Vagal TRPV1 activation exacerbates thermal hyperpnea and increases susceptibility to experimental febrile seizures in immature rats

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ABSTRACT

Thermal hyperpnea, a pattern of breathing during hyperthermia that is characterized by an increase in tidal volume as well as breathing frequency, is known to lead to respiratory alkalosis. Thermal hyperpnea-induced respiratory alkalosis is linked to febrile seizures (FS). The heat-sensitive transient receptor potential vanilloid-1 (TRPV1) receptors are localized in, and implicated in the heat sensitivity of peripheral and central structures involved in the respiratory response to hyperthermia. We, therefore, hypothesize that TRPV1 activation increases susceptibility to experimental FS (EFS) in immature rats due to an exacerbated thermal hyperpnea. We found that peripheral, but not central TRPV1 activation had pro-convulsant effects. These pro-convulsant effects were associated with an increased rate of expired CO_2 due to an exaggerated ventilatory response to hyperthermia. The TRPV1 antagonist, AMG-9810, and TRPV1 deletion abolished the pro-convulsant effects, while exposure to 5% CO_2, bilateral vagotomy and DREADD (designer receptor exclusively activated by designer drugs)-mediated inhibition of TRPV1-containing cells in the vagal nodose ganglia significantly attenuated these effects. These findings suggest that vagal TRPV1-driven thermal hyperpnea likely increases susceptibility to FS in immature rodents. This identifies a novel peripheral anatomical and molecular target that should be considered when developing therapeutics for FS.

1. Introduction

From as early as the 1920s, respiratory alkalosis has been known to induce seizures (Rosett, 1924; Rotheram et al., 1964; Kilburn, 1966; Yang et al., 2014). This is possibly because of the nature of alkalosis to increase neuronal excitability (Balestrino and Somjen, 1988) and induce spontaneous epileptiform activity in the brain (Aram and Lodge, 1987). In fact, acidosis has been shown to decrease epileptiform discharges in rat entorhinal cortex-hippocampal brain slices (Velíšek et al., 1994) and decrease seizure susceptibility in carbonic anhydrase II deficient mice (Velisek et al., 1993). Respiratory alkalosis may have particular significance for pediatric febrile seizure (FS) genesis, as it is well recognized that the increase in body temperature that accompanies a fever, causes a rapid increase in respiration, especially in young children (O'Dempsey et al., 1993; Gadomski et al., 1994; Davies and Maconochie, 2009; Nijman et al., 2012). This increase in respiration can lead to alkalosis and decreased seizure thresholds (Shahar et al., 2004; Schuchmann et al., 2006; White, 2006).

Several studies have reported an association between hyperthermia-induced respiratory alkalosis and FS. Morimoto et al. (1996) showed that if rats are hyperventilated during hyperthermia such that respiratory alkalosis develops, the latency to FS onset is shortened. Subsequent studies showed that exposure to heated dry air results in hyperventilation and decreased FS thresholds due to respiratory-induced intra-cerebral alkalosis (Schuchmann et al., 2006; Schuchmann et al., 2008). Consistent with these animal studies, it was recently shown that children with FS are more likely to have an associated respiratory alkalosis compared to children with fever but no seizures, who tend to have metabolic acidosis (Schuchmann et al., 2011). These studies point to a potential link between respiratory alkalosis and FS, however the precise mechanism involved is unknown.

The transient receptor potential vanilloid-1 (TRPV1) receptor is a thermosensory cation channel that plays an important role in thermoregulation in response to changes in environmental and core body temperature (Romanovsky et al., 2009). TRPV1 receptors are expressed in central and peripheral locations involved in regulating the

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respiratory response to elevated temperatures including the hypothalamus, brainstem (Pleschka and Wang, 1975; Inomoto et al., 1983; Tryba and Ramirez, 2003; Khatibi et al., 2011; Fan-Xin et al., 2012; Zhao et al., 2016), carotid sinus nerve (CSN) and vagus nerve (Richards, 1968; Gleeson and Brackenbury, 1984; Fadic et al., 1991; Weller et al., 2011; Roy et al., 2012; Korobkin et al., 2013; Sato et al., 2014). TRPV1 has also been shown to underlie the heat-sensitivity of the CSN and vagus nerve (Ni et al., 2006; Roy et al., 2012), and could, therefore, possibly be involved in mediating the hyperthermia-induced respiratory alkalosis that has been linked to FS. Furthermore, recent studies suggest a role for TRPV1 in seizure genesis in various adult rodent seizure models. TRPV1 antagonists have been associated with anti-convulsant effects, while, TRPV1 agonists have been associated with both pro- and anti-convulsant effects, depending on the route of administration, drug dosage and seizure model (Manna and Umathe, 2012; Chen et al., 2013; Gonzalez-Reyes et al., 2013; Khom et al., 2013). There is, however, a paucity of studies investigating the effects of TRPV1 on seizure genesis in immature animals and specifically in pediatric FS.

