HHS Public Access

Author manuscript

Respir Physiol Neurobiol. Author manuscript; available in PMC 2021 June 16.

Published in final edited form as:

Respir Physiol Neurobiol. 2020 June ; 277: 103438. doi:10.1016/j.resp.2020.103438.

P2X3 receptor antagonism reduces the occurrence of apnoeas in newborn rats

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Abstract

Hyperreflexia of the peripheral chemoreceptors is a potential contributor of apnoeas of prematurity (AoP). Recently, it was shown that elevated P2X3 receptor expression was associated with elevated carotid body afferent sensitivity. Therefore, we tested whether P2X3 receptor antagonism would reduce AoP known to occur in newborn rats. Unrestrained whole-body plethysmography was used to record breathing and from this the frequency of apnoeas at baseline and following administration of either a P2X3 receptor antagonist - AF-454 (5 mg/kg or 10 mg/kg s.c.) or vehicle was derived. In a separate group, we tested the effects of AF-454 (10 mg/kg) on the hypoxic ventilatory response (10 % FiO_2). Ten but not 5 mg/kg AF-454 reduced the frequency of AoP and improved breathing regularity significantly compared to vehicle. Neither AF-454 (both 5 and 10 mg/kg) nor vehicle affected baseline respiration. However, P2X3 receptor antagonism (10 mg/kg) powerfully blunted hypoxic ventilatory response to 10 % FiO_2. These data suggest that P2X3 receptors contribute to AoP and the hypoxic ventilatory response in newborn rats but play no role in the drive to breathe at rest.

Keywords

Apnoea of prematurity; P2X3 receptors; Carotid body; Neonates

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Declaration of Competing Interest

Both A.P.F. and J.G. were employees of Afferent Pharmaceuticals. The other authors declare no competing interests.

1. Introduction

Apnoea of prematurity (AoP) is highly prevalent in human infants born before 32 weeks of gestation, and its incidence is inversely related to gestational age and weight at birth (Henderson-Smart, 1981; Zhao et al., 2011). It is defined as an interruption of breathing for at least 20 s accompanied by both a desaturation of oxygen (SpO_2) to ≤ 80 % and bradycardia (heart rate lower than 2/3 of the baseline) for at least four seconds (Eichenwald et al., 2016; Ren et al., 2015; Zhao et al., 2011).

As with sleep apnoea, AoP is classified as either central (characterized by cessation of breathing effort), or obstructive where the airflow is obstructed usually at the pharyngeal level, or mixed (Eichenwald et al., 2016). In the short-term it may lead to cerebral hypoperfusion and eventually to ischemic brain injury (Pichler et al., 2003; Zhao et al., 2011). Indeed, persistence of AoP has been associated with neurodevelopmental impairment (Horne et al., 2017; Janvier et al., 2004).

The mechanisms underlying AoP are not fully understood, which makes the condition difficult to treat. Potential mechanisms causing AoP include: an immaturity of the brainstem respiratory rhythm generator, decreased central CO_2 sensitivity and increased peripheral chemoreceptor sensitivity to hypoxia (Martin and Wilson, 2012). An increase in carotid body sensitivity to hypoxia can trigger respiratory instability through an increase in chemical loop gain (Dempsey et al., 2014; Younes, 2014). In preterm infants with a high frequency of AoP, Nock et al. (2004) reported an exaggerated ventilatory response to hypoxia (15 % fraction of inspired O_2, FiO_2) consistent with CB hyperreflexia. Moreover, Cardot et al. (2007) found that acute hyperoxia (100 % FiO_2), used to reversibly block peripheral chemoreceptors, caused the greatest reduction in minute ventilation in the preterm infants that had the highest number of apnoeic episodes. These data suggest that peripheral chemoreceptor hyperreflexia may exist in infants with AoP.

Hyper-excitability of the carotid body has been found in animals with heart failure (Schultz et al., 2013). Our recent studies also demonstrated peripheral chemoreceptor hyperreflexia in the spontaneously hypertensive rat (SHR), which is caused by upregulated expression and activation of P2X3 receptors in chemoreceptive petrosal ganglion neurones (Pijacka et al., 2016). We showed that selective P2X3 receptor antagonism abolished aberrant tonic discharge of chemoreceptive petrosal neurones, and normalised the peripheral chemoreceptor reflex sensitivity measured from reflex evoked increases in sympathetic activity in the SHR *in vivo* (Pijacka et al., 2016). Finally, expression of P2X3 receptors can be regulated by the stage of development in rats. They have been shown to be higher in the intrinsic neurons of the myenteric plexus during the post-natal period but decline as maturity ensues (Xiang and Burnstock, 2004). Based on these findings, we tested the hypothesis that selective P2X3 receptor antagonism would reduce the frequency of the AoP in newborn rats.

