethanol, divinyl ether and ethylene, especially when under stress (Stumpe *et al.*, 2008; Fammartino *et al.*, 2010; Finer, 1965; Dillard, 1930). For example, Kimmerer and Kozlowski (1982) reported that stressed pine and birch seedlings, as well as eleven other analyzed plants, release ethylene, ethane, acetaldehyde, and ethanol; all having anesthetic actions in animals and humans (Koppanyi, 1945; Eger and Laster, 2001). In fact, plants emit huge amounts of low molecular mass hydrocarbons and other volatile compounds (Sharkey, 1996; Niinemets *et al.*, 2004; Loreto *et al.*, 2006), and many of these compounds have anesthetic properties (Eger and Laster, 2001; Baluška *et al.*, 2016). Interestingly, plants release volatile compounds not only to cope with stress but also to perform better in plant competition, many acting as allelochemicals (Kegge and Pierik, 2009). Plants produce both general and local anesthetics such as diverse alkaloids and flavonoids, menthol, cocaine, atropine, monoterpenes, and phenylpropanes (Ghelardini *et al.*, 2001; Ruetsch *et al.*, 2001; Facanha and Gomes, 2005; Watt *et al.*, 2008; de Lima Silva *et al.*, 2013; Behcet, 2014; Tsuchiya, 2017). We can expect that this list will get much longer in future. Recently,

dozens anesthetics and anesthesia-related compounds isolated from plants were reviewed, including essential oils, terpenoids, and alkaloids (Tsuchiya , 2017).

In addition to plant stress adaptation, other plant processes are known to be under control of endogenous compounds having anesthetics actions. Ethanol and other anesthetics overcome dormancy of *Panicum dichotomiflorum* and other plant species seeds and this effect can be reversed by pressure of > 1 MPa during the seed exposure to these anesthetics (Taylorson and Hendricks, 1979, 1980). Moreover, secondary dormancy induction via high temperature incubation of giant foxtail (*Setaria faberi*) seeds was prevented by anesthetics (Taylorson, 1982). The pressure reversal of anesthetics impacts on plants was confirmed also in their effects on lipid composition in barley root cells (Jackson and John, 1984). As in cells of animal and humans, anesthetics fluidize membranes and pressure reverses these impacts. This feature strongly suggests that anesthetics induced genuine anesthetic actions on plants as pressure is well-known to reverse all anesthetics actions, including loss of consciousness, in animals and humans (Johnson and Miller, 1970; Włodarczyk *et al.*, 2006; Heimburg and Jackson, 2007; Græsbøll *et al.*, 2014).

In the present study, we have used a wider range of plants as in studies published until now. We have shown that anesthetics stop both action potentials and plant movements. Moreover, anesthetics tested affect endocytic vesicle recycling and ROS homeostasis. These results suggest that the action of anesthetics is similar in plants and animals and that they target some general molecules and/or processes related to cellular membranes rather than specific

receptors. Finally, anesthetics affect also plant-specific aspects such as chlorophyll accumulation and seed germination.

Materials and Methods

Plant Materials

Sensitive plant (*Mimosa pudica* L.), Venus flytrap (*Dionaea muscipula* Ellis), Cape sundew (*Drosera capensis* L.), pea (*Pisum sativum* L.) and garden cress (*Lepidium sativum* L.) were obtained from a local garden store. These plants were maintained in a growth chamber at 23°C several days before the anesthetic experiments. Arabidopsis (*Arabidopsis thaliana* L.) Columbia wild type (Col-0) seeds were soaked in a sterilizing solution containing 12% sodium hypochlorite and 0.1% Triton X-100 for 15 min and washed at least five times with sterile distilled water. Sterilized seeds were planted on a 0.4% phytigel-fixed half-strength Murashige-Skoog (MS) growth medium without vitamin B. Petri dishes were incubated vertically at 23 C° under 16 hours/ 8 hours light and dark cycle.

