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A Single Hypoxic Event Ameliorates Pilocarpine Induced Hyperkinetic Movements in Planaria

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ABSTRACT

Intermittent hypoxia or hypoxia therapy is exposing an individual to oxygenation conditions that are below atmospheric levels in a planned or acute timeframe. Hypoxia therapy is a potentially novel therapeutic strategy for a variety of pathologies including: mitochondrial disorders, exercise training, and mild cognitive impairments. Mitochondrial dysfunction, hyperkinetic movements, and cognitive impairments are hallmarks of seizures and status epilepticus (SE). A seizure can be considered uncontrolled electrical activity in the brain and SE is a seizure lasting more than 30 minutes, or multiple seizures without regaining consciousness in between. We examined the possibility of using the Pilocarpine model for seizure like activity on brown planaria (*Dugesia tigrine*). Pilocarpine is a muscarinic acetylcholine receptor agonist capable of creating seizure related brain damage. We utilized 5 mM dosages of pilocarpine and then measured open field behaviour for 3 minutes. Mobility and aversive hyperkinetic movements were observed throughout the measurement phase. After exposure to 5 mM pilocarpine, the planaria displayed behaviours consistent with seizures (e.g. aversive hyperkinetic movements and decreased mobility). Additionally, we measured the effects of an acute hypoxic event on Planaria behaviour. We used 25% carbonated water to create a hypoxic environment for the planaria and then measured mobility and hyperkinetic movements for 3 minutes. We noted that exposure to the hypoxic environment produced no changes in behaviour. However, the aversive hyperkinetic movements produced with pilocarpine administration were completely absent when a brief (3 minutes) hypoxic episode followed the pilocarpine exposure ($p < 0.05$). Aversive behaviours remained present when the ordering of pilocarpine and hypoxia were counterbalanced. This ordering effect was consistent across 40 trials. Further evaluation of the pilocarpine seizure model and intermittent hypoxia on planarian behaviour is warranted.

1. INTRODUCTION

Hypoxia is a deficiency in the amount of oxygen reaching tissue that can be either generalized or limited to a specific region of the body [1]. Brain hypoxia occurs when there is a lack of oxygen at the level of the brain, and typically occurs because of: suffocation, choking, or an ischemic event. Previous studies have shown that brain hypoxia suppresses N-methyl-D-aspartate (NMDA) receptor responses in neonatal CA1 neurons of the hippocampus in rats [2]. The NMDA receptor subunit composition changes in response to seizure activity and may be responsible for the increase in excitability that often follows an initial seizure [3]. Individuals who experience seizures, or are diagnosed with epilepsy, have greater cognitive dysfunction than the general population [4]. Antiepileptic drugs (AEDs) are prescribed to those diagnosed with epilepsy. The action of AEDs results in global changes in excitation levels in the central nervous system and these changes often lead to cognitive and behaviour deficits [5]. More recently, it has been demonstrated that intermittent hypoxia, or hypoxia therapy, can be an effective therapeutic strategy for a variety of pathologies [6, 7]. Burtscher and colleagues [8] have illustrated the positive effects hypoxia therapy can have on elderly men's exercise tolerance, and Wang and colleagues [9] have demonstrated the powerful effects intermittent hypoxia therapy can have on treating individuals with mild cognitive impairment. The mechanisms by which these effects take place are still being elucidated. Lee and colleagues [10] have elegantly discussed the role of Hypoxia-inducible factor (HIF) in tissue protection and damage in acute and chronic exposure to hypoxic stress. The authors clearly outline potential cellular and downstream pathways as the targets of hypoxia therapy, identifying different pharmacological agents that could exacerbate or ameliorate the positive outcomes on different pathologies from hypoxia therapies.