2. Materials and methods

All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. Experiments were conducted on male Wistar rats on the day of birth (P0, total n = 60).

2.1. Experimental design

All perinatal pups exhibited apnoeas during the 10 min baseline recording within a range consistent with previous reports (Bairam et al., 2013a; Lefter et al., 2007). Two experimental protocols were performed in separate groups of animals. In the first, the effects of a selective P2X3 receptor antagonist - AF-454 on respiratory parameters and apnoea frequency were examined. In this protocol, pups were assigned randomly to one of three experimental groups: vehicle (n = 5), AF-454 (5 mg/kg, n = 5) or AF-454 (10 mg/kg, n = 7) all administered subcutaneously (s.c.). The experimenter was not blinded to these groups but data analysis was automated. In the second experimental protocol, the respiratory response to normobaric hypoxia (10 % FiO_2 in N_2) was assessed before and after AF- 454 (10 mg/kg s.c., n = 5).

In addition, to establish systemic exposure to AF-454, separate P0 rats (n = 34) were injected s.c. with either 5 or 10 mg/kg dose and subsequently killed by decapitation for blood and brain collection at three different time-points (10, 20 or 60 min after drug injection). The timings of collections were related to the time points at which respiratory variables were recorded. Blood was collected into lithium heparin tubes, processed in a refrigerated-centrifuge (10.000 rpm at 4 °C for 5 min) and the resultant plasma was stored at −80 °C. The brain was quickly extracted and placed into Eppendorf tubes, flash-frozen in liquid nitrogen and stored at −80 °C for subsequent analysis. Quantification of AF-454 was carried out by Afferent Pharmaceuticals (San Mateo, CA, USA) as described previously (Pijacka et al., 2016). The number of samples analysed for 5 mg/kg dose was: n = 5 at 10 and 20 min, and n = 4 at 60 min. For 10 mg/kg: n = 5 at 10 min, n = 7 at 20 min and n = 8 at 60 min.

2.2. P2X3 receptor antagonist

A P2X3 receptor antagonist, AF-454 (Afferent Pharmaceuticals, San Mateo, CA, USA), was freshly prepared on each experimental day. The drug was dissolved in propylene glycol (50 % propylene glycol in sterile distilled water) using an ultrasonic bath. Two different AF-454 doses were used in the present study: 5 mg/kg and 10 mg/kg. The drug was administered subcutaneously in a volume of 50–60 μl. An equivalent volume of propylene glycol (50 % in sterile distilled water) was used as a vehicle control.

2.3. Plethysmography

Unrestrained whole-body plethysmography (Emka Technologies, Paris, France) was used to record respiratory frequency, tidal volume and minute ventilation (Bairam et al., 2013a; Drorbaugh and Fenn, 1955). Newborn rats were placed inside an adapted, purpose-made, 45 mL animal chamber with an adjacent reference chamber that was connected to a differential pressure transducer and individual pneumotachographs. The temperature was kept constant

throughout the experiments by using a homoeothermic blanket set at 35 °C as previously described (Miller and Spear, 2010). Calibration of the plethysmography system was performed using a two-point method as recommended by the manufacturer. Humidity and temperature were recorded from the bias flow outlet. During all plethysmography experiments the bias flow was kept constant at 100 mL/min. After a 5 min period of adaptation to the plethysmograph, baseline data were acquired for 10 min followed by a dose of AF-454 (either 5 or 10 mg/kg s.c.) or vehicle, and breathing was recorded for a further 30 min.