In this study, we hypothesized that TRPV1 activation increases susceptibility to experimental FS (EFS) in immature rodents due to an exacerbated thermal hyperpnea (which in prior studies has been linked to respiratory alkalosis) (Fig. 1). We used the modified heated dry air model (Scantlebury et al., 2004) to induce EFS in postnatal day (P) 10 rats, TRPV1 KO and C57BL/6 control mice. We assessed the effects of TRPV1 agonists, piperine or capsaicin, and antagonist, AMG-9810, on seizure threshold temperature and latency following acute intraperitoneal (i.p.) or intracerebroventricular (i.c.v.) administration, to determine the site of action of the TRPV1 ligands. TRPV1 KO mice were used to confirm that the effects of the TRPV1 ligands were TRPV1-mediated. We used plethysmography to assess whether TRPV1 activation was associated with thermal hyperpnea, and we also determined whether the seizures were responsive to exposure to 5% CO_2. Finally, to identify the specific anatomical substrate of TRPV1-mediated thermal hyperpnea and subsequent seizure, we performed bilateral vagotomy or CSN denervation, and used designer receptor exclusively activated by designer drugs (DREADD) technology to inhibit TRPV1-containing cells, specifically in vagal nodose ganglia.

2. Materials and methods

2.1. Animals

Animal care and use conformed to the institutional policies and guidelines of the Health Science Animal Care Committee (HSACC), University of Calgary, Alberta, Canada. Experiments were performed on a total of 465 rats (220 females, 245 males), 41 mice; 12 TRPV1 KO (6 females, 6 males) and 29 C57BL/6 (20 females, 9 males) mice. All animals were kept on a 12 hour light/dark cycle with free access to standard rodent chow and water. Pregnant Sprague Dawley rats were obtained from Charles River Laboratories at E13 or E17 while TRPV1 KO (C57BL/6 background) and C57BL/6 mouse colonies were established in our mouse facility at the University of Calgary Health Science Animal Resource Center (HSARC) from TRPV1 KO (https://www.jax.org/strain/003770) breeding pairs and C57BL/6 (https://www.jax.org/strain/000664) breeding pairs obtained from Jackson Laboratories (Bar Harbor, ME, USA). The day of birth was considered as P0, and all experiments were performed at P10 or as indicated.

2.2. Agents used

Piperine (Sigma Aldrich, MO, USA) and AMG-9810 (Abcam Biochemicals, Cambridge, UK) were dissolved in a vehicle consisting of saline/DMSO/Tween-20 (7:2:1 ratio), while capsaicin (Sigma Aldrich, MO, USA) was dissolved in 100% ethanol/saline (1:1 ratio). The dose, route of administration and number of animals tested per group are outlined in Tables 1–4 or stated elsewhere. For the i.p. injections, 100 μL of piperine, AMG-9810 or vehicle was always administered 30 min prior to EFS or plethysmography, except in experiments where piperine-treated rats were pre-treated with AMG-9810. In that case, AMG-9810 was administered 30 min prior to piperine-treatment. For the i.c.v. injections, 5 μL of piperine, capsaicin, AMG-9810 or vehicle was administered 1 h prior to EFS or plethysmography. Since isoflurane can activate the TRPV1 receptor (Cornett et al., 2008), and takes ~7–9 min to be eliminated from the system in young rats (Chen et al., 1992), we waited 1 h after i.c.v. injection of the TRPV1 ligands before doing the EFS and plethysmography experiments, to ensure that the isoflurane was completely flushed from the system. In a separate experiment, we also assessed seizure thresholds 1 hour post-i.p. injection of 800 mg/kg piperine to ensure that piperine was still effective after 1 h. We used piperine instead of capsaicin for the peripheral studies because piperine is significantly less pungent (produces significantly less pain) than capsaicin (Ursu et al., 2010). Prior to drug injection, all pups were separated from the dam and allowed to acclimatize at room temperature (21–22 °C) for 30 min. Rectal temperature (T_{body}) was then measured in all animals just prior to drug injection (pre-injection T_{body}) and again after the specified period post-injection, before the start of EFS (post-injection T_{body}) using a RET-4 Type T thermocouple sensor (Physitemp Instruments Inc., NJ, USA) connected to the NI USB-TC01 thermocouple measurement device (National Instruments, TX, USA). Prior to EFS induction, animals were kept at room temperature before and after drug injection, unless stated otherwise.