Lee *et al.* [11] discussed the significant role adenosine plays in hypoxia signalling, where exposure to hypoxic stress triggers an increase in adenosine in the extracellular space which activates HIF activity. This process leads to a decrease in Adenosine signalling or inhibition of adenyl cyclase. Adenosine signalling is also highly associated with seizures [12], where increase in adenosine kinase (ADK) is implicated to act as an initiator and therapeutic target for spontaneous recurrent seizures. When ADK activity is high, there is an increase in seizures, but when ADK is low there appears to be resistance to seizures. Pairing these ideas together pointed to an acute episode of hypoxia as a potential therapeutic for seizure like events. To test this, we aimed to develop a planarian seizure model. To initiate a seizure like state in planaria (defined as high amounts of hyperkinetic movements), we administered Pilocarpine. Pilocarpine is a muscarinic acetylcholine agonist capable of producing seizures and cell damage that is similar in many respects to damage observed in the brain of a human epileptic patient [13]. Pilocarpine-induced seizures are initiated via muscarinic receptors and further mediated via NMDA receptors [14]. In animal models such as rats and mice, Pilocarpine has been systematically administered to induce epilepsy which can develop into temporal lobe epilepsy [15]. The common brown planaria, *Dugesia tigrina*, is a type of aquatic flatworm found in lakes, ponds, streams and springs where they occupy bottom habitats [16]. The advanced nervous system of the planaria is separated into distinct sensory and motor pathways [17]. Planaria express and utilize neurotransmitters and when exposed to specific drugs or stimuli, they display mammalian-like responses, such as a change in motility, enhanced stereotypical activity, behavioral sensitization and drug seeking and withdrawal [18]. Glutamate and aspartate are the major excitatory neurotransmitters involved in learning, memory, movement disorders, drug addiction, and other normal and abnormal physiological processes and behaviours [19] suggesting the potential involvement of these systems in seizure states. In planarian, acetylcholine causes hyperkinesia leading to prolonged tonic contraction causing an apparent shortening of the entire body length [20].

Here, we aimed to identify whether the pilocarpine model of epilepsy, as described in higher order biological systems, could elicit hyperkinetic movements in planaria. Furthermore, we aimed to identify what effect brief exposure to a hypoxic environment could produce in planaria exposed to pilocarpine or not. Our goal is to develop the pilocarpine seizure model in planaria, and observe if hypoxia has any enhancing or ameliorative effect on any hyperkinetic movements observed in planaria.

2. MATERIALS AND METHODS

2.1. Preparation of Pharmacological Conditions

Pilocarpine from Sigma Aldridge was used in these experiments. Earlier investigations identified that 5 mM was the optimal dosage of Pilocarpine to administer to the planaria. It was observed that 5 mM pilocarpine was the most effective concentration at producing hyperkinetic movements, higher concentration caused severe damage to the planaria and often resulted in their death. Next, the optimal concentration of carbonated water was tested. We created varying concentrations of carbonated and spring water based on ratios of volume (*i.e.*, v/v). We tested five different concentrations, 1%, 5%, 10%, 25% and 50%. These five concentrations were chosen to create a concentration curve. We wanted to find a concentration that didn't kill the planaria but produced a behavioural effect on the planaria. For each concentration, planaria were exposed to the solution for three minutes and open field behaviours were recorded. At 50% concentration, planaria died. At 25% concentration, we found that planaria didn't die from exposure and exhibited adverse behaviour different from pilocarpine exposure. At 10% concentration there was not enough adverse behaviour exhibited to prove that the carbonated water was affecting the planaria. We decided to continue our experiment with 25% carbonated water concentration. Data from all hypoxic conditions can be seen in Figure 1.

The hypoxic condition – 25% carbonated water—consisted of 5 mL of carbonated water and 15 mL of spring water. The exposure for every condition was 3 minutes (e.g. 3-minutes of pilocarpine followed by 3 minutes of carbonated water).

Each planaria was exposed to 1 of 5 different conditions: 1) 5 mM pilocarpine, or 2) 25% carbonated water, or 3) 5 mM pilocarpine followed by 25% carbonated water, or 4) 25% carbonated water followed by 5 mM pilocarpine, or 5) water. There were 25 - 40 planaria is each condition.