2.4. Hypoxia exposure

After a five-minute adaptation period, restful breathing was recorded for 5 min in normoxia (21 % FiO_2 balance N_2; baseline). Subsequently the inlet flow was changed to a hypoxic mixture (10 % FiO_2 in N_2; hypoxia) for 3 min. A 10 % FiO_2 was reached within approximately 30 s from the start of its infusion and from this time onwards the ventilatory responses to hypoxia were measured. Then, the inlet flow was reverted to normoxic gas for a five min recovery period. All gases were humidified prior to animal exposure and the bias flow at both normoxia and hypoxia was kept constant at 100 mL/min. These procedures – baseline normoxic, hypoxic exposure and recovery – were performed three times: before AF-454 and at 10 and 20 min after AF-454 (10 mg/kg s.c.) administration.

2.5. Immunofluorescence

A separate group of pups (n = 4) were overdosed with ketamine (60 mg/kg, Vetalar, Zoetis, London, UK). The common carotid artery bifurcations were removed and the carotid bodies (CBs) were dissected under a stereo microscope (Leica M80) and fixed in ice-cold methanol/DMSO (4:1). Immunocytochemical labelling of P2X3 receptors and tyrosine hydroxylase was carried out as described previously (Pijacka et al., 2016). Briefly the carotid bifurcations were incubated with 10 % goat serum/0.1 % Saponin (Sigma-Aldrich, UK) and then transferred to the primary antibodies (rabbit anti- P2X3 receptor 1:25; APR026AN0202, Alomone, Israel; mouse anti–tyrosine hydroxylase 1:25; F-11, sc-25269, Santa Cruz Biotechnology). Antibody selectivity was confirmed as previously described using pre-absorption in anti-genic peptide (Pijacka et al., 2016). P2X3 receptors were visualised by goat anti-rabbit Alexa Fluor 488 (Thermo Fisher Scientific, UK) whereas tyrosine hydroxylase was visualized using the goat anti-mouse Alexa Fluor 594 (Thermo Fisher Scientific, UK). CBs were whole-mounted on slides using mounting medium (Vectashield, H-1000, Vector Laboratories) and later examined under a Leica DFC365FX (Leica Microsystems, UK) widefield microscope.

2.6. Plethysmography data analysis

Plethysmography data were acquired at a sampling frequency of 1000 Hz and analysed using IOX 2.9.5.28 software (Emka technologies, Paris, France). Respiratory flow was calculated from changes in chamber pressure taking into account the humidity and temperature of the air inside the chamber as described by Drorbaugh and Fenn (1955). Respiratory flow waveforms were imported into Spike2 (CED, Cambridge, UK) for analysis of the apnoeic events. We defined apnoeas as cessation in breathing lasting at least 3 breaths as proposed previously (Nanduri et al., 2012; Rieusset et al., 2013; Sheikhbahaei et al.,

2017). For automated apnoea detection, a running average of expiratory time (TE) in 1-minute bins was calculated; apnoeas were then identified as breaths having > 3-fold the average TE. Defining apnoeas based on the entire respiratory cycle based on automated detection could be tricky due to the presence of sighs. The frequency of apnoeas was counted over a 10 min epoch at baseline, 10 and 20 min following administration of either AF-454 or vehicle and expressed as number of apnoeas/10 min. Respiratory minute volume ($\dot{V}_E$) was calculated from respiratory frequency (f_R) and tidal volume (V_T); (i.e. f_R x V_T). In addition, Poincare plots and analysis of short-term variability (SD1) and long-term variability (SD2) were performed to assess the regularity of breathing before and after drug exposure as described previously (Brennan et al., 2001; Peng et al., 2011). Briefly, a Poincare scattergram was constructed by plotting each breath-to-breath interval (BB_n) against its subsequent interval (BB_{n+1}). The analysis was carried out by fitting an ellipse to the scattergram, where the centre of the ellipse represented the average BB_n. SD1 is the standard deviation of the scattergram perpendicular to the line of identity, whereas SD2 is the standard deviation of the scattergram along the line of identity. 200 breaths were used in these analyses.

2.7. Statistical analysis

Statistical analyses were conducted using SigmaStat 3.5 software (Systat Software Inc., San Jose, CA, USA). Normality of the data distribution was tested by Kolmogorov-Smirnov test. The statistical test performed is indicated in the Results or in the Figure legends. Data are presented as mean ± SEM, with a significance level of $p < 0.05$.

3. Results

3.1. Plasma and brain exposure of AF-454

Plasma and brain concentrations of AF-454 were measured in separate pups (n = 34) and data are presented in Fig. 1. The timings of blood samples were related to the time points at which respiratory variables were recorded.