Impacts of Anesthetics on Plant Movements

These plants were acclimated in a glass chamber under a fume hood. A certain volume of diethyl ether (Carl Roth GmbH, Germany) reaching 15% of vapor was poured into a small beaker, and *Mimosa* and *Dionaea* were treated for 1 hour in the sealed glass chamber. The same procedure was made for the pea plant experiment. The volume of diethyl ether was approximately calculated using ideal gas constant (standard state of gas as 22.4 L/mol). For instance, to obtain 15 % of vapor in 1 L-size of test chamber, liquid phase of 700 µL diethyl

ether (74.12 g/mol, 0.71 g/cm³) was poured in beaker and allowed to evaporate inside the sealed glass chamber. There are no toxic impacts of this concentration of diethyl ether, as well as of the other anesthetics used, and the effects are fully reversible after their removal.

For the lidocaine experiment, *Mimosa* root was gently washed to remove soil and cultured in water-filled Erlenmeyer flask for several days for adaptation to hydroponic growth condition. A one percent lidocaine hydrochloride monohydrate (Sigma-Aldrich, Germany) solution was replaced with water in the flask, and only the roots of *Mimosa* were treated for 4 hours. Leaves of *Mimosa* were stimulated with a paintbrush by stroking along petioles. Trigger hairs in traps of *Dionaea* were touched at least twice with the tip of the metal needle. The movement of trap was recorded with a single-lens reflex camera (Canon EOS Kiss X7i, Japan). The sequences of plant responses were followed in same individuals. The responses of anesthetics-treated plants were observed at least three times and the representative pictures and movies are shown here.

The Cape sundew plants (*Drosera capensis* L.) were enclosed in a small jar (5L volume) in an atmosphere of 15% diethyl ether. At the same time the control plants were enclosed in another jar without diethyl ether. After one and half hours, 3 crushed dead fruit flies (*Drosophila melanogaster*) were put on individual sticky adhesive traps of sundew in control and diethyl ether treated traps. In this way, 4-5 traps were fed on the same plant. We used dead crushed prey because in a diethyl ether atmosphere, the flies were anesthetized (they do not move and do not provide mechanical stimuli) in comparison to control plants. The plants were kept for another 3 hours in an atmosphere of diethyl ether. The effectiveness

of the anesthetic was observed and quantified as tentacle and trap bending reaction. After 3 hours, the diethyl ether was removed to observe speeds of recoveries after treatments.

Measurement of Action Potentials

Venus flytrap plants were incubated in 15% diethyl ether for 2 hours in polypropylene bag with attached electrodes inside. The bag was then opened and the trigger hair was touched repeatedly every 100 seconds. The action potentials 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 (VUP, Prievidza, Slovakia) commonly used in electrocardiography. The reference electrode was taped to the side of the plastic pot containing the plant submerged in 1–2 cm of water in a dish beneath the pot. The electrodes were connected to an amplifier made in-house (gain 1–1000, noise 2–3 μV , bandwidth [-3 dB] 105 Hz, response time 10 μs , input impedance $10^{12} \Omega$). The signals from the amplifier were transferred to an analog-digital PC data converter (eight analog inputs, 12-bit-converter, $\pm 10 \text{ V}$, PCA-7228AL, supplied by TEDIA, Plzeň, Czech Republic), collected every 6 ms.

Effects of Anesthetics on Seed Germination and Chlorophyll Accumulation

Garden cress seeds were directly placed on damp double-layered filter paper with 4 mL of distilled water in 9 cm round-shaped petri dish. Lidocaine was dissolved in distilled water. For gaseous compounds, diethyl ether or ethyl vinyl ether was applied as liquid phase into a small container made from a lid of 1.5 mL eppendorf tube, and the petri dish was immediately

