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An oxygen-rich atmosphere or systemic fluoxetine extend the time to respiratory arrest in a rat model of obstructive apnea

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ABSTRACT

Audiogenic seizure-prone mice can be protected from seizure-associated death by exposure to an oxygen atmosphere or treatment with selective serotonergic reuptake inhibitors (SSRIs). We have shown previously in a rat model that epileptic seizure activity can spread through brainstem areas to cause sufficient laryngospasm for obstructive apnea and that the period of seizure-associated obstructive apnea can last long enough for respiratory arrest to occur.

We hypothesized that both the oxygen-rich atmosphere and SSRIs function by prolonging the time to respiratory arrest, thus ensuring that seizure activity stops before the point of respiratory arrest to allow recovery of respiratory function. To test this hypothesis, we evaluated each preventative treatment in a rat model of controlled airway occlusion where the times to respiratory arrest can be measured.

Adult male Sprague Dawley rats (median age = 66 days) were studied in the absence of any seizure activity. By directly studying responses to controlled airway occlusion, rather than airway occlusion secondary to seizure activity, we could isolate the effects of manipulations that might prolong respiratory arrest from the effects of those manipulations on seizure intensity. All group sizes were ≥ 8 animals per group.

We found that both oxygen exposure and fluoxetine significantly increased the time to respiratory arrest by up to 65% ($p < .0001$ for 5 min oxygen exposure; $p = .031$ for 25 mg/kg fluoxetine tested 60 min after injection) and, given that neither treatment has been shown to significantly alter seizure duration, these increases can account for the protection of either manipulation against death in sudden death models. Importantly, we found that 30 s of exposure to oxygen produced nearly the same protection as 5 min exposure suggesting that oxygen exposure could start after a seizure starts ($p = .0012$ for 30 s oxygen exposure). Experiments with 50% oxygen/50% air mixtures indicate that the oxygen concentration needs to be above about 60% to ensure that times to respiratory arrest will always be longer than a period of seizure-induced airway occlusion. Selective serotonin reuptake inhibitors, while instructive with regard to mechanism, require impractical dosing and may carry additional risk in the form of greater challenges for resuscitation.

We conclude that oxygen exposure or SSRI treatment prevent seizure associated death by sufficiently prolonging the time to respiratory arrest so that respiratory function can recover after the seizure abates and eliminates the stimulus for seizure-induced apnea.

1. Introduction

The growing body of clinical and preclinical research and clinical evidence from cases of sudden death in epilepsy (SUDEP) and near miss

cases has led to more focused ideas about (1) the underlying mechanism (s) and (2) potential biomarkers that may help to stratify risk (e.g. (Devinsky et al., 2016; Jansen and Lagae, 2010; Lacuey et al., 2018; Poh et al., 2012; Stewart et al., 2017; Vilella et al., 2018)).

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Although SUDEP is the most common cause of death among persons with epilepsy (e.g. (Barot and Nei, 2019; Devinsky et al., 2016)), deaths are thankfully rare. Most deaths occur at night when the individual is in bed at home (e.g. (Clark and Riney, 2016; Massey et al., 2014; Purnell et al., 2018; Ryvlin et al., 2013; Shorvon and Tomson, 2011)). The rarity of events and their circumstances create most of the challenge for collecting detailed physiological records during seizure events that could be used to accelerate the search for mechanism and biomarkers. Preclinical studies permit manipulations and monitoring that (1) can be impossible to obtain from persons with epilepsy, and (2) can suggest specific mechanistic features to focus clinical studies.

We have shown previously that epileptic seizure activity can spread through lower brain areas to activate laryngomotor efferent fibers and cause laryngospasm (i.e. not via reflex mechanisms). The laryngospasm can be sufficient to completely obstruct the airway (obstructive apnea) and that the period of obstructive apnea can last long enough for respiratory arrest to occur (Nakase et al., 2016; Stewart et al., 2017). Our definition of respiratory arrest is the point at which the individual stops attempting to draw a breath. We consider this to be equivalent to the “onset” of terminal apnea as defined by Ryvlin et al. (Ryvlin et al., 2013).

Associated with the period of airway occlusion is a progressive oxygen desaturation, and this loss of oxygen can contribute to seizure termination (Hotta et al., 2009; Nakase et al., 2016; Stewart et al., 2017). As the seizure ends, on its own or from hypoxia, it also ceases to be a stimulus for laryngospasm, and the airway can open. If respiratory arrest occurs before the airway reopens, the individual will progress from respiratory arrest to cardiac arrest and death unless mechanically resuscitated. If the airway reopens before respiratory arrest occurs, the individual will spontaneously resume breathing and recover from the event.

In mouse studies, DBA/2 mice experiencing audiogenic seizures that would normally be fatal with nearly 100% mortality were protected by experiencing those seizures in an oxygen atmosphere (Venit et al., 2004; Willott and Henry, 1976). Similarly, selective serotonergic reuptake inhibitors (SSRIs) such as fluoxetine or 5-HT_{2A} agonists have been shown to protect against death in multiple strains of audiogenic seizure-prone mice (Buchanan et al., 2014; Faingold et al., 2014; Faingold et al., 2011b; Zeng et al., 2015), suggesting that these drugs protect the brainstem circuits critical for respiration (Bateman et al., 2010; Blum, 2009; Buchanan et al., 2014; Faingold et al., 2014; Faingold et al., 2011a; Feng and Faingold, 2017; Richerson and Buchanan, 2011; Tupal and Faingold, 2006; Uteshev et al., 2010).