3.2. Effect of AF-454 on baseline respiration

In normoxia, no differences in baseline respiratory frequency or tidal volume (and hence minute ventilation) were found after vehicle or either dose of AF-454 (5 or 10 mg/kg doses; Table 1).

3.3. Effects of AF-454 on apnoea frequency

AF-454 (10 mg/kg s.c., n = 7) reduced the occurrence of apnoeas from 6.3 ± 1.7 apnoeas/10 min at baseline to 3.1 ± 0.9 apnoeas/10 min at 10 min post drug administration (P = 0.027). By 20 min after drug delivery this was further decreased to 2.4 ± 0.7 apnoeas/10 min (P = 0.024; Fig. 2B). We noted that the apnoeas were not preceded by any change in ventilation relative to baseline. In contrast, both the lower dose of AF-454 (5 mg/kg s.c. n = 5) and vehicle (n = 5) did not change the frequency of occurrence of apnoeas (Fig. 2B).

At the higher dose, P2X3 receptor blockade also increased the regularity of respiration in newborn rats. Fig. 3C represents the grouped data of SD1 (short term variability) and SD2

(long term variability). AF-454 (10 mg/kg, n = 7) decreased SD1 at 10 (P = 0.011) and 20 min (P = 0.041) post drug administration indicating a lower inter-breath variability. However, AF-454 did not change SD2 at either time point (NS). Both SD1 and SD2 were unaffected in pups receiving the lower dose of AF-454 (5 mg/kg, n = 5).

3.4. Effects of AF-454 on the response to hypoxia

Fig. 4A shows the changes in respiratory frequency, tidal volume and minute ventilation in response to hypoxia (10 % FiO_2) before, 10 and 20 min after AF-454 (10 mg/kg s.c.) administration (n = 5). The reflex increases in respiratory frequency ($P < 0.001$) and minute ventilation ($P < 0.01$) in response to hypoxic exposure were blunted significantly after AF-454. Although tidal volume response to hypoxia presented a trend to a decrease, it was not significant (P = 0.055).

3.5. Immunofluorescence

Fig. 4B demonstrates co-localization of P2X3 receptors and tyrosine hydroxylase immunofluorescence in the carotid body from a newborn rat. The presence of these receptors in the carotid body was observed in three additional rat pups.

4. Discussion

The data presented demonstrate that P2X3 receptor antagonism reduced the frequency of occurrence of AoP in newborn rats. After acute antagonism of P2X3 receptors (10 mg/kg AF-454), the frequency of AoP was reduced significantly by 51 % and 61 % at 10 and 20 min post-treatment, respectively. Both tested doses of AF-454 (5 and 10 mg/kg) did not alter baseline respiratory parameters evaluated (f_R, V_T and $\dot{V}_{E)}$ indicating that P2X3 receptors are functionally inactive and do not play a role in the drive to breathe at rest under normoxic conditions. In contrast, the ventilatory response to hypoxia (f_R and $\dot{V}_{E)}$ was abolished after P2X3 antagonism with 10 mg/kg AF-454 suggesting that P2X3 receptors are crucial for the hypoxia-induced hyperventilation in newborn rat pups. We also confirmed that P2X3 receptors are expressed in the carotid body of P0 rats. Thus, our data suggest that P2X3-mediated endogenous signalling may contribute to AoP in this rat model. However, we cannot exclude a potential contribution from other signalling pathways within the carotid body or other afferent systems such as pulmonary/laryngeal mechanoreceptor afferents, which may also express P2X3 receptors (Cardot et al., 2007; Martin and Wilson, 2012). Given the finding of the antagonist within the brain (Fig. 1), we cannot rule out an effect within the central nervous system including an action on the central chemoreceptors, for example. However, this appears unlikely as despite numerous P2X receptor sub-types being identified in the ventrolateral medulla, which included the ventral respiratory column, P2X3 receptors were not identified (Thomas et al., 2001). Brain penetrance of this compound likely reflects a leaky blood brain barrier in these immature animals as AF-454 has not be found within the brain of mature rodents when administered systemically (A.P. Ford – personal communication). However, a caveat here is that the blood was not removed from the cerebral circulation so the brain samples did contain the drug within brain vasculature.