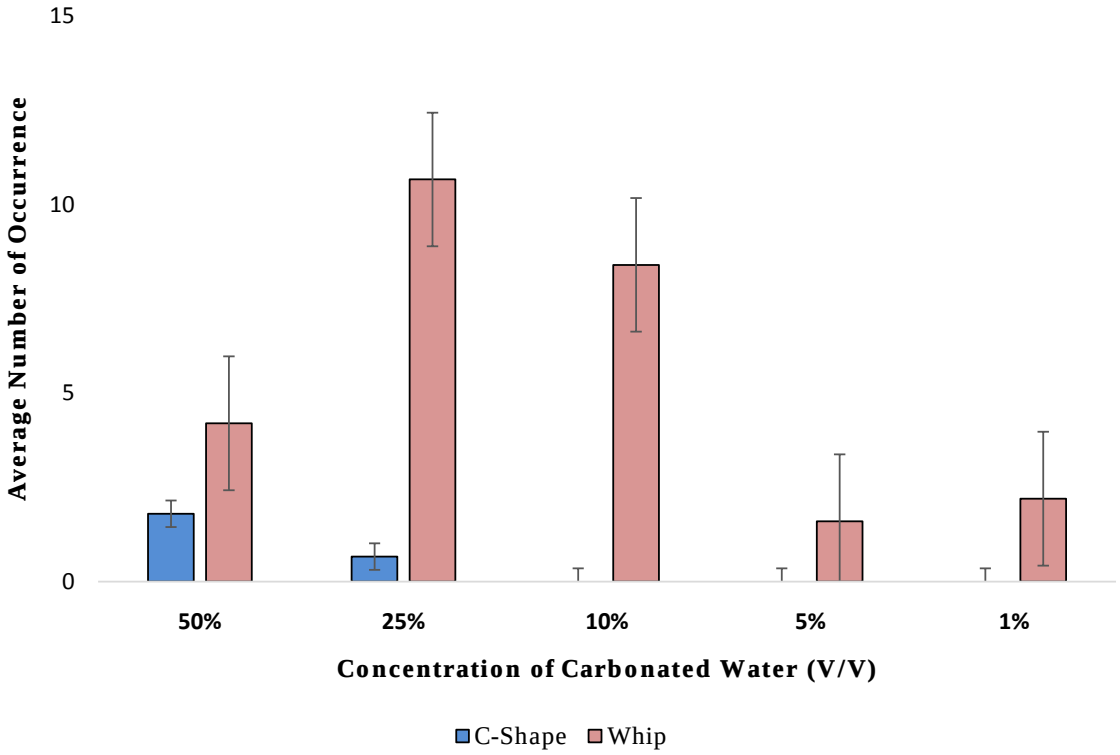

Figure 1. Average number of occurrences for all five behaviours for each concentration of carbonated water mixed with spring water (Hypoxic condition). Means and SEMs presented.

2.2. Planaria Open Field Measurements

All Planaria were purchased from Boreal Science. In total, 149 planaria were tested throughout the duration of this experiment over a total of four months. Planaria were kept in a dark fridge with the temperature set at 4˚ Celsius in a plastic container with PC brand spring water. Before experimentation, planaria were acclimated to the testing room for a minimum of 30 minutes. To observe the effect of each experimental manipulation, we observed open field behaviour of the planaria after being treatment exposure. The purpose was to observe the effect of an acute hypoxic episode following a seizure like episode. In order to accommodate order effects, we included a reverse condition exposure (20 mL of 25% carbonated water for three-minutes followed by exposure to 20 mL of 5 mM pilocarpine for three-minutes). After exposure to one of the five conditions, the planaria were placed in a petri dish containing 20 mL of spring water on top of 0.5 cm grid paper for 3 minutes. Using a freely available counter application for a cell phone, the number of 1 cm gridlines the planaria crossed and adverse behaviours such as c-shaping, head and tail whips, corkscrew and all others were recorded. The planaria were observed for a total 180 seconds.