sealed to obtain 15% of vapor inside the dish. For xenon treatment, xenon (Sigma-Aldrich, Germany) and oxygen (Sigma-Aldrich, Germany) were premixed with the blend ratio of 80%: 20% in a 60 mL plastic syringe. The xenon gas was then insufflated into the tightly sealed Petri dish through silicon tube. The dishes were incubated for 24 hours at room temperature under continuous light condition. For recovery experiment, the anesthetic compounds were removed either by exchanging air or distilled water. For chlorophyll extract, the leaf samples were collected from the 2-days-old anesthesia treated-seedlings (24 hours anesthesia treatment followed by 24 hours incubation). The sample was weighed and ground in 1.5 mL of cold acetone. Crude extract was centrifuged at 10,000 x g for 5 min. The supernatant was collected and mixed with 2.5 mM K-phosphate buffer (pH 7.4) to obtain 80% of acetone-extract solution. Absorbance of the extracted solution was measured at the wavelength of 663.6 nm and 646.6 nm with spectrophotometer. The content of chlorophyll *a* and chlorophyll *b* in each sample was calculated using the equations by Porra *et al.*, 1989).

Histochemical ROS Staining

Nitro blue tetrazolium salt (Sigma-Aldrich, Germany) was dissolved in 0.1 M Tris-HCl buffer (pH 9.5) with 0.1 M NaCl and 0.05 M MgCl₂ to obtain 5 mM of stock solution. For maize and Arabidopsis roots, 500 μM and 5 mM of NBT was used for staining, respectively. Roots were treated with xenon (Xe 80% : O₂ 20%) or 15% diethyl ether or 1% lidocaine (w/v) in the same manner as described above for 1 hour at room temperature. The samples were then incubated in NBT solution for 15 min prior to microscopic observation.

Effects of Anesthetics on Endocytic Vesicle Recycling in Root Apex Cells

Five days after germination, *Arabidopsis* seedlings were stained with 4 μ M of N-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide (FM4-64, Sigma-Aldrich, Germany) dye dissolved in distilled water for 10 min. Seedlings were then rinsed and soaked in either 0 or 1% lidocaine solution for 30 min. For gaseous anesthetic treatments, seedlings were placed back into 1/2 MS plates after FM4-64 staining and treated. For diethyl ether treatment, approximately 25 μ l of diethyl ether was dropped onto the surface of phytigel and the lid of petri dish was quickly closed to obtain 15% of diethyl ether vapor. For xenon treatment, xenon and oxygen were mixed with the blend ratio of 80% : 20% in a 60 mL syringe. The xenon gas was then insufflated into the tightly sealed Petri dish with seedlings. In total, 200 mL of gas was used for each dish. The treatments of diethyl ether and xenon were conducted for 30 and 90 min, respectively. After the treatment with those anesthetics, all seedlings were treated with 35 μ M Brefeldin A (BFA, Sigma-Aldrich, Germany), which blocks endocytic vesicle recycling (Lippincott-Schwartz *et al.*, 1991; Baluška *et al.*, 2002), for 30 min.

Confocal Microscopy

Images of FM4-64 stained BFA-treated cells were taken by confocal laser microscopy (Fluoview FV1000, Olympus, Germany). The FM4-64 was excited at 545 nm by He-Ne laser. The fluorescence emissions were collected between 630 and 700 nm. The summation of the BFA-compartments areas visualized with FM4-64 fluorescence in unit area (50 μ m² square) was calculated from the confocal image of root epidermal cells. The square contains

about 20 - 30 BFA compartments. They were then averaged from independent root samples with ImageJ software (Mac OSX version 1.43r).

Statistical Analysis

For the statistical analyses of the quantification of chlorophyll contents (Fig. 3B) and BFA-compartment size (Fig. 4D), *p*-values were calculated with two-tailed Student's *t*-test.

P-values less than 0.05 were considered significant.

Results

Effects of Anesthetics on Plant Organ Movements

Diethyl ether vapor immobilized the leaf-closing reaction of sensitive plants *Mimosa pudica* L. (Fig. 1A). The leaf closure was observed by gently stroking the petiole with a paintbrush (Video S1). After 1 hour of 15% diethyl ether treatment, the *Mimosa* plants completely lost their response to touch stimuli (Video S2). Once diethyl ether was removed by exchanging the air in a treatment chamber, leaf response was gradually recovered, and returned to normal 7 hours after removal of diethyl ether (Video S3).