We hypothesized that both the oxygen-rich atmosphere and the SSRIs function by prolonging the time to respiratory arrest, thus ensuring that seizure activity could stop on its own before the individual was at risk for respiratory arrest. To test this hypothesis, we evaluated each preventative treatment in a rat model of controlled airway occlusion that permits (1) controlled complete airway closure, (2) control of the atmosphere available to the animal, and (3) continuous monitoring of physiological variables including airway pressures associated with normal spontaneous breathing and attempts to breathe during occlusion. This last element enables the precise determination of the point of respiratory arrest. This direct measure of the impact of SSRIs on the time to respiratory arrest would also be free from the confound of the drugs' effects on the intensity of seizure activity (Buchanan et al., 2014).

2. Materials and methods

Methods and implants were similar to those described in detail previously (Mooney et al., 2019; Nakase et al., 2016; Villiere et al., 2018). All procedures were approved by an Animal Care and Use Committee and conducted in accordance with the United States Public Health Service's Policy on Humane Care and Use of Laboratory Animals. Adult male Sprague Dawley rats (AGE median = 66, mean = 88 days;

WEIGHT median = 306, mean = 330 g; Envigo Chicago, IL) were housed in AAALAC-accredited facilities and maintained on a 12 h light:dark cycle with an average temperature of 23 °C and humidity of 55%, monitored daily, and had unrestricted access to water and food.

Urethane (1.5 g/kg ip) was used for anesthesia in all animals. Euthanasia at the end of experiments included overdosing animals with mixture of pentobarbital (150 mg/kg) and phenytoin (19 mg/kg; Euthasol, Virbac AH, Fort Worth, TX).

2.1. Monitoring/recording

ECG recordings. Limb-lead ECG was recorded using copper strips coated with conductive gel wrapped around both forelimbs and either the base of the tail or the left hindlimb. Signals were amplified and filtered to pass 1 Hz to 1 kHz (Model 1800 AC amplifier with headstages; A-M Systems, Sequim, WA) and digitized at 2 kHz (Micro1401 controlled by Spike 2 software; Cambridge Electronic Design, Cambridge, UK). Rhythm was assessed by reviewing P waves and associated QRS complexes for variations in wave shape, beat-to-beat intervals, and atrial-ventricular coupling.

Pulse oximetry. Arterial oxygen saturation was measured by using a clip sensor on the thigh (TDR-43C, Med Associates, St Albans, VT) coupled to a pulse oximeter (CANL-425SV-A, Med Assoc.). The raw pulsatile waveform of the pulse oximeter was digitized (2 kHz) with other signals.

Inspiratory airway pressures. A pressure transducer (CyQ, Columbus Instruments, Columbus, OH) was connected to the sidearm of a T-shaped tracheal tube (see below). Inspiratory pressures during complete airway occlusion were measured relative to those observed during baseline breathing. Signals were digitized along with other signals recorded at the same time.

2.2. Other manipulations/implants

Tracheal implants. A T-shaped tracheal tube was placed through an incision between cartilaginous rings of the trachea after the trachea was exposed in the neck. The distal arm was “sealed” in the trachea with suture around the trachea, compressing it against the outside wall of the implant. The proximal arm was secured with suture to the outside of the proximal trachea for additional mechanical stabilization.

Controlled airway occlusion. Obstructive apnea was produced with controlled airway occlusion. The exterior arm in the straight path with the implanted arm served breathing or could be occluded. A pressure transducer on the tracheal tube sidearm recorded forces developed during either normal breathing with the tracheal tube open to the atmosphere or during complete closure of the open port. In prior work, we showed that during occlusion, respiratory effort exerted to inspire progressively increased with each attempt until effort ceased and that this point of respiratory arrest was associated with acute left ventricular dilatation, hypokinesia, and asystole (Nakase et al., 2016). To control the atmosphere available to the animal, the open port was connected to a stream of air, oxygen, or an air/oxygen mixture that flowed continuously past the open port and could be switched to control the amount of time that the animal had access to a particular gas.

SSRI pretreatment. Animals were injected intraperitoneally with fluoxetine (25 mg/kg made as a 2 mg/ml solution in saline to ensure complete solubilization) or with an equivalent volume of saline. Injected volumes were 12.5 ml/kg and averaged 4.25 ± 1.00 ml/animal (mean \pm standard deviation). Fluoxetine hydrochloride (cat# 1279804) was obtained from Sigma Aldrich Inc. (St. Louis, MO).

2.3. Experimental design

Oxygen-rich atmosphere exposure. Animals were randomly assigned to one of 4 groups: (1) exposure to an oxygen atmosphere for 5 min prior to airway occlusion, (2) exposure to an air stream for 5 min

prior to airway occlusion, (3) switching to an oxygen stream for only 30 s prior to airway occlusion, or (4) switching to a mixed stream (50% oxygen/50% air) for only 30 s prior to airway occlusion. Gas was streamed at ~4 l/min. In all animals, the occlusion was left in place until 10 s after the point of respiratory arrest, which was defined as the time of the peak of the last breath attempt. Each animal was resuscitated as described previously (Villiere et al., 2018), but only the first occlusion event in each animal was analyzed in this study (repeated occlusions change the response profile to occlusion as described in (Mooney et al., 2019)). Planned group sizes were 8 animals per group.

SSRI pretreatment. Animals were randomly assigned to one of 2 groups: (1) systemic injection of fluoxetine (25 mg/kg IP) 60 min prior to occlusion testing, or (2) intraperitoneal saline of an equivalent volume based on animal weight 60 min prior to occlusion testing (e.g. (Tupal and Faingold, 2006; Zeng et al., 2015)). Occlusion testing was identical to that described for the oxygen exposed animals. Group sizes were 8 animals per group.