2.3. Statistical Analyses

One-way analysis of variance, t-tests, and non-parametric mean analyses were conducted to observe any group differences. All statistics were performed on Windows PC with SPPS 20.

3. RESULTS

The results from the *oneway* ANOVA conducted on grids crossed resulted in a significant difference between treatment conditions ($F(4, 148) = 2.761$, $p < 0.5$, $\eta^2 = 0.07$). This can be seen in **Figure 2**. The significant differences being between the control, 5 mM pilocarpine and 25% carbonated water followed by 5 mM pilocarpine. In controlled conditions, planaria are very mobile moving around the petri-dish resulting in many grids crossed. The results from a post-hoc comparisons of groups found that in both the 5 mM pilocarpine condition and the combination condition of 25% carbonated water followed by 5 mM pilocarpine there is a significant decrease in the average number of grids the planaria cross compared to the control condition. This decrease indicates that these conditions are distressing to the planaria, the lack of mobility can be a result from an increase in adverse behaviours as seen in **Figure 3** and **Figure 4** or ultimately because they succumb to the condition.

When examining c-shape behaviour the results of a *oneway* ANOVA revealed significant differences between treatment conditions ($F(4, 148) = 16.707$, $p < 0.5$, $\eta^2 = 0.32$). The significant differences were between control, 5 M pilocarpine and 25% carbonated water followed by 5 mM pilocarpine. Post-hoc comparisons of groups revealed that planaria exhibited a significantly greater average number of c-shape behaviour in both the 5 mM pilocarpine condition as well as the combination condition of 25% carbonated water followed by 5 mM pilocarpine in comparison to the control. These results can be seen in **Figure 3**. The control condition has a low average number of c-shape because this behaviour is classified as adverse and planaria will not exhibit it under positive circumstances. The increase in the adverse c-shape behaviour seen in both the 5 mM pilocarpine and 25% carbonated water followed by 5 mM pilocarpine conditions indicate that these conditions are negative environments for planaria. Similarly, the results from a *oneway* ANOVA examining whip behaviour showed significance between the treatment conditions ($F(4, 148) = 13.258$, $p < 0.5$, $\eta^2 = 0.27$). The significant differences were between control, 5 M pilocarpine and 25% carbonated water followed by 5 mM pilocarpine. The data from post-hoc comparisons of groups revealed that the occurrence of whips was again significantly higher in planaria exposed to 5 mM pilocarpine and 25% carbonated water followed by 5 mM pilocarpine when compared to the control. Corresponding data is presented in **Figure 4**.

4. DISCUSSION

We've showed that planaria exposed to the pilocarpine seizure model demonstrated a reduction in