2.3. Surgical procedures

All surgical procedures were conducted using sterile techniques. Animals were anesthetized with 3% isoflurane in 100% O_2, but ketamine/xylazine (100/10 mg/kg; CDMV, QC, Canada) was also used where indicated.
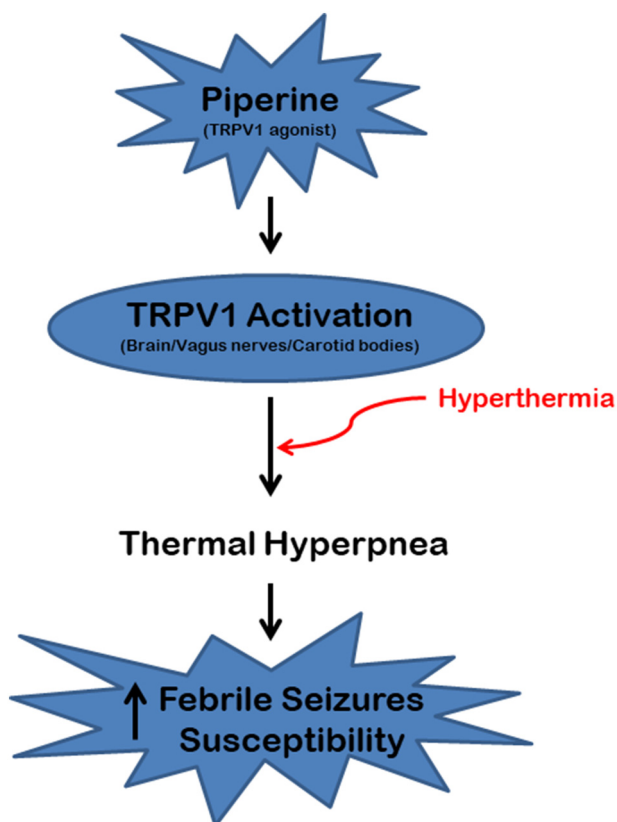

Fig. 1. Schematic of hypothesis.

Table 1

Baseline characteristics of postnatal day 10 rats subjected to experimental febrile seizure following i.p. or i.c.v. injection of the TRPV1 agonists, piperine (PIP) or capsaicin (CAP) and/or antagonist, AMG-9810 (AMG), with or without 5% CO_2 exposure. The data are presented as mean $\pm$ SEM. There were no differences between naïve and vehicle-injected pups and therefore these two groups were combined into one control group. In the i.p. dose response studies, rats treated with 200 mg/kg AMG were smaller than rats in the other treatment groups (*$p < 0.05$); one way ANOVA followed by Holm-Sidak post hoc comparison. Pre-injection body temperature (T_{body}) was lower in rats treated with 200 mg/kg AMG + 800 mg/kg PIP compared to controls (***$p < 0.001$), and post-injection T_{body} was lower than pre-injection T_{body} in most treatment groups (*$p < 0.05$; ***$p < 0.001$). Significant differences in post-injection T_{body} compared to controls (#$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$); two way repeated measures ANOVA followed by Holm-Sidak post hoc comparison. Significant differences in ΔT_{body} post-i.p. injection compared to controls (*$p < 0.05$); one way ANOVA followed by Holm-Sidak post hoc comparison. In the i.c.v. dose response studies, post-injection T_{body} was lower with 50 μg CAP compared to the pre-injection T_{body} (***$p = 0.001$); two way repeated measures ANOVA followed by Holm-Sidak post hoc comparison. Significant difference in ΔT_{body} post-i.p. injection compared to controls (*$p < 0.05$); one way ANOVA followed by Holm-Sidak post hoc comparison. In the 5% CO_2 exposure study, post-injection T_{body} was lower in all groups compared to pre-injection T_{body} (**$p < 0.01$, ***$p < 0.001$). Significant differences in post-injection T_{body} compared to vehicle (###$p < 0.001$) and compared to vehicle/CO_2 (!!!$p < 0.001$); two way repeated measures ANOVA followed by Holm-Sidak post hoc comparison. Significant differences in ΔT_{body} compared to vehicle (***$p < 0.001$), compared to vehicle/CO_2 (###$p < 0.001$) and compared to PIP (!$p = 0.023$); one way ANOVA followed by Holm-Sidak post hoc comparison.