2.4. Data analysis and statistics

Analyses were done with Spike 2 software (Cambridge Electronic Design, Ltd., Cambridge, England) and Microsoft Excel. Data are reported as means \pm standard deviation in the text and figures unless otherwise noted. All statistics were computed with GraphPad Prism 8 software or IBM SPSS Statistics (version 24). Independent samples *t*-tests, one-way and two-way analyses of variance were used for group comparisons. A $p < .05$ was predefined, after appropriate post-hoc correction for multiple comparisons where appropriate, to be statistically significant. The specific tests and post-hoc corrections are given in the text with the results and in the figure legends.

3. Results

Fifty-four adult male animals were used. Thirty-eight was the final number of animals in the oxygen atmosphere series and 16 animals were used in the SSRI series.

3.1. Oxygen-rich atmosphere exposure

Exposure to an oxygen-rich atmosphere prior to airway occlusion prolonged the time to respiratory arrest. Examples of individual experiments on animals exposed to either an air stream or an oxygen stream for 5 min prior to airway occlusion are shown in Fig. 1 and multiple measures are summarized in Fig. 2. Key features of the responses are (1) a significantly longer time to respiratory arrest in animals exposed to oxygen, (2) ECG changes indicative of hypoxia (ST segment elevation, rate decreases due to missed QRS complexes, premature ventricular beats) preceding the point of respiratory arrest in all animals, (3) a pattern of inspiratory attempts with rapidly increasing effort as the animal approaches the point of respiratory arrest in all animals, and (4) minimum pulse oximetry levels that were comparable across all groups ($37\% \pm 11$ for oxygen animals, $n = 12$, $30\% \pm 9$ for air animals, $n = 8$; mean, standard deviation). Interestingly, the breath attempts made during the first half of the occlusion period during oxygen exposure show an initial stable pattern of moderate effort that transitioned into the pattern of increasing effort seen during occlusions in air (Fig. 1, tracheal pressure traces). Even clearer is the sustained high level of oxygen saturation in the oxygen-breathing rat that persists for about 30 s before the gradual decline with similar slope and endpoint to the air-breathing rat (Fig. 1, pulse oximetry traces). Specific additional comparisons are illustrated in Fig. 2.

Whereas oxygen exposure for 5 min prior to airway occlusion led to a 65% increase in the time to respiratory arrest (88.4 vs. 53.6 s), we assessed shorter oxygen exposures (30 s) and short exposures to more modest enrichment (50% oxygen/50% air for 30 s). As shown in Fig. 2,

exposure to 30 s of oxygen immediately prior to airway occlusion ($n = 10$) increased the time to respiratory arrest by 52% (81.6 vs. 53.6 s) and the number of breath attempts (Fig. 2 E, F) was not significantly different from the results obtained with and exposure time of 5 min. A one-way ANOVA for the times to respiratory arrest was significant ($F(3, 34) = 9.972$; $p < .0001$) and Dunnett's multiple comparisons of each oxygen group against air are shown in Fig. 2E. For the numbers of breath attempts, a one-way ANOVA was also significant ($F(3, 34) = 9.939$; $p < .0001$) and Dunnett's multiple comparisons of each oxygen group against air are shown in Fig. 2F. When the concentration of oxygen was reduced by mixing oxygen and air 1:1, and applied for 30 s, the time to respiratory arrest trended to a higher value, increasing by 24% (66.4 vs. 53.6 s) compared with air ($n = 8$ rats; $p > .05$ after post-hoc correction for multiple comparisons, but $p = .014$ in a straight comparison by *t*-test) and the number of breath attempts was significantly increased (Fig. 2F).

3.2. SSRI pretreatment

The results of experiments on animals receiving either fluoxetine or saline are shown in Fig. 3. Raw data examples are not shown because the raw data closely resemble the raw data illustrated in Fig. 1, with modest increases in the time to respiratory arrest. Fluoxetine delivered intraperitoneally and evaluated at 1 h post-injection produced an average increase in the time to respiratory arrest of 28% (63.8 vs. 49.9 s, $n = 8, 8$), but the number of breath attempts was almost identical for the two groups (Fig. 3D), indicating a lower rate of attempts to inspire in the SSRI-treated animals.

When we evaluated the impact of systemic fluoxetine on the pre-occlusion respiratory rate, we found that the largest effect sizes for fluoxetine was on altering the rate of breathing (Fig. 3E, F). Two-way ANOVA was significant for fluoxetine/saline ($F(2, 28) = 283.5$; $p < .0001$), pre-injection/post-injection/occlusion ($F(1, 14) = 30.20$; $p < .0001$), or the product of drug X condition ($F(2, 28) = 9.536$; $p = .0007$). The corrected *p* values for multiple comparisons (Tukey) are shown in Fig. 3E. Tracheal pressure measures do not give information on tidal volumes. SSRI treatment was shown to not alter ventilation in DBA/1 mice (Zeng et al., 2015).

The respiratory rate was significantly slowed by fluoxetine. The large rate differential between pre-injection and pre-occlusion (Fig. 3E, F) suggest that fluoxetine did impact the respiratory brainstem as suggested by others (e.g. (Bateman et al., 2010; Blum, 2009; Devinsky et al., 2016; Faingold et al., 2011b; Feng and Faingold, 2017; Massey et al., 2014; Richerson and Buchanan, 2011; Tupal and Faingold, 2006; Zeng et al., 2015)). The rate of breath attempts during occlusion was lower than the pre-occlusion rate of actual respiration, but the magnitude occlusion-associated drops were similar between the saline and fluoxetine-treated animals. This lower rate of breath attempts during occlusion did not significantly alter the rate of desaturation, nor was there any measurable effect on the magnitudes of tracheal pressure changes during inspiration attempts. Also observed was a tendency for lower minimum oxygen saturation levels after SSRI treatment, but average oxygen saturation minima were not statistically different (34% for fluoxetine group, 37% for saline group).