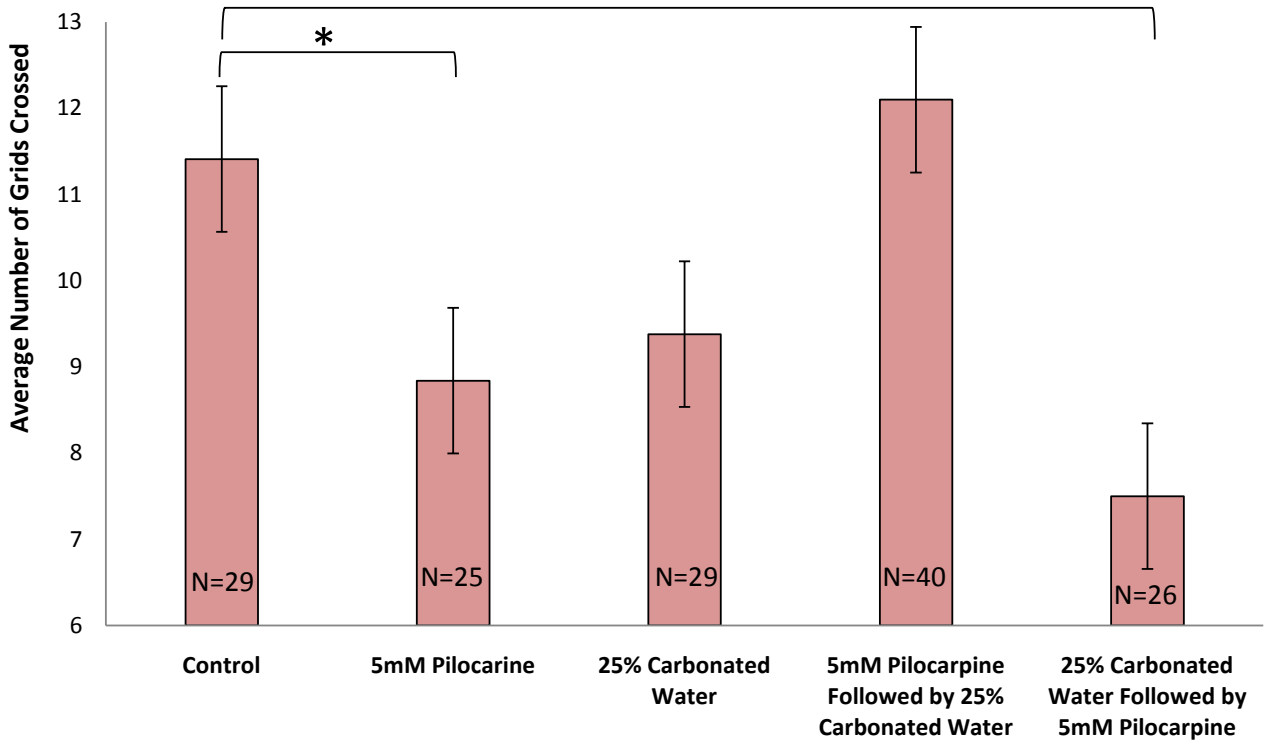

Figure 2. The average number of grids planaria crossed compared across the five different conditions of exposure. Means and SEMs presented (* = $p < 0.05$).