Another moving plant, Venus flytrap (*Dionaea muscipula* J. Ellis), was also introduced in order to monitor the effect of diethyl ether. As shown in figure 1B, control plants closed the leaf trap after stimulation of the trigger hair inside the leaves 2-3 times (Video S4). One hour of 15 % diethyl ether treatment completely abolished the response even though trigger hairs

were stimulated many times (Video S5). The response recovered 15 min after the removal of diethyl ether (Video S6).

Cape sundew plant (*Drosera capensis* L.) is a well-known carnivorous plant that captures its prey by moving leaves covered with sticky tentacles. As shown in figure 1C, all leaves tested in the control plants have a strong leaf and tentacle bending reaction within 1 hour in response to being fed crushed dead fruit flies. On the contrary, leaves treated with 15% of diethyl ether showed no bending reaction. The reaction was not merely slowed down but it was completely inhibited. After the removal of diethyl ether, the leaf bending reaction was recovered within a few hours (not shown).

In normal conditions, growing pea tendrils show a rotating trajectory in free space (Fig. 1D and Video S7). When the pea plants were exposed to 15% of diethyl ether, tendrils completely stopped their autonomous circumnutations immediately and were immobilized in a curled shape (Video S8). Interestingly, the application of a 1% lidocaine solution only to the root part (local anesthesia) of sensitive plant leaves also abolished the response to touch stimulus after 5 hours of treatment (Video S9). Leaf responsiveness recovered 17 hours after the solution was replaced with distilled water (Video S10).

Loss of Action Potentials in Trigger Hairs of Venus flytrap under Diethyl Ether

15% of diethyl ether treatment completely attenuated action potentials in response to trigger hair stimulation (Figure 2A). After removal of diethyl ether, the amplitude of recorded

action potential gradually recovered (recorded every 100 seconds) and returned to the normal state after 900 s. Importantly, this recovery time of action potential is remarkably consistent with the observation of leaf movements previously shown in figure 1B.

Anesthetics Induce ROS Exaggerated Production in Arabidopsis and Maize Root Apices

We used the NBT histochemical staining procedure for detecting superoxide production in *Arabidopsis* root apex. The treatment of roots with diethyl ether for 1 hour promoted exaggerated generation of superoxide in the meristem and the root apex transition zones (Figure 2B). Similar effects on high ROS productions were scored also in maize root apices under the lidocaine and xenon 1 hour exposures. The purple-blue color represents the area of superoxide generation (Figure 2C).

Anesthetics Inhibit Dormancy Breaking and Chlorophyll Accumulation

Intriguingly, seeds of garden cress (*Lepidium sativum* L.) failed to break dormancy under the treatment of anesthetics. Seeds were incubated on moist filter paper under continuous light. Petri dishes were tightly sealed and filled with individual treatments of 15% diethyl ether, 15% ethyl vinyl ether or 80% xenon with 20% oxygen. For lidocaine treatment, filter paper was moistened with 1% lidocaine solution instead of distilled water. As figure 3A shows, dormancy was prolonged for 24 hours under all the anesthetics whereas the mock treatment showed seed germination. Once the anesthetics were removed, all seeds broke their dormancy over the following 24 hours. Cotyledons of germinated seedlings treated with anesthetics exhibited a distinct yellowish color. The treatment of diethyl ether, lidocaine and xenon

reduced the contents of chlorophyll in leaves (Fig. 3B). These results suggest that anesthetics here used impede dormancy breaking and chlorophyll synthesis and/or assembly in the thylakoid membranes. As anesthetics impair mitochondrial functions too (Sanchez *et al.*, 2011; Boscolo *et al.*, 2012), these compounds might also have negative effects on the formation of thylakoid membranes of chloroplasts.

