Behavioural and electrophysiological characterization of an unknown water soluble anaesthesia-inducing agent found in common adhesive tape

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Abstract

Immersion of a ubiquitously used brand of translucent pressure sensitive adhesive tape (TLPSAT), in normal water was found to confer anaesthetic-like effects to the resultant solution (TLPSAT A). We characterised this anaesthetic-like property by assessing its dose-dependence, behavioural effects and toxicity in zebrafish (*Danio rerio*) in comparison to other commonly used anaesthetics, namely 2-phenoxyethanol (2-PE) and tricaine methanesulfonate (TMS). Toxicity tests showed that it is comparable to TMS and safer than 2-PE in terms of mortality. We also observed differences in the nature of opercular movement due to the three compounds with differences in the power spectrum. Electrophysiological investigations using grasshopper (*Hieroglyphus banian*) as a model system led us to conclude that nicotinic acetylcholine (nACh), γ -aminobutyric acid (GABAergic) mechanisms, or voltage gated calcium (Ca^{2+}), sodium (Na^+) and potassium (K^+) channels in insects are not affected by this anaesthetic at the concentrations effective in fish. Electroretinography (ERG) measurement from honeybees (*Apis mellifera*) in the presence of the different compounds yielded abolishment of the receptor potential under TMS or 2-PE perfusion whereas all ERG components were preserved under TLPSAT A perfusion, indicating that the Ca^{2+} dependent channels and photo-transduction machinery in bees are not potentially affected by TLPSAT A. Thus, the unknown compound(s) in the TLPSAT aqueous extract likely induce(s) anaesthesia in zebrafish via a mechanism unlike that of TMS and 2-PE.

Keywords: Translucent Pressure Sensitive Adhesive Tape; Anaesthesia; zebrafish; local field potential; ERG; phototransduction; receptor potential; slow plasticity; Antennal lobe

Introduction

Anaesthesia and more specifically general anaesthesia, has been defined as a drug-induced, reversible state of unconsciousness in animals, characterised by immobility, sedation, hypnosis, akinesia and amnesia (Brown et al., 2010; Grasshoff et al., 2005).

Contrary to previous notions that anaesthetics follow a unitary mechanism of action, increasing evidence suggests that different classes and subclasses of anaesthetics have distinct molecular targets (Grasshoff et al., 2005). Over the last few decades, a repertoire of different ion channels have been implicated for their direct involvement in the molecular mediation of general anaesthesia, some of the most important of them being GABA_A, voltage-gated sodium (Na_v), TREK-1 potassium channels, acetylcholine and NMDA receptors (Grasshoff et al., 2005; Zhou et al., 2012).

In different planes of anaesthesia, the level of arousal of the organism and corresponding brain states change, mediated by changes in both activity of certain nuclei and cell types and the functional connectivity between them in different regions of the brain (Roth, 1979; Perouansky, 2007; Mashour et al., 2013; Leffa et al., 2016). Understanding how anaesthesia works in different model organisms at a cellular, molecular and circuit level thus becomes essential to find clues about the differential effects of anaesthetics on different species.

Our lab has serendipitously discovered that an aqueous extract of TLPSAT shows anaesthesia-like effects on zebrafish (*Danio rerio*). We did check with a variety of other adhesive tapes (another brand of TLPSAT, a brand of transparent pressure sensitive adhesive tape (TSPSAT), a brand of double sided tape, two different brands of insulation tapes) but we did not observe any effect on zebrafish. The exact composition of the TLPSAT tape is not available in the public domain and there are no reports of the anaesthetic properties of the compounds present in it. In this context, we set out to characterise the properties of the active agent mediating this anaesthesia-like effect using zebrafish, honeybees (*Apis mellifera*) and a grasshopper species native to South Asia (*Hieroglyphus banian*) as model systems. The implications of this study are twofold. First, it is an inexpensive product and a commodity used by a large number of people daily, meaning that people are in frequent contact with this active agent whose effects on humans are unknown. Second, the active compound might be novel or a known compound with unknown anaesthetising properties.

We set out to first characterise our solution in terms of its dose-dependent effects on induction and emergence times to and from a defined surgical plane of anaesthesia using TMS and 2-PE as comparisons. We also tested the comparative toxicity of our solution in two cases, immersion in solution and intubation-based perfusion. Behavioural differences during anaesthesia were also evaluated in terms of opercular movement frequency changes for all the agents used for comparison. The neurophysiological action of the active agent was also assessed by means of intracellular and local field potential (LFP) recordings from the grasshopper brain and electro-retinogram recordings from the honeybee.