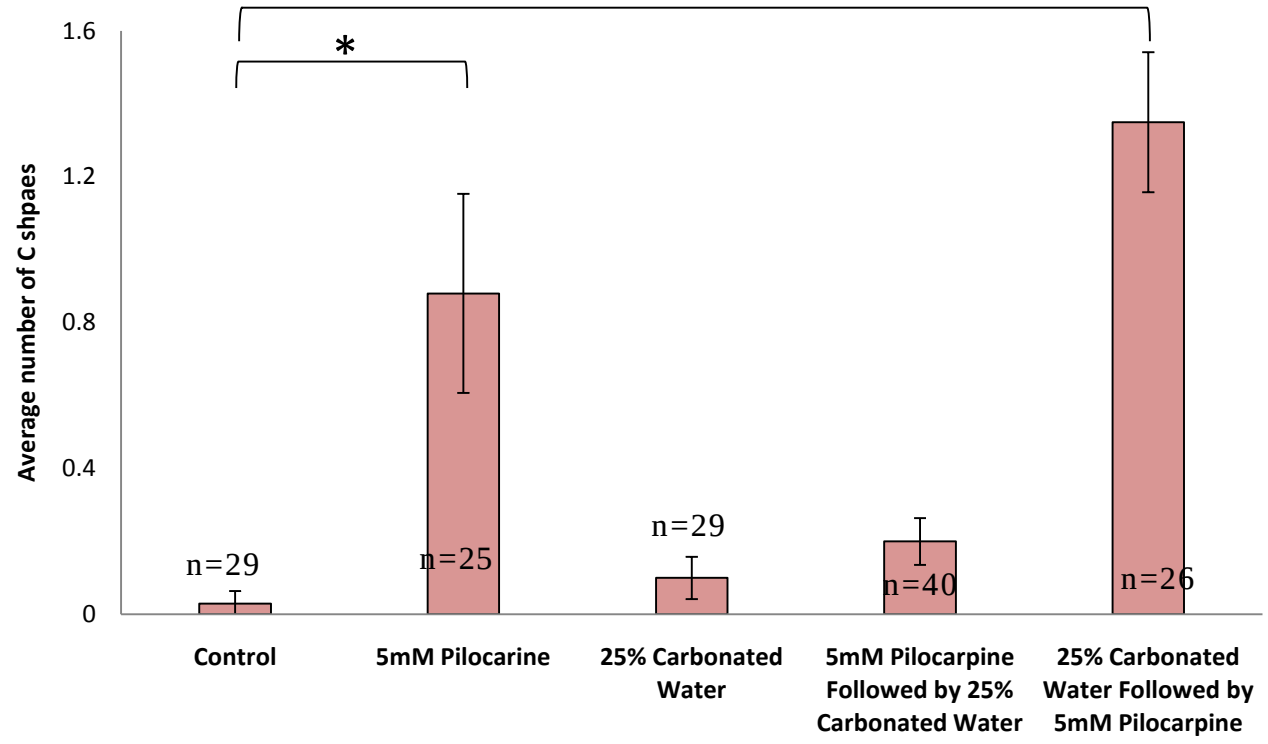

Figure 3. The average number of c-shapes planaria exhibited compared across the five different conditions of exposure. Means and SEMs presented (* = $p < 0.05$).