It has been suggested that peripheral chemoreceptor gain is increased in AoP and can trigger exaggerated ventilatory responses to hypoxia (Martin and Wilson, 2012). The resulting ventilatory overshoot would drop $PaCO_2$ below the central apnoeic threshold thereby eliciting a subsequent apnoeic episode (Al-Matary et al., 2004; Cardot et al., 2007). Such a mechanism is comparable to the heightened chemical loop gain thought to underpin sleep disordered breathing in adult humans (Dempsey et al., 2014; Eckert, 2018; Edwards et al., 2016). In this context, the reduction of peripheral chemoreceptor sensitivity with hyperoxia has lowered the incidence of sleep apnoeas (Edwards et al., 2014) and this supports the notion of the carotid body as a potential therapeutic target. Additionally, our study confirms that pharmacological antagonism of P2X3 receptors, which may include those within the carotid body, is a viable treatment strategy for rat AoP. Barrington et al. (1986), found that the respiratory stimulant – doxapram, reduced AoP in newborn humans. Although it is well established as a stimulant of carotid bodies, doxapram also activates central chemoreceptors (Osaka et al., 2014) so the mechanism of the response described by Barrington et al. (1986) remains equivocal.

P2X3 receptors in the carotid body appear to mediate its hyper-excitability in hypertensive rats, consisting of both hyperreflexia and aberrant tone generation (Pijacka et al., 2016). However, given the lack of a change in baseline respiration after P2X3 receptor antagonism, it appears that the carotid bodies of P0 rat pups do not generate aberrant tone, at least in afferent fibres connected to brainstem circuits controlling breathing (Zera et al., 2019). In contrast, Niane et al. (2011) suggested that P2X3 receptors could contribute to both the baseline drive to breathe and chemoreflex gain during hypoxia in newborn rats. These authors found that both suramin, a non-selective P2X receptor antagonist and A-317491, a selective P2X3 antagonist, decreased baseline ventilation and the responses to hypoxia in newborn rats. However, Niane et al. (2011) studied newborn rats at P4, P7, P12 and P21 while in the present study we only used newborn rats on the day of birth and a different selective P2X3 receptor antagonist. All told, that the contribution of P2X3 receptor signalling for baseline ventilation may change with development.

An interesting aspect of our study was the robust reduction in the ventilatory response to hypoxia after P2X3 receptor blockade in P0 day rats. These findings are in agreement with the results of Niane et al. (2011), who demonstrated that P2X3 antagonism caused a significant reduction in the respiratory responses to hypoxia in P4, P7 and P21 rats. Although the role of ATP in the carotid body during development of the rat is not fully explored, some studies have shown that ATP contributes to the hypoxic ventilatory response through P2X receptors in newborn rats from four days old and later (Bairam et al., 2013b; Niane et al., 2012, 2011). We speculate that at birth there is either enhanced ATP release to hypoxia and/or the P2X3 receptors are upregulated at this time to sensitise the detection of hypoxia, which may be a protective mechanism to ensure initiation and maintenance of respiration at the onset of air breathing. Thereafter these receptors may down regulate.

Given the lack of an effect of P2X3 receptor blockade on ventilation at rest, the question prompted is how blocking these receptors attenuates the AoP in newborn rats. We speculate that the P2X3 receptor antagonist was acting, in part, at the level of the carotid body. We did not observe any hyperventilation preceding the AoP suggesting that disturbances in the

partial pressure of carbon dioxide and chemical loop gain, known to trigger sleep apnoea in adults (Dempsey et al., 2014; Younes, 2014), are unlikely and therefore cannot explain the AoP recorded in the present study. However, we do not believe that this rules out a role for the carotid body in mediating AoP. As we recently reviewed (Zera et al., 2019), functionally separate sub-populations of glomus cells connected to distinct reflex pathways exist. We propose that one line of chemoreceptive petrosal neurone transmission contains P2X3 receptors that are connected to glomus cells that release episodically ATP and project to post-inspiratory neuronal circuits causing prolongation of their activity resulting in inspiratory off-switching and increasing expiratory time causing the apnoeas. Alternatively, we do not rule out mechanisms involving immaturity of other reflexes such as the Hering-Breuer deflation/inflation reflexes (Dutschmann et al., 2009, 2014; Hannam et al., 1998; Thach and Franz, 1978).