Our results indicate that the dose-dependent effects of the agent are similar to those of TMS and 2-PE. The toxicological profile in terms of mortality rates was found to be similar to that of TMS and safer than that of 2-PE. Results of electrophysiological recordings targeting the olfactory pathway in grasshopper indicated that voltage-gated sodium channels (Na_v), voltage-gated potassium channels (K_v), voltage-gated calcium channels, or nicotinic acetylcholine (nACh) receptors homologous to that of vertebrates may not be mediating the pharmacological action of the active compound. Our ERG results revealed that TLPSAT A does not affect the ERG response and that all the ERG components were preserved as in normal saline perfusion whereas both TMS and 2-PE reliably abolished the receptor potential indicating that unlike TLPSAT A, they are potentially interfering with the phototransduction machinery. We are, as of now, unable to isolate the active compound due to the varied spectra observed in the results which made it quite impossible to predict the compounds present in the mixture.

Methods

Animal Housing and Maintenance

Indian wild caught adult zebrafish (*Danio rerio*) of mixed ages and sex and adult grasshoppers (*Hieroglyphus banian*) used for the experiments were kept at optimal conditions. The zebrafish of mixed ages and sex were kept in-house at the zebrafish facility, Dr. Reddy's Institute of Life Sciences, University of Hyderabad Campus. Glass fish tanks (18cm X 6cm X 12cm) were constantly bubbled with ambient air and were maintained at room temperature (~30°C). Habitat water was replaced at regular intervals and maintained at a pH of ~7.1-7.5. Fish were fed dry fish feed twice a day. Grasshoppers were bred in-house at the Centre for Neural and Cognitive Sciences and were maintained at relative humidity: 70%; temperature: 29°C and 14:10h light/dark cycle. Honeybees (*Apis mellifera*) were maintained in hive boxes within the University of Hyderabad campus and foragers were collected in the afternoon for subsequent experiments. All procedures and experiments involving zebrafish have been performed following prescribed guidelines with prior approval from the institutional animal ethics committee (DRILS/IAEC/KC/2019-2).

Preparation of Anaesthetic Agents

TMS (Sigma-Aldrich), 2-PE (SRL Chemicals) and TLPSAT solution were used for the experiments. Chosen dilutions of each agent were used to check for dose-dependence and behaviour. A stock solution of the TLPSAT aqueous extract was prepared in a beaker by putting ~5m of it, cut into pieces, in 100ml of habitat water. The beaker was covered with Parafilm and kept at 4°C for 24h, after which the tape pieces were removed.

Stages of Anaesthesia

Characterisation of the stages of anaesthesia was done according to the criteria mentioned in Collymore et al. (2014) and Matthews & Varga (2012) with minor modifications.

Induction times, emergence times and time taken for partial/complete loss of equilibrium were manually scored by analysing recorded videos. Induction time to reach level 5 anaesthesia is defined here as the time taken to reach shallow/no opercular movement, and loss of tactile and tail pinch responses. Induction time to reach level 4 anaesthesia is characterised by time to reach all level 5 parameters except tail pinch response. Emergence time has been defined as the time taken to regain normal swimming behaviour. Partial loss of equilibrium has been defined as the state in which the fish rests or swims laterally on its side after losing equilibrium. Complete loss of equilibrium is said to be achieved when the fish flips 180° with its ventral side upwards. Partial/complete loss of equilibrium was scored only if the fish maintained the respective state for >3 seconds.

LC-MS of TLPSAT solution

To prepare the TLPSAT sample for LC-MS, 5m of TLPSAT was directly immersed in 100ml ddH₂O and kept undisturbed except for intermittent stirring for ~24h after which the tape was discarded. The resultant solution was then slowly heated at ~50-55°C so that the water is vaporised and the active compound, if volatile, does not escape the solution. We also checked the efficacy of the solution after heating till 50% evaporation and observed that the anaesthetic effect is preserved and the fish recovered after anaesthesia. This showed that the active compound did not evaporate. After the final volume had reached ~1ml, we sent the sample for spectral analysis using an LC-MS system in the School of Chemistry, University of Hyderabad, India.

Survival Assay using TLPSAT A

Two groups (N=10) of zebrafish were taken. One group was transferred to a petri dish containing 40ml of stock concentration of TLPSAT A till stage 5 anaesthesia was reached, after which the fish were transferred to a habitat with water. Another group which acted as a control was sham-treated with habitat water in a petri dish and transferred to a different habitat with water. Both groups were kept under observation for 45 days to check for morbidity and mortality.

Protocols for toxicity tests

To check for acute toxicity effects in fish, two approaches were followed: one by direct immersion into the anaesthetic solutions and another where the fish were perfused with the agents for specific time periods using an intubation based system.

In the first approach, three sets of fish, one set each for TLPSAT A (N = 11), TMS (N = 4), and 2-PE (N = 6) were immersed one at a time in the respective solutions for 10min.

For the intubation-based perfusion test, two sets of fish were used for perfusion, one set each for TLPSAT A and TMS.