Anesthetics Affect Membrane Dynamics and Vesicle Trafficking in Root Apex Cells

It is currently proposed that many anesthetics are likely to interfere with lipid membranes. We have checked the effect of anesthetics on *Arabidopsis* root cells in terms of membrane trafficking, which is based on an elaborate maintenance of cellular membrane dynamics. 15% diethyl ether and 1% lidocaine slowed the rate of endocytic vesicle recycling, a process that was, in particular, completely diminished in the presence of diethyl ether (Fig. 4A, B). On the contrary, xenon treatment enhanced the formation of BFA-induced compartments in root epidermal cells. Although the mechanism is still unclear, these results indicate that anesthetics alter normal membrane properties and vesicle trafficking in plant root cells.

Discussion

The fact that plant cells responded to these compounds in a similar manner as animals and humans is very intriguing. It has previously been demonstrated that *Mimosa* plants lose their leaf-closing response to touch stimulus under a lidocaine and xenon exposures (Weigl, 1968; Milne and Beamish, 1999). Importantly, as we show here, it is enough to expose only roots of *Mimosa* seedlings to lidocaine to block the movements of their shoots. Plant sensitivity

to anesthetics might help to reveal still elusive mechanisms of their actions. It is still really puzzling how such chemically and physically diverse compounds; including chemically inert gas xenon, volatile organic solvent ether, and water soluble molecule like lidocaine; could all induce very similar impacts on both plants and animals.

Our present data not only expand plant systems tested for anesthetics by using pea tendrils, seeds of garden cress and cape sundew leaf traps; but also shows for the first time that the immobilization of plant organ movements is based on inhibition of action potentials. In other words, as in animal and humans, bioelectricity and action potentials animate not only humans and animals, but obviously also plants. In Venus flytrap, action potentials are also necessary to close the trap and to initiate the digestive processes (Böhm *et al.*, 2016). The number of action potentials is translated via gland cells into the touch-inducible jasmonate signaling that promotes the formation of acidic secretory vesicles which drive development of this ‘green stomach’ (Scherzer *et al.*, 2017; Pavlovič *et al.*, 2017). The ultimate prediction from our present data is that anesthetics will prevent also the Venus flytrap closure and its subsequent transformation into the ‘green stomach’. The fact that not only animals/humans but also plants are animated via action potentials is of immense importance for our ultimate understanding of the elusive nature of plant movements and plant-specific cognition / intelligence based plant behavior (Pollan, 2013; Gagliano, 2014; Gagliano *et al.*, 2016; Trewavas 2016, 2017; Baluška and Levin, 2016; Gross, 2016; Calvo *et al.*, 2016; van Loon, 2016; Krausko *et al.*, 2017). It should be not surprising that plants are sensitive to anesthetics as they express and use similar critical proteins which are discussed as possible targets of anesthetics in animals

and humans, including glutamate and GABA receptors (Price et al., 2012; Ramesh et al., 2015; Chen et al., 2016; De Bortoli et al., 2016; Ramesh *et al.*, 2017; Weiland *et al.*, 2015; Žárský, 2015). Saltveit (1993) reported that anesthetics halothane and methoxyflurane reduced chilling injury in cucumber cotyledons, cucumber hypocotyls and tomato pericarp. The relative effectiveness of the anesthetics in reducing chilling injury was similar to their relative effectiveness in inducing anesthesia in animals and their relative lipid solubility.