Interestingly, and consistent with the tendency for lower minimum oxygen saturation levels after SSRI treatment, it was more difficult to resuscitate animals in the SSRI group after respiratory arrest. We resuscitated animals with bolus gas delivery for lung inflation as described previously (Villiere et al., 2018). SSRI-treated animals required more attempts on average than saline treated animals to effect resuscitation (2.00 ± 0.76 vs. 0.50 ± 0.53 five milliliter air bolus treatments for resuscitation; mean \pm standard deviation; $p = .0004$). All SSRI-treated animals required lung inflation for resuscitation, whereas 4/8 saline treated animals spontaneously restarted breathing after the occlusion was stopped (occlusion was stopped 10 s after the point of respiratory arrest to confirm that respiratory arrest had

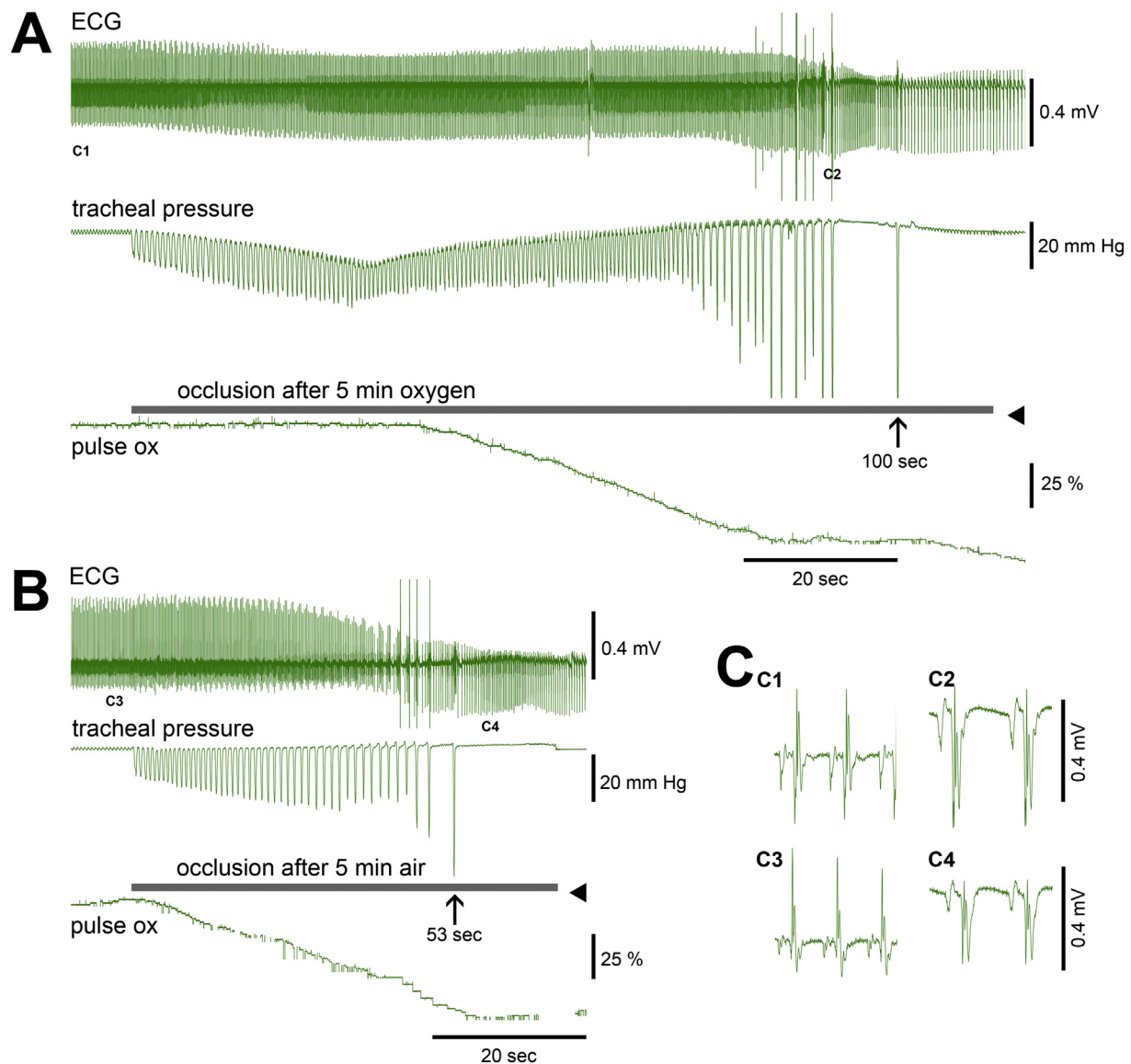


Fig. 1. Examples of prolongation of the time to respiratory arrest from oxygen exposure. Panel A: Controlled airway occlusion after breathing oxygen for 5 min. Time of occlusion is marked by a heavy horizontal line. Last breath attempt (i.e. the point of respiratory arrest) occurred at 100 s after occlusion onset (marked by arrow). Top trace shows limb lead ECG with initial normal sinus rhythm that showed significant ST segment elevation indicative of hypoxic changes in the heart, bradycardia, and premature ventricular contractions starting at about 80 s post-occlusion. The forces associated with breath attempts during airway occlusion are shown in the center trace, and the pulse oximeter output is shown in the bottom trace of the panel. One hundred percent oxygen saturation is marked by the arrowhead at the right edge of the panel and coincides with the bottom of the horizontal bar marking the period of occlusion. Panel B: Similar to panel A, but from an animal that was breathing air. Note the significantly shorter time to respiratory arrest (53 s) and shorter times to significant cardiac rhythm irregularities (about 40 s). Panel C: Higher temporal resolution illustrations of ST changes in the ECG from panels A and B during pre-occlusion and occlusion phases of the study. Times are marked in panel A as C1 (pre-occlusion) and C2 (occlusion) and in panel B as C3 (pre-occlusion) and C4 (occlusion). Each segment in 0.5 s in duration.