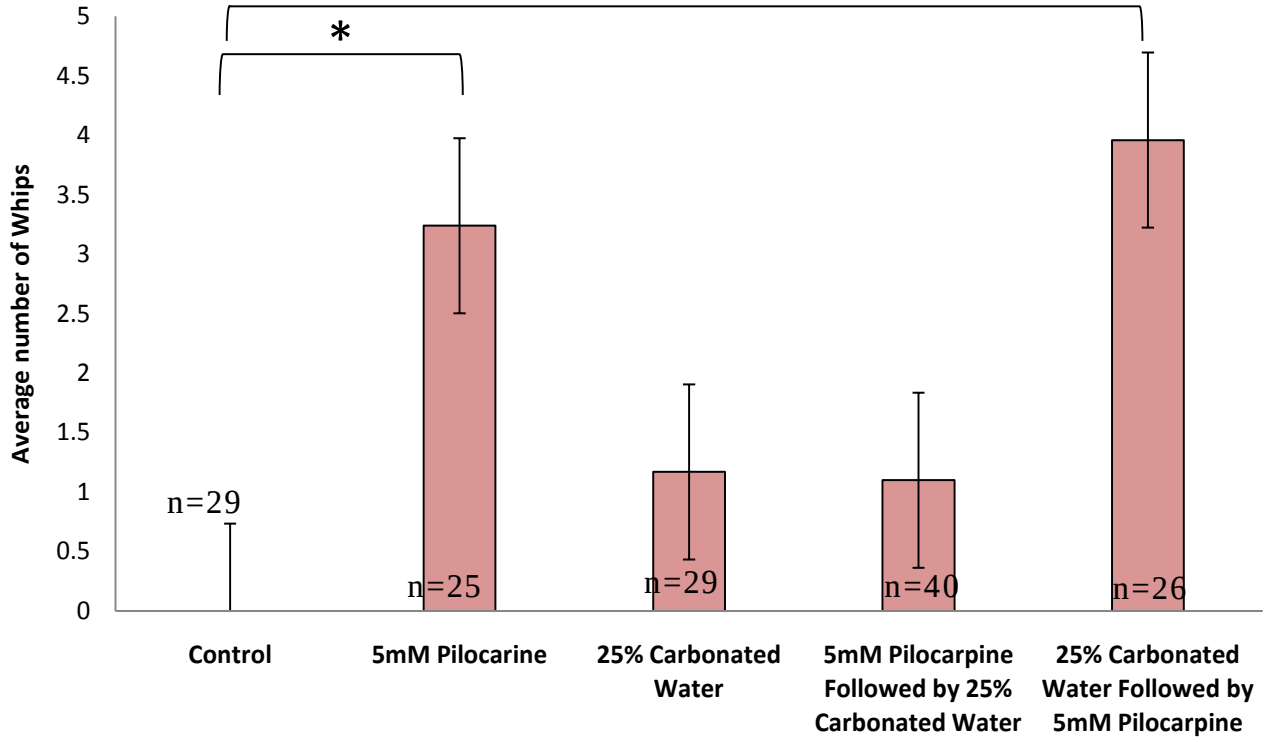

Figure 4. The average number of whips the planaria exhibited compared across the five different conditions of exposure. Means and SEMs presented (* = $p < 0.05$).

motility (as measured by a decrease in the number of gridlines crossed) and an increase in aversive (hyperkinetic) movements. Results (Figure 2) demonstrated that 5 mM pilocarpine alone significantly decreased mobility of planaria when compared to controls. While Figure 3 and Figure 4 demonstrate the obvious pattern of 5 mM pilocarpine increasing aversive hyperkinetic behaviour in planaria when compared to controls. These results confirm the hypothesis that exposure to pilocarpine can produce motor deficits in planaria, with both mobility impairments and the presence of aversive hyperkinetic movements occurring. This pattern of motor deficits was not seen in the hypoxic condition (25% carbonated water).

We then compared the combination of hypoxia and pilocarpine to observe any differences in planaria behavior. Much like 5 mM pilocarpine alone, when planaria were exposed to our hypoxic condition and then 5 mM pilocarpine there were reductions in gridlines crossed and increases in aversive hyperkinetic behaviours (Figures 1-3). Perhaps the most intriguing effect occurred when the hypoxic condition was applied after exposure to 5 mM pilocarpine. In this condition, there were no significant differences in open field behaviour as compared to control planaria. In other words, the application of a single hypoxic episode ameliorated any negative effects the pilocarpine had on planaria. All of the aversive hyperkinetic movements seen in the 5 mM pilocarpine condition were absent, and there was no difference in mobility of the animal. This amelioration was only seen when the hypoxic event was presented in this order (pilocarpine → hypoxia), when the order of exposure was reversed (hypoxia → pilocarpine) no such improvement was observed.

The exact mechanism by which these improvements occur remains to be elucidated. Work by Lee and colleagues [19] point to adenosine as a potential mediator of hypoxia-related improvements in seizures behaviours. The termination of adenosine signalling through the activity of HIF provides a rational hypothesis as to how the planaria become resistant to future hyperkinetic movements (*i.e.*, seizures). That an ameliorative effect occurred when hypoxia followed pilocarpine—and not the inverse—points to the potential therapeutic capacity of hypoxia therapy. This reversal of order serves as a useful positive control.

5. CONCLUSION

Here we demonstrated that administration of 5 mM Pilocarpine was a reliable model to develop hyperkinetic movements in brown planaria. This is the first demonstration that the pilocarpine epilepsy model seen in higher vertebrates is useful with planaria. In addition, we have shown that when the specific order of 5 mM pilocarpine followed by 25% carbonated water was applied to planaria the adverse effects (*i.e.*, reduction in mobility and increase in hyperkinetic movements) found when planaria were exposed to 5 mM pilocarpine only were completely absent. More treatment conditions are required to make any definitive statements about the efficacy of hypoxic conditions in higher order vertebrates, but we have reliably demonstrated the production of hyperkinetic movements in planaria when exposed to pilocarpine. That a single hypoxic event completely removes any aversive behaviour is an intriguing development. It is limiting not knowing the exact mechanism as to how hypoxia ameliorates the adverse behaviour. Longer hypoxic durations and additional pharmacological agents should be tested in tandem with this protocol to fully understand hypoxia's potential as a therapeutic intervention. More work with planaria should follow, then additional measures with higher order vertebrates can be pursued.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest regarding the publication of this paper.

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