The data presented support peripheral P2X3 receptor antagonism as a potential novel approach to suppress AoP in newborn rats although penetrance into the brain may present undesirable off target effects. The treatment for AoP in human infants includes methylxanthines such as caffeine and theophylline (Henderson-Smart and De Paoli, 2013). However, methylxanthine therapy has diverse side-effects such as ta-chycardia, emesis and jitteriness (Eichenwald et al., 2016) and has been associated with necrotizing enterocolitis (Cox et al., 2016). Because of this, new therapies for the treatment of AoP are needed and P2X3 receptor antagonism could fulfil such a role.

Acknowledgements

This work was funded by grants from São Paulo Research Foundation (FAPESP; Grant #2016/02184-0) and British Heart Foundation. APA was funded by NIH (1R01AT008632-01). JFR is funded by a programme grant from the Health Research Council of New Zealand and the Royal Society of New Zealand. We thank Afferent Pharmaceuticals for the generous gift of AF-454.

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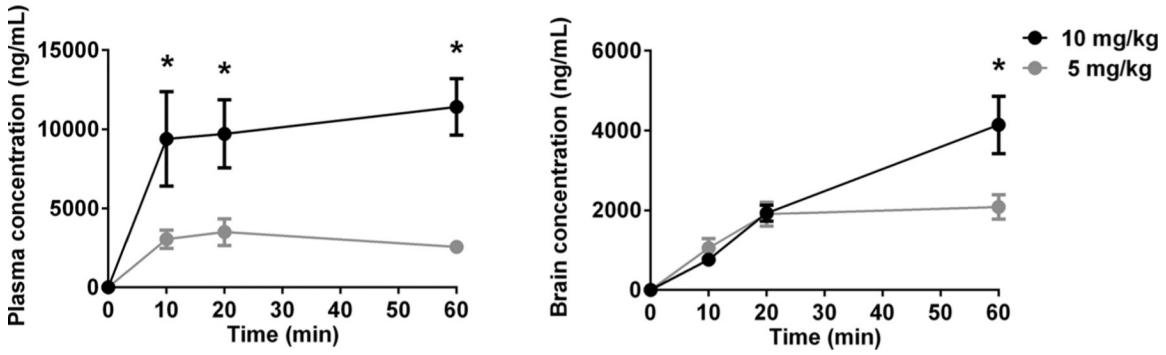

Fig. 1.

Pharmacokinetic profile of the two doses of AF-454 used in this study as detected in the plasma and brain from newborn rats at the day of birth (P0). Gray symbols, 5 mg/kg at 10 min (n = 5), 20 min (n = 5) and 60 min (n = 4); Black symbols, 10 mg/kg at 10 min (n = 5), 20 min (n = 7) and 60 min (n = 8). Data were analysed by two-way ANOVA with Tukey post hoc test. *P < 0.05 vs 5 mg/kg at the same time point.