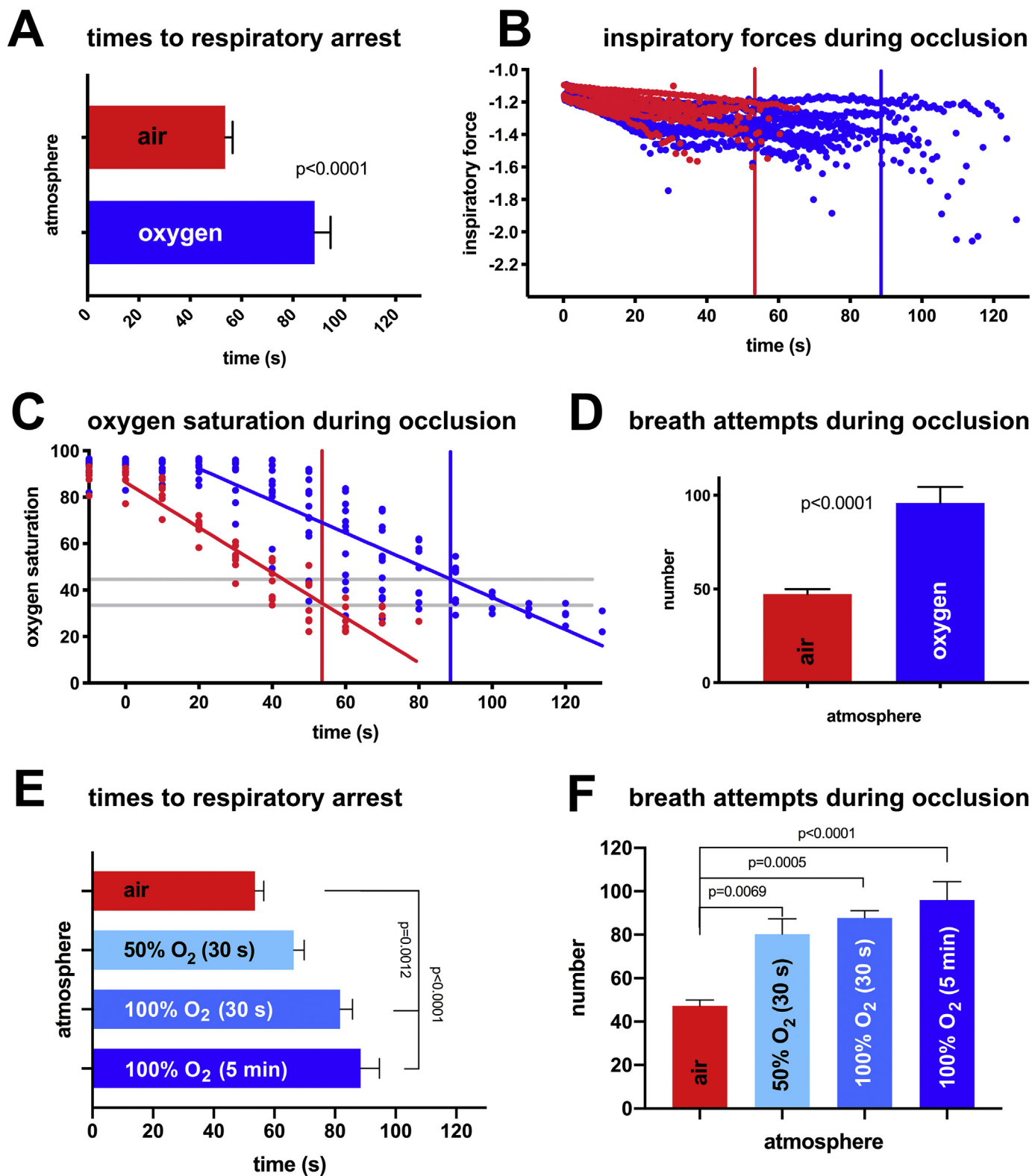
occurred).

4. Discussion

We used a rat model in which obstructive apnea was caused with controlled airway occlusion to evaluate two interventions that have been shown to prevent death in animal models of sudden death in epilepsy. We determined the times to respiratory arrest in animals breathing oxygen vs. air, and animals treated with the selective serotonin receptor uptake inhibitor, fluoxetine. We found that both oxygen exposure and fluoxetine significantly increased the time to respiratory arrest and, given that neither has been shown to alter seizure duration, these increases can account for the protection of either manipulation against death in sudden death models.

Importantly, we found that 30 s of exposure to oxygen just prior to airway occlusion produced nearly the equivalent protection as 5 min exposure up to the point of occlusion, suggesting that oxygen exposure could start after a seizure starts. Experiments with 50% oxygen/50% air mixtures indicate that the concentration needs to be above about 60% (the result of mixing equal quantities of pure oxygen with air) to ensure times to respiratory arrest that are sufficiently long that they will always be longer than the seizure and any period of seizure-induced airway occlusion.

Selective serotonin reuptake inhibitors, while instructive with regard to mechanism require impractical dosing and carry additional risk in the form of greater challenges for resuscitation.