Dose-dependent effects of the anaesthetics

Fish were transferred from the home tank and placed in a Petri dish containing 40ml of the respective anaesthetic and on reaching stage 5 anaesthesia, were transferred to another Petri dish containing habitat water for recovery. In those cases where the fish did not reach Stage 5 anaesthesia, they were exposed to the solutions for a minimum of 10min before transfer to habitat water for recovery.

Concentrations of 300mg/L, 150mg/L, 75mg/L and 37.5mg/L were used for TMS and 0.2%, 0.1%, 0.05% and 0.025% were used for 2-PE respectively. For the TLPSAT A, the stock concentration

(5m/100ml habitat water) was used along with dilutions of 2, 4, 5, 6, 7, 8 DFs respectively. All cases were video recorded using a Sony Handycam for behavioural analysis. Mean $\pm$ SEM was computed for all the induction and emergence times. Sigmoid curves (expressed here as: $y = \frac{\gamma}{1 + e^{-\alpha(x - \beta)^\theta}}$, where x is the data, α , β are shifting and scaling parameters and γ and θ are the half-maximal dose and slope respectively) were fitted with the level 5 induction time data and the emergence times at all the dilutions in all groups wherever applicable.

Tracking opercular movements

The opercular movements of fish after level 5 anaesthesia was reached due to the three different compounds were tracked and analysed using NsysLab tracker, a video analysis programme developed in our lab (Miriwala et al., 2013). Each group (N=2) for X and X/2 concentrations of the three anaesthetics were taken for this analysis. 34 seconds of video after the fish had reached level 5 anaesthesia was used for analysis. Three areas of interest (AOIs) were defined: (i) Operculum, (ii) The body of the fish away from operculum and (iii) The background away from the fish. Means of the pixel values were calculated for these AOIs in each frame to get the respective time series for the AOIs. These time series data were further analysed to find the frequency components present in the operculum movement by using fast Fourier transform (FFT). The power was calculated in a window around the frequency of interest for each of the AOIs. We used a measure of power in the operculum as well as the body by normalising them with that of the background. X and X/2 data from each treatment group were collated (thus making the effective N=4 for each group) for the opercular power analysis.

Preparation of grasshoppers for electrophysiology

The procedure followed was as previously elucidated (Laurent and Naraghi, 1994; Singh and Joseph, 2019). In brief, grasshoppers were procured and restrained using electric tape on a plastic platform. The platform including the animal was placed on a layer of clay stuck to a petri dish. The antennae were threaded through thin PTFE tubing including removal of wings and limbs to minimise aberrant movements which can introduce artefacts in electrophysiological recordings. A wax cup was built around the head of the grasshopper so as to hold insect Ringer's solution (140 mM NaCl, 5 mM KCl, 5 mM CaCl₂, 4 mM NaHCO₃, 1 mM MgCl₂, 6.3 mM HEPES, pH 7.1; Laurent & Naraghi, 1994) which was applied at regular intervals after the cuticle was removed and superfused at a standardised rate during the recording process. The cuticle between the eyes was cut out and the fat bodies and air sacs beneath were carefully removed to expose the brain. The gut was removed by virtue of an incision made at the posterior end which was ligatured subsequently. A thin wire coated with a layer of wax was carefully positioned below the brain so as to elevate it and hold it still during the recordings. An optimal amount of protease was applied on the surface of the brain to de-sheath it for accessing the neurons.

Odour Delivery

Odour was presented to the antennae as previously elucidated (Brown et al., 2005). Dry air (~0.75L/min) was continuously passed to the antennae and odour was presented by means of a measured amount (~0.1L/min) of dry air passing into a vial containing the odour solution which was transferred due to the difference in pressure to another tube connected to the vial and leading to the PTFE tubing. 5ml of 1-hexanol (Sigma-Aldrich) at 10% dilution (in Mineral oil v/v) was used in this experiment, kept in a 30ml glass vial.

Electrophysiology

Extracellular recordings: Local field potential (LFP) was measured from the mushroom body (MB) calyx of the grasshopper brain *in vivo* using borosilicate glass capillaries filled with insect Ringer's.

Intracellular recordings: The projection neurons (PNs) of antennal lobe (AL) were targeted for the intracellular recordings from the grasshopper *in vivo*. 0.2M KCl-filled borosilicate glass capillaries that had an impedance of ~100M were used for the recordings in grasshopper.

The grasshopper was subjected to bath application of saline and TLPSAT A during the course of the experiment. LFP and intracellular activity in response to odour stimuli was first recorded under insect

saline and after the signals stabilised over a period of 15min, the saline was replaced with X concentration of TLPSAT A and recording was initiated after a period of ~2min of application. The naïve conditions were measured by recording after 15min of the last application of odour to check if the build-up of LFP oscillations with repeated presentation of odour is intact. Then the TLPSAT A was replaced by saline for 15min to wash out the anaesthetic. Again, a set of trials were recorded to check if the system recovered from the effects of anaesthetic if any.