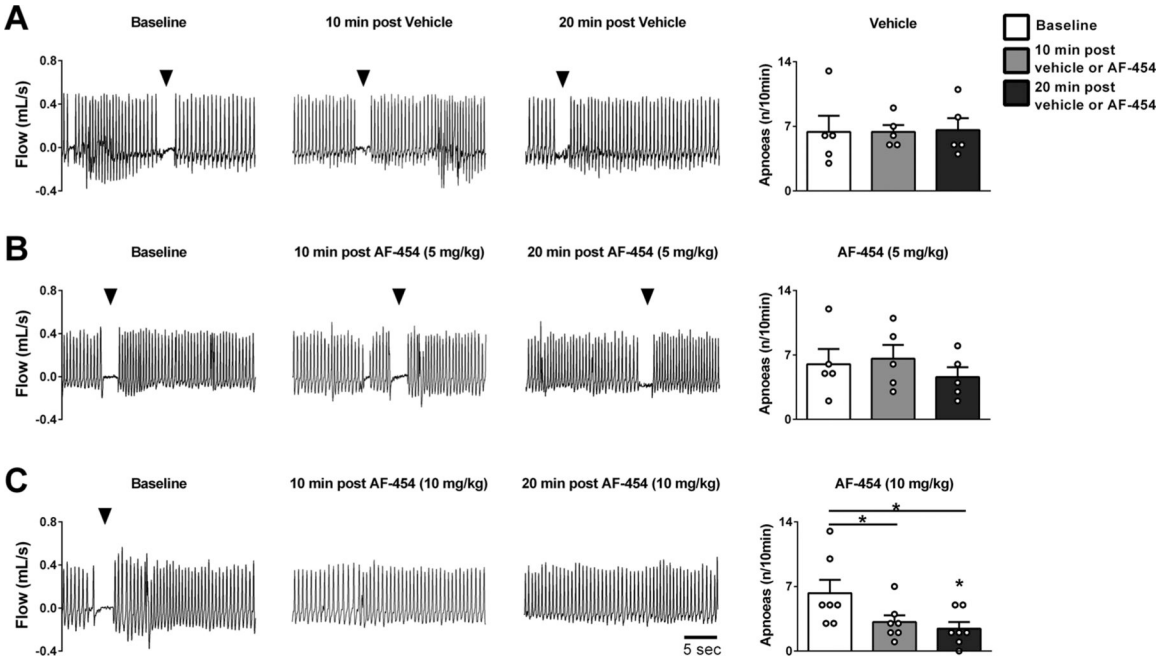

Fig. 2.

Representative plethysmographic recordings and grouped data showing the effects of vehicle or AF-454 on apnoea frequency at baseline, 10 and 20 min post injection. **A.** Vehicle (n = 5), **B.** AF-454 (5 mg/kg, n = 5) and **C.** AF-454 (10 mg/kg, n = 7). Arrows indicate an apnoea episode. Data were analysed by repeated measures one-way ANOVA with Tukey post hoc test. *P < 0.05 vs respective baseline values.

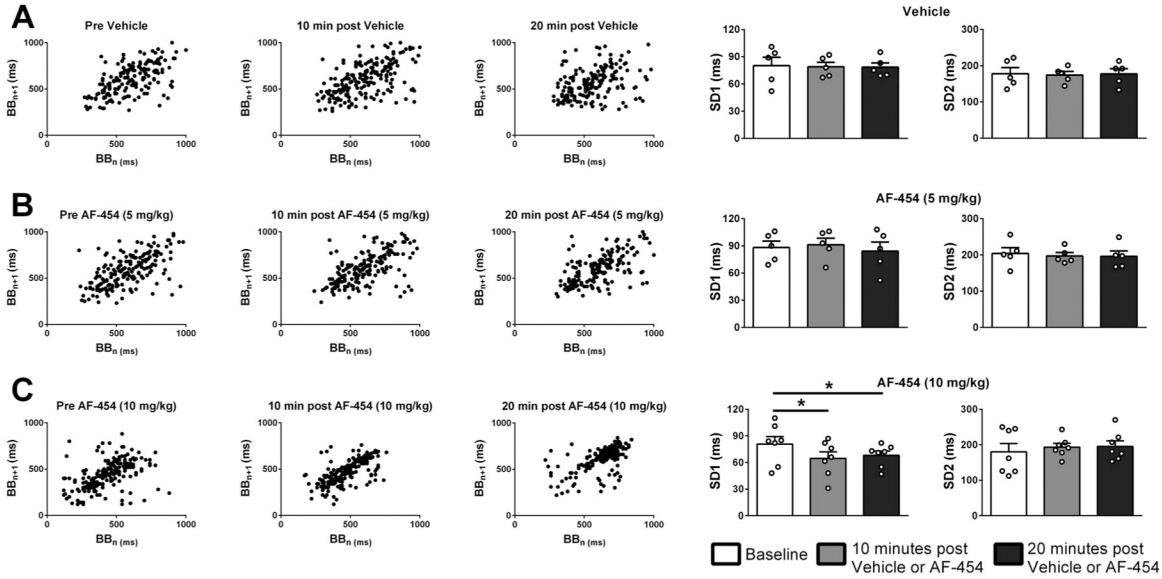

Fig. 3.

Representative Poincaré plots showing the consecutive breath-to-breath intervals and grouped data of SD1 (short term variability) and SD2 (long term variability) of breathing before, 10 and 20 min after vehicle or AF-454. **A.** Vehicle (n = 5), **B.** AF-454 (5 mg/kg, n = 5) and **C.** AF-454 (10 mg/kg, n = 7). Data were analysed by repeated measures one-way ANOVA with Tukey post hoc test. *P < 0.05 vs respective baseline values.