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5. Apnea and respiratory arrest

The discrimination between central and obstructive apnea can be challenging without clear evidence of attempts or the lack of attempts to breathe. With monitoring such as tracheal pressure recordings, the effort associated with attempts to breathe during airway occlusion are

evident (e.g. (Nakase et al., 2016; Stewart et al., 2017)). In mice experiencing audiogenic seizures, however, death is associated with intense tonic convulsive activity marked by hindlimb extension that can easily mask or prevent attempts to inspire during obstructive apnea. Differentiating between central and obstructive forms of apnea in such circumstances may not be possible with observation, plethysmography,

Fig. 2. Impact of oxygen exposure on times to respiratory arrest in a rat model of complete airway occlusion. Panel A: Rats exposed to room air ($n = 8$ rats) experienced respiratory arrest about 50 s after the onset of controlled airway occlusion (a method to simulate laryngospasm-based complete airway closure). By contrast, rats breathing for 5 min in an oxygen enriched atmosphere before airway occlusion lasted nearly twice as long before respiratory arrest occurred ($n = 12$ rats). P value is after correction for multiple comparisons (see Panel E). Panel B: Each attempted breath and its associated inspiratory force are plotted. Animals breathing air are shown in red and animals breathing oxygen are shown in blue. The large increases in inspiratory effort just before respiratory arrest are seen near the mean times for respiratory arrest (vertical red and blue lines). Because large inspiratory effort against a closed airway is biophysically favorable for the generation of pulmonary edema, the persistence of smaller inspiratory breath attempts protects longer against the development of pulmonary edema. Panel C: Oxygen shifts the desaturation profile to later times. There is nearly a 40 s delay in desaturation onset in animals breathing oxygen vs. those breathing air. This delay in the onset of desaturation and the time for respiratory arrest is the basis of the protection against death. The gray horizontal lines indicate the intersection of the linear regression line with the mean time to respiratory arrest. Linear regression lines are fitted only to the falling phase of the oxygen saturation curves and are meant for comparison of the rates of desaturation. They are not meant to summarize the entire multi-phasic saturation profile. Panel D: The total number of breath attempts made during complete airway closure is significantly greater in animals that breathed oxygen prior to airway occlusion. This is another representation of the protective role of oxygen. P value is after correction for multiple comparisons (see Panel E). Panel E: comparison of briefer oxygen exposure times and lower concentrations of oxygen on the times to respiratory arrest. Brief (30 s; $n = 10$ rats) exposure to oxygen is as protective as longer (5 min; $n = 12$ rats) exposures. Brief exposure to 50% oxygen ($n = 8$ rats) did not retain statistical significance when compared to air after post hoc correction for multiple comparisons (because the group sizes were small), but in a straight *t*-test comparing only air and 50% oxygen the time to respiratory arrest was prolonged ($p = .0135$). Mean times were: 53.6 (air), 66.4 (brief 50% O₂), 81.6 (brief O₂), and 88.4 (oxygen) seconds. Illustrated *p* values are after correction for multiple comparisons after one-way analysis of variance (see text for additional details). Panel F: Breath attempts for all oxygen conditions showing the impact of brief and 50% oxygen. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

or electrical recordings that include both convulsive and inspiratory EMG. Plethysmography can offer clues based on the transitions into or out of apneic episodes (Villiere et al., 2017). If one considers the point of respiratory arrest as the onset of a form of central apnea (which we do), the same masking challenges exist. The advantage of the current study is that we were able to directly determine the point of respiratory arrest before and after specific manipulations that have been shown to prevent death.

6. Relation of our oxygen findings to those in mice

Sufficient access to oxygen is the one manipulation that has been shown to protect absolutely against death in any animal model of sudden death. That oxygen nearly doubled the time to respiratory arrest is not surprising given other experiences with oxygen, e.g. breath holding (Godfrey and Campbell, 1968; Godfrey and Campbell, 1969). As we discussed previously (Nakase et al., 2016), breathing oxygen prior to a breath holding dive prolonged the dive time by nearly two-fold. In our own experience, oxygen does not alter seizure durations and we are unaware of any evidence in any model where oxygen does alter seizure durations. Oxygen exposure shifted the desaturation curve to longer times, but once desaturation started, it followed a time course that resembled that for air breathing animals (Figs. 1 and 2C). The safety factor provided by oxygen with regard to the time to respiratory arrest completely explains why oxygen treatment ensures survival of 100% of animals (Venit et al., 2004; Willott and Henry, 1976).

7. Relation of our SSRI findings to those in mice

We found that systemic fluoxetine (25 mg/kg given 1 h prior to airway occlusion) significantly prolonged the time to respiratory arrest in a measure that was free from additional effects of the drug such as reducing the severity of seizure activity (Buchanan et al., 2014). More pronounced was fluoxetine's action to decrease the pre-occlusion respiratory rate, which is an indication of its actions on respiration referred to in the Introduction. Whereas the percent change in breath attempt frequency caused by airway occlusion was equivalent in fluoxetine-treated or saline control animals (Fig. 3F), the absolute frequency of breath attempts was lower in fluoxetine-treated animals (Fig. 3E). The lower frequency is consistent with the slightly slower rate of oxygen desaturation (not statistically significant; Fig. 3C). Fluoxetine may also have led to a significant or subtle hyperventilation effect to prolong the time to respiratory arrest similar to what has been described for breath holding, but we have no direct evidence of hyperventilation (Godfrey and Campbell, 1968; Marks et al., 1997). We did not measure tidal volume in animals together with tracheal

pressure. In fact, in DBA/1 mice, there was no evidence of an effect of SSRI treatment on ventilation, but they did show that respiratory arrest did not occur in treated animals in association with seizure activity (Zeng et al., 2015). That the impact on respiratory arrest was consistent in both models suggests that this action of SSRI treatment is not dependent on the change in respiratory rate that we observed or that Zeng et al. did not observe.