Raster plots, peri-stimulus time histograms (PSTH), inter-spike interval histograms (ISI) and spike shapes were all computed using custom programmes written in MATLAB. The bin duration for the PSTHs was taken at 100ms, averaged over 10 sweeps and converted into spikes per second for each condition. The bin duration for the ISIs were taken at 10ms and averaged over 10 sweeps for each condition.

Electroretinography (ERG): *A. mellifera* foragers were collected from the hive boxes and cooled in a refrigerator at 4 °C for immobilisation for a brief duration and mounted in plastic Eppendorf tubes using adhesive tape to restrain them. A wax cup was made over the head but excluding the compound eye to be recorded from, so as to hold bee saline (37 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.4 mM KH₂PO₄, Ph 7.2) (Kirschner et al., [2006](#)) but not immerse the compound eye of interest. The cuticle was cut open to access the inside for perfusion. The preparation was placed in the electrophysiology setup and a glass microelectrode was placed, just touching the surface of the compound eye. The eye being recording from, received 1-s white light stimulus during each trial lasting 10s. Bee saline-filled borosilicate glass capillaries of impedance <10MΩ were used for the recordings. 2X, 600mg/L and 0.4% concentrations of TLPSAT A, TMS and 2-PE respectively mixed with bee saline were used for the set of ERG experiments. N=3 animals per experimental group were used.

Statistical analysis

All statistical analyses were carried out using MATLAB (Mathworks, MA) and Microsoft Excel.

Results

LC-MS results too noisy to be interpreted

It was not possible for us to interpret the LC-MS results because there was an abundance of peaks with different m/z ratios making it extremely difficult to predict the components present in the mixture. This data in essence suggests that what we are dealing with is a highly complex mixture of different compounds.

Rate of Induction to Level 5 Anaesthesia is comparable across agents

The dose-dependence of the rate of induction due to TLPSAT A is comparable with that of the other two anaesthetics used in this experiment (Figure 1). Induction times at X concentrations were not significantly different (One way ANOVA: $F(2, 15) = 3.55$, $p = 0.059$) but were significantly different at X/2 concentrations (One Way ANOVA: $F(2, 15) = 4.71$, $p = 0.029$). Multiple comparisons at X/2 concentrations showed a significant difference between the induction times due to 2-PE and TMS ($p = 0.04$) but not between those due to TLPSAT A and 2-PE or TMS. The detailed mean $\pm$ SEM values of induction and emergence times at all the concentrations are given in Table 1.

Dilutions of used agents	Induction Time (s)			Emergence Time (s)
	Partial Loss of Equilibrium (Level 3)	Complete Loss of Equilibrium (Level 4)	Loss of tail pinch response (Level 5)	
TLPSAT A (N=6)				
x	16.5 ± 1.76	18.17 ± 2.07	35.67 ± 2.64	319.83 ± 126.7
x/2	20.17 ± 2.33	29.67 ± 2.45	98.67 ± 12.35	162 ± 35.21
x/4	198.67 ± 16.6	228.17 ± 20.74	-	114.83 ± 15.37
x/5	381.5 ± 29.75	-	-	93.33 ± 21.45
x/6	>15 min (n=2)	-	-	-
TMS (N=5)				
0.3mg/mL	18.8 ± 2.75	23 ± 2.28	50 ± 9.54	202 ± 99.61
0.15mg/mL	31 ± 7.91	77 ± 18.52 (n=3)	103 ± 7.75	124.2 ± 10.56
0.075mg/mL	441 ± 136.19 (n=4)	662 ± 0 (n=1)	-	48 ± 23.69
0.0375mg/mL	-	-	-	-
2-PE (N=5)				
0.2%	9.6 ± 0.85	14.2 ± 3.46	28.2 ± 3.38	112 ± 16.95
0.1%	33.6 ± 7.49	37.4 ± 8.26	60.8 ± 9.56	164 ± 33.04
0.05%	43.4 ± 2.36	58 ± 9.52	-	115.8 ± 14.66
0.025%	-	-	-	-

Table 1. Table showing the mean ± SEM of the induction times to reach partial loss of equilibrium, complete loss of equilibrium and Level 5 anaesthesia including the emergence times for each wherever applicable.

Figure 1. Rate of anaesthesia induction in adult zebrafish using TLPSAT A, TMS and 2-PE. Colour coded '+', 'o', 'x' represent the rates of induction for individual animals for TLPSAT A (red), TMS (green) and 2-PE (blue).

Distinct nature of opercular movement due to the different agents

The power of the frequencies at around 2.5Hz is higher for TMS compared to either TLPSAT A or 2-PE. The power of ~4Hz frequencies when anaesthetised with TLPSAT A is higher than that with 2-PE whereas the power of ~2.5Hz frequencies are similar with either TLPSAT A or 2-PE (Figure 2(a-d)).