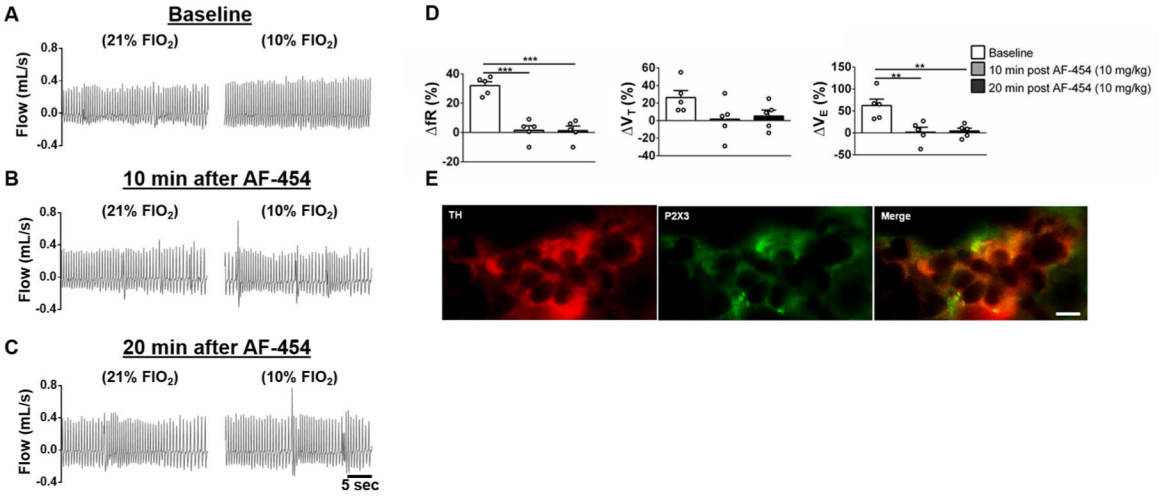

Fig. 4.

Effects of AF-454 (10 mg/kg, n = 5) on the acute ventilatory responses to hypoxia (10 % FiO_2) and P2X3 receptor immunostaining in the rat carotid body. **A, B** and **C.** Representative plethysmographic recordings during normoxia (21 % FiO_2) and hypoxia (10 % FiO_2) at baseline (A), 10 (B) and 20 min after AF-454 (10 mg/kg) administration. **D.** Grouped data showing the changes in respiratory frequency, tidal volume and minute ventilation in response to 10 % FiO_2 exposure at baseline, 10 and 20 min after AF-454 (10 mg/kg) administration. Data were analysed by repeated measures one-way ANOVA with Tukey post hoc test, **P < 0.01; ***P < 0.001 vs Baseline. **E**: P2X3 receptor and tyrosine hydroxylase (TH) immune-colocalization in the carotid body of newborn rats. Scale bar represents 10 μm.

Table 1

Respiratory parameters before and following AF-454 or vehicle administration.

	AF-454 (5 mg/kg) (n = 5)			**AF-454 (10 mg/kg) (n = 7)**			**Vehicle (n = 5)**		
	Baseline	**10 min**	**20 min**	**Baseline**	**10 min**	**20 min**	**Baseline**	**10 min**	**20 min**
f_R (breaths min $^{-1}$)	102 ± 3	101 ± 2	99 ± 4	104 ± 5	104 ± 5	101 ± 7	99 ± 6	94 ± 4	90 ± 6
V_T (ml kg $^{-1}$)	9.1 ± 0.3	9.2 ± 0.2	8.9 ± 0.4	8.5 ± 0.5	9.1 ± 0.8	9 ± 1	9.5 ± 0.9	10.1 ± 0.8	10.4 ± 1.5
$\dot{V}_E$ (ml kg^{-1} min $^{-1}$)	928 ± 44	926 ± 42	884 ± 53	892 ± 88	945 ± 127	940 ± 152	953 ± 112	984 ± 114	984 ± 172

Abbreviations: f_R: Respiratory frequency, V_T: Tidal volume, $\dot{V}_E$: Minute ventilation. For each time point, 10-minute epochs were considered for analysis. Data were analysed by repeated measures one-way ANOVA. No differences were found between the different time-points within each treatment group.