The doses of selective serotonergic reuptake inhibitors used in this model and in the mouse models are, however, medically impractical (roughly ten times the clinical doses used for depression). More significantly, their use may permit respiratory arrest at lower overall oxygen saturations, which can make resuscitation more difficult.

8. Hypoxia as cause or consequence of apnea

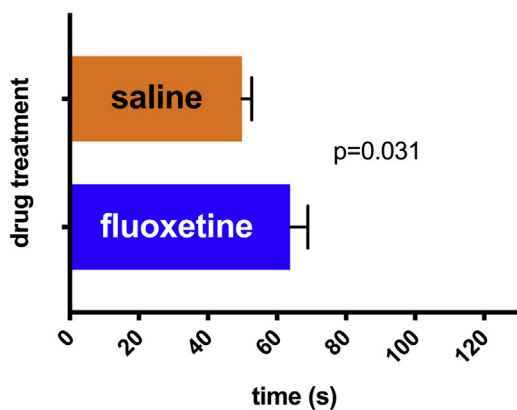
The mechanisms that have been proposed to account for death in the same mouse models that can be protected with oxygen exposure or SSRIs generally point to brainstem dysfunction, particularly involving serotonergic respiratory brainstem neurons, caused by seizure activity (e.g. spreading depolarization (Aiba and Noebels, 2015)). This dysfunction manifests as central apnea, which drives desaturation and progresses to respiratory arrest. The protection afforded by SSRI or serotonergic receptor agonist pre-treatment has been viewed in the context of this mechanism as protective against the possibility of central apnea.

As described in the Introduction, our view has been that seizures "disrupt" brainstem activity by activating brainstem regions, rather than inactivating them. In our view, seizure activity drives laryngospasm and thus obstructive apnea. The obstructive apnea persists as long as the laryngospasm persists, establishing a temporal competition for whether the seizure terminates before respiratory arrest or vice versa. In this view, the central form of apnea (i.e. respiratory arrest) is a consequence of hypoxia, not its initial cause.

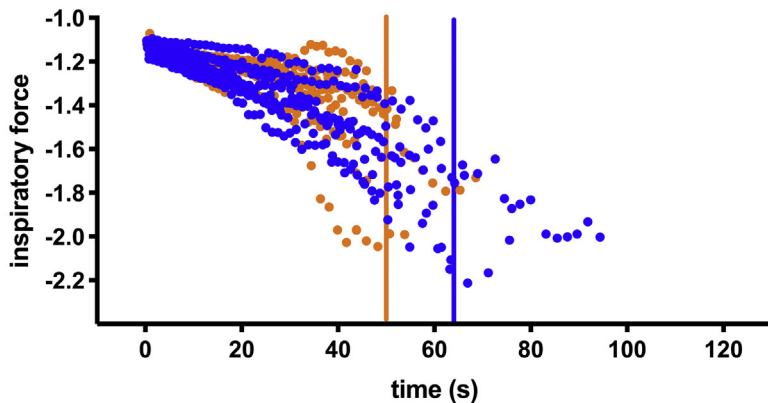
Survival of audiogenic seizure-prone mice after exposure to oxygen is difficult to account for in the first view (seizure-associated central apnea), but readily accounted for in the second (seizure-associated obstructive apnea). If oxygen exposure prevents death by preventing the brainstem dysfunction and central apnea of the first viewpoint, then it is unclear what brainstem consequences are directly due to seizure activity. In our view, all of the seizure-driven events (including laryngospasm and obstructive apnea) still occur, with or without an oxygen-enriched atmosphere, but the temporal competition between seizure termination and respiratory arrest is eliminated because of the much longer times to respiratory arrest.

Whereas oxygen exposure increased both the number of breath attempts during occlusion (Fig. 2 D, F) and the time to respiratory arrest