The frequency spectrum with TMS was much broader than that with 2-PE or TLPSAT A. A distinct peak at around 4Hz was quite prominent in case of TLPSAT A whereas a peak at around 2.5Hz was observed with 2-PE.

Figure 2. Nature of the opercular movement under level 5 anaesthesia is distinct with different anaesthetics. Nature of the opercular movement due to 2-PE (a.i-ii), TLPSAT A (b.i-ii), or TMS (c.i-ii). (a.i), (b.i) and (c.i) Mean pixel values of the colour-marked three different AOIs represented as a time series exhibit periodicity of opercular movement compared to that of the body and background with 2-PE, TLPSAT A, or TMS. (a.ii) FFT of the time series with 2-PE reveals that the power of ~2Hz and ~4.5Hz frequencies of the opercular movement are greater than that of the body and the background. (b.ii) FFT of the time series with TLPSAT A reveal the power of ~4Hz frequencies to be more than that of the other two AOIs. (c.ii) FFT of the time series for TMS reveal the power of ~2Hz frequencies to be more than that of the other two AOIs. The opercular movement in this case seems to exhibit a broad range of frequencies. (d) The normalised power of the opercular movement at around 4Hz (± 0.3 Hz) is higher than that of the body. It is the highest in case of TMS, moderately high with TLPSAT A and is the lowest with 2-PE. (f) The normalised power of the opercular movement at around 2.5Hz (± 0.3 Hz) is higher than that of the body. It is quite high with TMS and quite low with either 2-PE or TLPSAT A. (e, g) Normalised power of the body is minimal with any of the three agents at both around 4Hz and 2.5Hz frequencies. Note: Figures a-c are representative analyses of one fish each at each of the treatment conditions. N=4 for each treatment condition in figures d-g.

Survival with TLPSAT A is comparable to that with TMS or 2-PE in short-term exposure

The TLPSAT A-treated group showed similar mortalities compared to the sham (water)-treated group (Figure 3). This suggests that it is as safe as 2-PE or TMS, considering their individual toxicities (Neiffer & Stamper, 2009). This result suggests that a single exposure via immersion till induction to level 5 anaesthesia does not affect fish mortality compared to the control group. It does not indicate anything about potential long-term biochemical and physiological effects of the TLPSAT A but merely suggests that it is as safe as other commonly used anaesthetics when short-duration treatments are considered.

Figure 3. Survival curve with TLPSAT A shows minimal and similar mortality as the sham-treated group. Figure shows the number of fish (N = 10) surviving over 45 days after treatment.

TLPSAT A toxicity is comparable to that of TMS and is lower than 2-PE in terms of mortality

Tables show percentage mortality with each of the agents under two different methods: by immersion (Table 2) and perfusion (Table 3). The results suggest that while TLPSAT A is not as efficacious as TMS, it is safer than 2-PE. Although the efficacy of the TLPSAT A in long-term anaesthesia is not as robust as that of TMS, in principle, it is possible to keep the fish alive even at X concentration of TLPSAT A. This observed toxicity can also due to impurities and/or other compounds present in the solution acting directly or potentially exacerbating the effect of the unknown active compound(s) in the mixture. It is therefore necessary that the active compound is identified and isolated from the mixture before we can comment more on this.

Table 2: Toxicity Test with anaesthetics by immersion for 10min.

Agents Used (X/2 concentration)	Number survived	Mortality %
TLPSAT A (N = 11)	8	27.27
TMS (N = 4)	3	25
2-PE (N = 6)	0	100

Table 3: Toxicity tests with TLPSAT A or TMS by perfusion for 30min or 1h.

Duration of exposure to used agents	Survival for chosen concentrations			
	X	Mortality %	X/2	Mortality %
TLPSAT A				
1h	1 (n = 3)	66.66	2 (n = 5)	60
30min	2 (n = 4)	50	4 (n = 6)	33.33
TMS				
1h	-	-	3 (n = 3)	0
30min	-	-	3 (n = 3)	0

TLPSAT A does not dramatically change the nature of LFP or intracellular response in grasshopper

In the olfactory circuitry of the grasshopper, the projection neurons (PNs) of the antennal lobe are largely excitatory in nature, synapsing on to the inhibitory local neurons (LNs) via cholinergic synapses. LNs, in turn, form a feedback circuit where most LNs synapse on to PNs using GABAergic synapses. Both receive input from the olfactory receptor neurons (ORNs) and form excitatory synapses with the Kenyon cells (KC) of the mushroom body (Gupta & Stopfer, 2015). Previous extracellular recordings from the MB calyx have shown oscillations (~20Hz in locusts) to mediate odour recognition and discrimination which in turn is facilitated by synchronous spiking in populations of PNs and LNs (Laurent and Davidowitz, 1994; Bazhenov et al., 2001; Kay, 2015). Following these findings, studies in different model systems have shown that frequent exposure of a particular odour invokes oscillatory synchronisation of spiking in PNs which is followed by a brief period of relative quiescence (Stopfer, 2014). Immunohistochemical analyses of MBs of various insects suggest that acetylcholine (ACh) mediates neuronal transmission between the PNs and MB (Heisenberg, 1998). The LNs in locust and *H. banian* have been shown to be GABAergic in nature via immunohistochemical analyses (Singh and Joseph, 2019). The LNs exhibit Ca^{2+} spikes mediated by voltage gated calcium channels (Laurent and Davidowitz, 1994; Bazhenov et al., 2001; Kay, 2015).