Although there is a strong consensus that anesthesia results in loss of consciousness, it is still a mystery how different kinds of chemical compounds bring about the same anesthetic state. It is logical to expect that any cellular system that is affected or disrupted by an anesthetic compound must be important for maintaining neural activities. Extensive work has been performed to investigate specific receptors or mechanisms perceiving anesthetic compounds (Franks, 2008). However, there are still many controversies, for example, lipid (membrane) theory *versus* protein (receptor) theory (Rinaldi, 2014). One of main reasons making this problem difficult is the limited access to living tissues under anesthesia treatment. Here we showed that the sensitive plant, Venus flytrap and Cape sundew plants, as well as pea tendrils, were immobilized with diethyl ether. Under anesthesia treatment, Venus flytrap lost the ability to generate action potentials in response to touch; whereas pea tendrils stopped their autonomous searching movements and were immobilized in a curled stature. Xenon was effective at different levels of responses, such as seed germination, chlorophyll accumulation, ROS production and vesicle recycling. Importantly, also seismonastic movements of Mimosa leaflets are based on action potentials in pulvinus motor cells (Volkov *et al.*, 2010a,b). These

electrical signals in pulvinus motor cells are closely associated with the actin cytoskeleton and calcium signaling (Yao *et al.*, 2008; Kanazawa *et al.*, 2006; Visnovitz *et al.*, 2007). In future, patch clamp analysis of these excitable motor cells might be very useful. All these results suggest that critical molecules and/or lipids of membranes are targets of anesthetics. As in animals, they block action potentials due to their actions on membranes and their lipids also in plants.

Although the current mainstream prefers the receptor theory of anesthetics actions, the fact that all life can be anesthetized (Bernard, 1878; Wolfe *et al.*, 1998; Eckenhoff, 2008; Sonner, 2008; La Monaca and Fodale, 2012; Perouansky, 2012; Sonner and Cantor, 2013; Rinaldi, 2014; Baluška *et al.*, 2016) and the general validity of high pressure reversal of anesthesia speak strongly against this anesthetic receptor theory (Johnson and Miller, 1970; Taylorson and Hendricks, 1979, 1980; Wlodarczyk *et al.*, 2006; Heimbürg and Jackson, 2007; Græsbøll *et al.*, 2014). Physical action of anesthetics was also supported recently by Turin and co-workers (Turin *et al.*, 2014) who discovered connections between electron spin and anesthesia. It is possible that the ultimate target of anesthetics will be shown to be electronic structure of critical proteins embedded within lipid bilayers of membranes (Ueda *et al.*, 1977; Kamaya *et al.*, 1981; Heimbürg and Jackson, 2007; Booker and Sum, 2013; Græsbøll *et al.*, 2014). Anesthetics impact lipid bilayer thickness and mechanical properties, both of which are well known to control protein functions (Andersen and Koeppe, 2007). Especially lipid rafts, which have central roles in intracellular communication and signaling (Lingwood and

Simons, 2010; Simons and Sampaio, 2011; Head *et al.*, 2014; Sezgin *et al.*, 2017), emerge to be particularly sensitive to anesthetics (Morrow and Parton, 2005; Bandejas *et al.*, 2013).

Importantly, *Arabidopsis* is expressing homologue of the lipid raft organizer flotillin Flot1 which is involved in clathrin-independent endocytic pathway (Li *et al.*, 2012; Yu *et al.*, 2017) in root apex cells of *Arabidopsis* (Li *et al.*, 2012). Interestingly in this respect, lipid rafts are very abundant at the *Arabidopsis* root apex cross walls (Zhao *et al.*, 2015a,b), and are active in the endocytic vesicle recycling (Zhao *et al.*, 2015b), which is typical feature for this root apex zone (Baluška *et al.*, 2002, 2010; Baluška and Mancuso, 2013). *Arabidopsis thaliana* respiratory burst oxidase homolog D (RbohD) was reported to be localized to lipid rafts and we have shown that reactive oxygen species regulate endocytic vesicle recycling (Yokawa *et al.*, 2016). As our present data show that anesthetics inhibit endocytic vesicle recycling in *Arabidopsis* root apex cells and disrupt ROS homeostasis, one possible scenario would be that anesthetics target primarily the physical properties of membranes, especially lipid rafts. Consequently, this would then cause aberrant functions of membrane proteins and vesicle trafficking, inhibiting action potentials, and precluding plant organ movements.