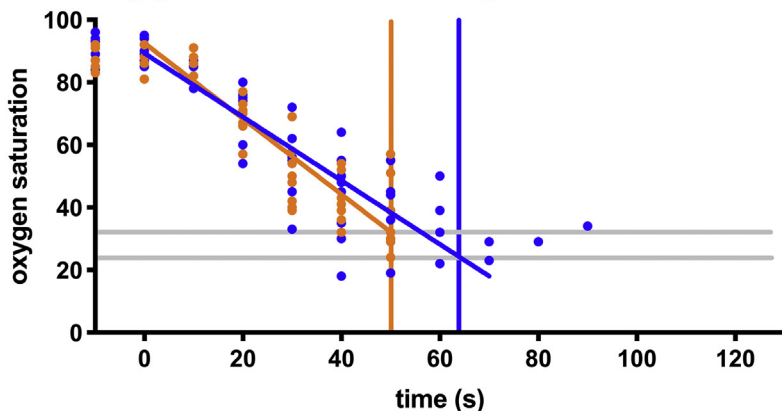
A times to respiratory arrest



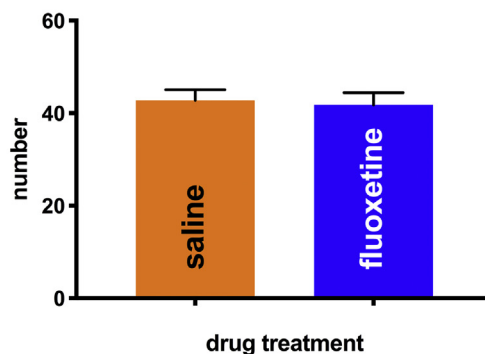
B inspiratory forces during occlusion



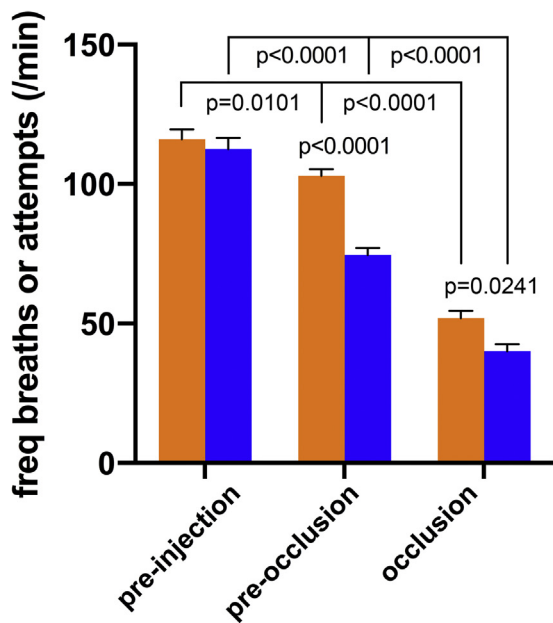
C oxygen saturation during occlusion



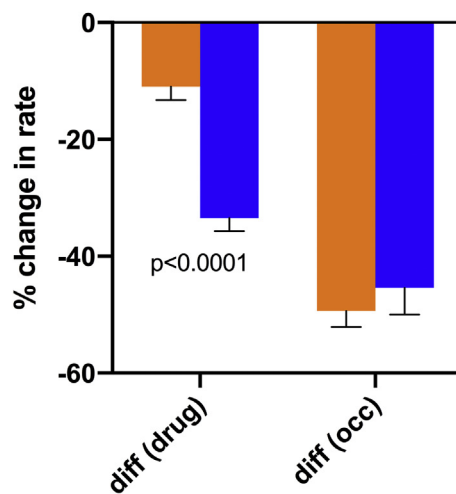
D breath attempts during occlusion



E frequency of breaths or attempts



F Δ freq breaths or attempts



(caption on next page)

Fig. 3. Impact of systemic fluoxetine on times to respiratory arrest in a rat model of complete airway occlusion. Panels A-D resemble those shown in Fig. 2. Panel A: Rats exposed to systemic fluoxetine (25 mg/kg IP; n = 8 rats) experienced respiratory arrest at an average time after occlusion onset of 63.8 s. Saline injected control rats (n = 8) arrested at an average time of 49.8 s after occlusion onset (p = .031). Panel B: There were no apparent differences in the inspiratory forces generated during breath attempts while occluded (the mean times to respiratory arrest are shown as vertical orange and blue lines). Panel C: The slopes of the linear regression lines did not differ statistically. The gray horizontal lines indicate the intersection of the linear regression line with the mean time to respiratory arrest. Panel D: There was no difference in the number of breath attempts between the two groups, but the increased time to respiratory arrest for the fluoxetine group indicated that the rate of breath attempts was reduced with the drug. Panel E: Frequency of breath attempts pre-injection with fluoxetine or saline, post-injection (i.e. pre-occlusion), and during occlusion are compared. There was no difference between saline and fluoxetine groups in the pre-injection measures, but these groups differed from each other at later times (pre-occlusion p < .0001, occlusion p = .0241). P values shown on the graph are from a two-way ANOVA with repeated measures after Tukey's multiple comparison test to correct for multiple comparisons. Other details are in the text. Panel F: The largest differential in respiratory (or respiratory attempts) rate was between baseline and drug or saline. Fluoxetine showed a much larger effect on the spontaneous breathing rate (p < .0001). The differential rate between pre-occlusion and occlusion was comparable for fluoxetine and saline treated animals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Fig. 2E), the increased time to respiratory arrest after fluoxetine treatment (Fig. 3A) was not associated with an increased number of breath attempts (Fig. 3D). The frequency of breath attempts made by fluoxetine-treated animals was less than that by saline-treated animals before and after airway occlusion (Fig. 3 E, F). As the contributions of muscular effort (forces generated during breath attempts and/or the number of breath attempts) are associated with the rate of oxygen desaturation, a lower respiratory rate would be expected to be associated with a longer time to respiratory arrest and protection from seizure-associated obstructive apnea. Whereas our study eliminates the complication of SSRI impact on seizure intensity, it does not address the possibility of SSRI impact on the intensity of laryngospasm as a cause of airway occlusion. Our study also does not permit us to address the potential SSRI impact on seizure-associated central apnea, a phenomenon that we have also argued is due to brainstem activity (activation of the diving reflex; Villiere et al., 2017).

9. Translation to SUDEP prevention in epilepsy patients

We speculate that the remarkable protections against death that have been demonstrated by Willott et al. (Willott and Henry, 1976) and Venit et al. (Venit et al., 2004) can be translated into practical protections for epilepsy patients. Relatively brief access to an oxygen atmosphere just prior to airway occlusion led to a significant prolongation of the time to respiratory arrest. Our data indicate that as little as 30 s of oxygen immediately prior to airway closure can cause enough of an extension of the time to respiratory arrest that it would improve survival odds if seizure activity caused obstructive apnea. Thirty seconds would be within the time window of a seizure itself, suggesting that oxygen could be protective even if supplied after a seizure starts. Fitting epilepsy patients for use in bed with either nasal cannula for oxygen delivery alone or a continuous positive airway pressure (CPAP) mask which provides oxygen plus provides pressure to prevent obstructive sleep apnea could prevent SUDEP (CPAP has even been suggested to reduce seizure frequency (Devinsky et al., 1994)).

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Declaration of Competing Interest

None of the authors (SM, RK, RG, JT, AB, KS, JBS, MS) have any conflicts of interest to declare.

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