Taking advantage of this well-characterised circuitry, we simultaneously recorded intracellular activity from the PN of the antennal lobe and LFP from the ipsilateral side of the MB calyx while odour (1-hexanol) was presented for 1s to check whether our solution affects the aforementioned circuitry in terms of the spectral changes in LFP, the firing properties of the PN at different time points, spike shape and nature of PN subthreshold activity.

Figure 4. Protocol for electrophysiology experiments showing the perfusion, odour application and analysis time points.

Figure 5(a-c). Nature of PN spiking in response to TLPSAT A. (a.i-iv.) Representative raw spike traces (1st, 5th and 10th trails) (top left), raster plots (top middle) and peri-stimulus time histograms (top right) with perfusion under saline after ~6min in saline block (i), TLPSAT A at 12min in the TLPSAT A block (ii), odour-naïve condition under TLPSAT A after 12min interval (iii) and odour-naïve condition under saline after 12min interval during the recovery phase (iv). **(b.i-iv.)** Inter-spike interval histograms with perfusion under saline (i), TLPSAT A (ii), odour-naïve condition under TLPSAT A (iii) and odour-naïve condition under saline (iv). **(c)** Colour-coded amplitude-normalised mean spike

shape for all the aforementioned conditions. (Scale bars/windows: pink, 1s of odour delivery; vertical black, 20mV)

The protocol was per the schematic shown in Figure 4. Bath application of our solution did not seem to have a dramatic effect on the grasshopper brain, as is observed in Figures 5 and 6. Intracellular activity of the PN also showed no prominent changes in the number of spikes elicited. The raw traces (top left) and their corresponding raster plots (top middle) across the four conditions (Figure 5.a.i-iv) were found to be more or less similar. No obvious differences were observed in the peri-stimulus time histograms (Figure 6.a.i-iv, top right). The inter-spike intervals (Figure 6.b.i-iv) approximately follow a Poisson distribution in both TLPSAT A and saline conditions, including in the naïve blocks suggesting that the PN response is unlikely to have been desynchronised over the different experimental conditions. The spike shape in all the conditions (Figure 5.c) was found to be similar except for some shifts that do not correlate with any particular treatment. Such shifts can be due to impedance changes during recording.

Figure 6(a-b). Nature of LFP from the calyx of the mushroom body with or without the application of TLPSAT A. (a) Representative traces of raw LFP during odour stimulus: (top-bottom) for saline bath (black) and TLPSAT A bath (green) during continuous odour application. **(b)** Average (8-10th trials) amplitude spectrum of 1.4s (initial 0.5-1.9 seconds of the recording) before (top) and 1.3s during stimulus presentation (initial 2.1-3.4s of the recording) (bottom) in each of the colour-coded conditions. (Scale bars: horizontal, 1s of odour delivery from 2-3s of each trail; vertical, 100 μ V)

The LFP frequency components recorded from the calyx of the mushroom body before or after the application of TLPSAT A in developed condition remained intact (Figure 6a-b). In the initial blocks under saline perfusion, the power spectrum shows a prominent peak around ~16Hz observed during odour stimulus. This is expected since oscillations in insects like locusts (Laurent & Davidowitz, 1994) and grasshoppers (Singh and Joseph, 2019) range around 16-30Hz after repeated exposure of a particular odour stimulus. This peak was still preserved after insect saline was replaced with TLPSAT A perfusion and exposed to it for ~2min. A larger sample size is needed to reach a definite conclusion.