In contrast to all other anesthetics used in our study, water soluble lidocaine is local anesthetic known to act in animals via inhibition of the non-selectively voltage-gated sodium channels (Bean *et al.*, 1983; Chevrier *et al.*, 2004; Fozzard *et al.*, 2011). Relevance of our data in this respect is still unclear and will await further experimental studies.

Arabidopsis model systems, with its excellent tools and mutant collections, represent an ideal model object in our endeavor to illuminate the elusive mechanisms underlying both anesthetic actions as well as the phenomenon of consciousness (Trewavas and Baluška 2011; Mashour and Alkire, 2013; Calvo *et al.*, 2017). As plants generally, but especially the model plant *Arabidopsis thaliana*, are very suitable to experimental manipulation (they do not run away) and allow easy electrical recordings; we propose them as ideal model objects to study anesthesia and to serve as a suitable test system for human anesthesia.

Author Contributions:

K.Y. and F.B. conceived the work and prepared the draft. K.Y. and T.K. conducted a majority of the experiments presented in the paper. A.P. performed the experiment using sundew plants and measured the action potential in Venus flytrap plants. S.G. recorded the time-lapse movie of the movement of Pea tendrils. M.W. conducted the treatment of diethyl ether and lidocaine. S.M. provided the apparatus necessary for xenon treatment and evaluated the procedures and results. All authors edited the manuscript.

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Figure Legends

Figure 1

Effects of a volatile anesthetic agent, diethyl ether, on plant movements.

(A) The leaf-closing movement of *Mimosa pudica* under 15% of diethyl ether. After 1 hour of treatment, leaves completely lost the response to touch stimuli. All leaves gradually recovered closure movement after 7 hours following the removal of diethyl ether. Arrows indicate closed leaves. (B) The rapid trap movement of *Dionaea muscipula* disappeared after 1 hour of 15% diethyl ether treatment. The arrow indicates the leaf stimulated. (C) Sundew plant (*Drosera capensis*) showed no prey reaction under 15% diethyl ether atmosphere. Arrows show normal trap bending reaction. (D) The movement of tendrils disappears with 15% diethyl ether. The movies are available as supplemental material.

Figure 2

Disappearance and recovery of action potentials in *Dionaea muscipula*, and the production of reactive oxygen species in *Arabidopsis* roots under anesthesia.

(A) Recovery of diethyl ether inhibition of action potential on leaves of Venus flytrap in response to trigger hair stimulation recorded every 100 seconds after removal of diethyl ether. (B) The NBT histochemical staining for detecting superoxide production in *Arabidopsis* root apex. The treatment with anesthetics promoted the generation of superoxide in between the meristem and transition zones. (C) The NBT staining in maize roots under lidocaine and xenon treatment. The purple-blue color represents the area of

superoxide generation. The black arrows indicate the position of strong NBT staining pattern. The representative pictures are shown from 7 – 9 of stained samples.

Figure 3

Inhibition of dormancy breaking and chlorophyll accumulation under anesthetics.

(A) The inhibition of cress seed germination under anesthetic treatment. For 24 hours, all anesthetics inhibited germination. Seed germination completely recovered 24 hours after removal of anesthetics. (B) The anesthetic treatment attenuates chlorophyll accumulation. Chlorophyll was extracted from the leaves of cress seedlings germinated after 24 hours of anesthetic treatment. The values are averaged from 5 independent treatments. The error bars represent the standard deviation. (*** $p < 0.001$; ** $p < 0.01$)

Figure 4

Anesthetics Modify Vesicle Recycling in *Arabidopsis* Root Epidermal Cells.

(A) 15% of diethyl ether for 30 min. (B) 1% Lidocaine for 30 min. (C) 80% Xenon mixed with 20% oxygen for 90 min. (D) A comparison of the size of BFA-induced compartments between mock and treated cells. The total area of BFA-induced compartments located inside a square of 50 μm^2 was calculated and averaged from 7-9 independent roots. The error bars represent the standard deviation. (*** $p < 0.001$; ** $p < 0.01$)