In light of these results, a few conclusions can be reached. Studies probing this circuitry in locusts (MacLeod & Laurent, 1996) have shown that application of picrotoxin, a non-competitive GABA_A antagonist in the antennal lobe abolished LFP at the MB while not suppressing the PN response. Picrotoxin-resistant GABA_B response of PN was also found to be effectively abolished using CGP54626, a GABA_B antagonist in *Drosophila* (Wilson & Laurent, 2005). In light of these studies, one would expect the synchronous nature of PN firing and MB LFP to be affected if the mechanism of TLPSAT A is GABA-mediated. However, both the PN response and MB calyx LFP in the grasshopper did not seem to be affected in presence of TLPSAT A during frequent odour administration. This indicates that the compound's action may not be mediated by GABA_A receptors. The slow inhibition thought to be mediated by GABA_B receptors remained intact as seen from the intracellular traces in different conditions. Comparative analyses of mammalian and insect GABA receptors have shown them to be more or less similar in terms of function although some of them do differ pharmacologically with bicuculline administration (Lummis, 1990, Hosie et al., 1997; Buckingham et al., 2005). Studies in zebrafish have confirmed that propofol and etomidate, both being agonists of GABA-R, manage to almost completely silence intracellular and LFP activity till the removal of the agents (Du et al., 2018). Therefore, it is expected that the profound GABA-mediated neuronal silencing would have been observed in the case of the grasshopper as well. Second, we can conclude that conserved voltage-gated Na⁺ channels (Na_v) are not affected, because action potentials are not affected by application of TLPSAT A as indicated by the consistently similar spike shape across the different bath conditions. TMS has been found to preferentially inhibit Na⁺ conductance by increasing the threshold for depolarisation (Frazier & Narahashi, 1975; Attili & Hughes, 2014) and has also been used to anaesthetise invertebrates (Cooper, 2011) indicating a certain degree of conservation. If the action of our active compound was Na_v mediated, we would have observed significant changes in the neuronal firing patterns and spike shapes. Voltage-gated potassium channels (K_v) in the insect brain homologous to that of vertebrate species also do not seem to be affected due to the similarity in the spike shapes across the different bath conditions. ACh mediation of the agent's action is also unlikely since AL-MB, as well as ORN-PN and ORN-LN connections are largely mediated by nicotinic type ACh receptors (Gupta and Stopfer, 2015) and some significant changes in the neuronal activity patterns should have been observed if it was otherwise. Further, these conclusions would also imply that voltage-gated Ca²⁺ channels are also not involved since they mediate synaptic transmission between neurons and in our experiments the synaptic transmissions remained intact. However, whether the observed effects in zebrafish are mediated by AMPA or NMDA-R type receptors cannot be either inferred or speculated from these results since the assay does not allow us to check AMPA or NMDA-R mediated processes.

TLPSAT A does not affect the ERG unlike 2-PE and TMS

Visual transduction in invertebrates has been well-studied, especially in *Drosophila* where a number of mutants with visual defects have been identified using extracellular ERGs as a preliminary basis of identification (Heisenberg, 1970). Studies have also provided a framework for separating the ERG response into different components: the laminar potential, composed of the slow cornea-positive on transient and the cornea-negative off transient, and the receptor potential, the fast sustained negative potential mediated by the photoreceptor cells. Several studies have proved the G-protein-coupled phosphoinositide (PI) signalling pathway to be essential in phototransduction which culminates in phospholipase-C (PLC)-mediated Ca²⁺ influx inside the microvillar photoreceptors via the Ca²⁺-selective transient receptor potential (*trp*) and the non-selective *trp*-like (*trpl*) cation channels (Hardie, 1991; Hardie and Minke, 1992; Hardie & Juusola, 2015). However, in some species such as honeybees, inositol (1,4,5) triphosphate (InsP₃), which is a by-product of PLC-mediated hydrolysis of phosphatidyl-inositol (4,5) biphosphate (PIP₂), has been observed to play a more important role in inducing Ca²⁺ release from possibly intracellular Ca²⁺ stores called submicrovillar cisternae (SMC) containing InsP₃ receptors (Nasi et al., 2000; Hardie, 2001; Raghu et al., 2012). Nonetheless, the role of *trp* and *trpl* in honeybee phototransduction is still important as the process most likely involves both efflux and influx of Ca²⁺ (Nasi et al., 2000). Thus, by utilising the established role of Ca²⁺ in phototransduction, it is possible to further probe whether the mechanism underlying the observed

anaesthetic effects of TLPSAT A on zebrafish are Ca^{2+} mediated. We used *A. mellifera* as a model system to record ERGs under TLPSAT A perfusion *in vivo* along with the other anaesthetics, namely TMS and 2-PE.

Figure 7(a-b). Abolishment of receptor potential observed under TMS or 2PE but not under TLPSAT A. (a) Traces showing the ERG response under TLPSAT A (top), TMS (middle) and 2PE (bottom) (N=3 bees in each group). The ERG during the sequence of treatment, saline followed by the anaesthetic and then wash are indicated by red, green and blue respectively. Scale bars: horizontal, 1 s indicating when the light stimulus was on; vertical, 2mV. (b) Bar plots (Mean $\pm$ SD) including individual data points (N=3) showing the change in receptor potential under TLPSAT A (top), TMS (middle) or 2-PE (bottom) perfusion. TLPSAT A: Two way ANOVA: $F_{\text{perfusion type}_N}$: 2.97, $p = 0.162$. TMS: Two way ANOVA: $F_{\text{perfusion type}_N}$: 18.63, $p = 0.009$. Multiple comparisons between perfusion conditions showed that the receptor potential is significantly reduced under TMS perfusion ($p=0.01$) compared to under saline perfusion and that there is a significant recovery of the receptor potential when TMS was replaced with saline perfusion ($p=0.01$). 2-PE: Two way ANOVA: $F_{\text{perfusion type}_N}$: 16.21, $p = 0.012$. Multiple comparisons between perfusion conditions showed that the receptor potential is significantly reduced under 2PE perfusion ($p=0.011$) when compared to initial saline perfusion but does not significantly recover after saline wash. The post-treatment receptor potential is also significantly reduced compared to that under pre-treatment ($p=0.048$) after a 90-min wash. So recovery may be slower with 2-PE. * = $p < 0.05$.

TLPSAT A failed to elicit any observable changes in the ERG waveform where all the ERG components were preserved (Figure 7a-b, top panel). However both TMS and 2-PE significantly abolished the receptor potential while keeping the on- and off-transients intact (Figure 7a-b, middle and bottom panels). To the best of our knowledge, no previous studies have studied changes in the ERG waveform under these anaesthetics in bees. Our results, while not providing conclusive information regarding the mechanism of action of TLPSAT A, do show that the assay we describe can

functionally distinguish the physiological effects of such compounds. The results also suggest that both TMS and 2-PE can disrupt the phototransduction machinery. In summary, we report a serendipitous discovery of anaesthesia-like effects of a brand of Translucent Pressure Sensitive Adhesive Tape aqueous extract and in vivo characterisation of these effects.

Discussion

The findings from the above experiments suggest that safety and efficacy of TLPSAT A is comparable to that of 2-PE and TMS. The dose-dependence results point towards TLPSAT A acting like any other anaesthetic in terms of behaviour effects. The survival and toxicity results hint at the relative safety of TLPSAT A over 2-PE while not compromising on the robustness of the anaesthetic action. Further, electrophysiological investigations provide clues to exclude possible channels and receptors mediating the effects observed in adult zebrafish. The analysis of the nature of opercular movement hints towards possibly differential effects of each of the agents on respiratory behaviour. ERG results were clearly able to tease out a difference under TLPSAT A compared to TMS and 2-PE at the neurophysiological level, possibly pointing to a mechanism underlying its anaesthetising property.

The intracellular and LFP data recorded from the grasshopper, in essence, provide some insight regarding the possibility that GABA receptors might not be mediating the anaesthetising effect observed in zebrafish. However, the possibility of the effect being NMDA-R or AMPA-R-mediated could not be assessed in this study. With NMDA-R having a role in mechanisms of learning including formation and consolidation of memory (Collingridge, 1987; Xia et al., 2005; Li et al., 2009; Baez et al., 2018) it might well be possible that the active compound might affect these mechanisms if it turns out to be NMDA-R-mediated. However, TMS and 2-PE were not used in this experiment. 2-PE has been shown to have a very specific action on NMDA-R with apparently no observable non-specific interactions with other receptors *in vitro* (Müßhoff et al., 1999). To ensure the reproducibility of our results, more intracellular and extracellular recordings from the grasshopper brain need to be done.

The ERG results indicate that Ca^{2+} is not involved in mediating the anaesthetising action of the TLPSAT A. However, TMS and 2-PE effectively abolished the receptor potential, thus suggesting that they are involved in disrupting PI signalling either by directly/indirectly antagonising SMC InsP_3 receptors or by impeding Ca^{2+} influx in photoreceptors via the *trp* channels. In case of TMS, although it is known to be a Na_v antagonist, the abolishment of the receptor potential by TMS is unlikely because of Na^+ influx since *trp* and *trpl* channels are 100x and 4x more permeable to Ca^{2+} compared to Na^+ (Reuss et al., 1997; Hardie, 2001). In case of 2-PE, so far only NMDA receptors have been implicated as its main target and not much is known whether it affects other Ca^{2+} channels. Our results indicate it might affect *trp* channels and/or InsP_3 receptors as well, possibly by disrupting PI signalling, perhaps more strongly due to the bees' inability to optimally recover from 2-PE perfusion even after >90min as indicated by our results.

It was not possible to isolate the active compound using LC-MS alone. The results of LC-MS revealed that the solution is in fact a mixture of various compounds and that identification of the specific mass spectral signature of our compound of interest is not possible using this method alone without further fractionation. Therefore, the usage of separation techniques such as TLC and HPLC is necessary to fractionate the solution and identifying the peak(s) containing the active compound. Mass spectrometry of the fraction of interest might provide less noisy spectra which can potentially help us identify and isolate the compound for further analyses.

Recordings from the zebrafish nervous system are needed to further understand the underlying mechanism(s) which mediate this effect and how well it corresponds, if at all, to the mechanisms of other anaesthetics delineated in zebrafish. Currently, we do not know if this solution has anaesthetic properties in other vertebrates.

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