	N	Body weight (g)	T_{body}: pre-injection (°C)	T_{body}: post-injection (°C)	ΔT_{body}: post-injection (°C)
i.p. (mg/kg)					
25 PIP	6	20.5 ± 1.2	31.3 ± 0.4	28.8 ± 0.5***	−2.5 ± 0.7
50 PIP	6	19.8 ± 1.2	30.4 ± 0.4	27.3 ± 0.6***,##	−3.1 ± 0.6
100 PIP	6	19.7 ± 1.6	30.6 ± 0.3	27.6 ± 0.5***,#	−2.3 ± 1.1
200 PIP	6	20.8 ± 1.2	30.7 ± 0.2	27.7 ± 0.4***,#	−3.0 ± 0.3
400 PIP	6	19.2 ± 1.3	30.9 ± 0.3	26.8 ± 0.8***,###	−4.1 ± 1.0*
800 PIP	10	19.3 ± 1.1	30.3 ± 0.3	26.5 ± 0.4***,###	−3.8 ± 0.4*
100 AMG + 800 PIP	6	19.8 ± 0.7	30.4 ± 0.2	26.4 ± 0.1***,###	−4.0 ± 0.2*
200 AMG + 800 PIP	6	19.5 ± 0.5	28.7 ± 0.5***	26.0 ± 0.2***,###	−2.7 ± 0.6
Naïve control	6	19.0 ± 1.2	31.1 ± 0.4	29.3 ± 0.5	−1.9 ± 0.4
Vehicle-injected control	11	19.9 ± 0.5	30.8 ± 0.2	29.3 ± 0.3	−1.5 ± 0.3
Control (vehicle-injected + naïve)	17	19.6 ± 0.5	30.9 ± 0.2	29.3 ± 0.3***	−1.6 ± 0.3
25 AMG	6	21.7 ± 0.5	30.7 ± 0.7	30.5 ± 0.4	−0.2 ± 0.4
100 AMG	6	22.4 ± 0.4	30.9 ± 0.7	28.9 ± 0.5***	−2.0 ± 0.5*
200 AMG	5	19.0 ± 1.5*	28.5 ± 0.8	27.4 ± 0.5*,#	−1.1 ± 0.5
300 AMG	6	23.2 ± 0.7	31.2 ± 0.6	30.2 ± 0.6*	−1.0 ± 0.3
Naïve control	5	22.2 ± 0.4	29.9 ± 0.4	29.9 ± 0.5	0.1 ± 0.1
Vehicle-injected control	7	21.7 ± 0.5	30.2 ± 0.5	29.9 ± 0.5	−0.3 ± 0.5
Control (vehicle-injected + naïve)	12	21.9 ± 0.3	30.1 ± 0.3	29.9 ± 0.4	−0.2 ± 0.3
i.c.v. (μg)					
1 PIP	6	20.0 ± 0.9	30.1 ± 0.4	30.4 ± 0.3	0.3 ± 0.4
10 PIP	7	19.4 ± 1.3	29.6 ± 0.5	29.3 ± 0.5	−0.3 ± 0.4
25 PIP	7	19.4 ± 0.8	29.7 ± 0.3	29.6 ± 0.4	−0.1 ± 0.4
100 PIP	7	22.1 ± 0.6	28.5 ± 0.4	28.6 ± 0.4	0.1 ± 0.3
1 AMG	6	20.5 ± 0.8	29.5 ± 0.3	29.6 ± 0.4	−0.1 ± 0.3
10 AMG	6	20.5 ± 0.3	30.1 ± 0.2	29.7 ± 0.4	−0.4 ± 0.2
25 AMG	7	20.7 ± 0.6	30.3 ± 0.3	29.6 ± 0.5	−0.6 ± 0.3
100 AMG	8	21.8 ± 0.6	28.6 ± 0.6	29.4 ± 0.4	0.7 ± 0.5
Naïve control	6	19.8 ± 0.9	30.2 ± 0.2	30.0 ± 0.4	−0.3 ± 0.3
Vehicle-injected control	10	20.9 ± 0.8	29.7 ± 0.4	28.7 ± 0.4	−1.3 ± 0.3
Control (vehicle-injected + naïve)	16	20.5 ± 0.6	29.9 ± 0.2	29.2 ± 0.3	−0.7 ± 0.3
25 CAP	7	18.9 ± 0.7	27.9 ± 0.3	27.4 ± 0.5	−0.5 ± 0.3
50 CAP	7	18.3 ± 0.7	28.0 ± 0.3	26.5 ± 0.4***	−1.4 ± 0.4*
Control (vehicle-injected)	6	18.8 ± 0.9	27.7 ± 0.4	28.1 ± 0.6	0.2 ± 0.4
5% CO_2					
Vehicle	9	20.9 ± 0.5	32.9 ± 0.3	31.9 ± 0.3**	−1.0 ± 0.4
PIP (800 mg/kg)	9	21.1 ± 0.4	32.8 ± 0.3	29.2 ± 0.3***,###,!!!	−3.6 ± 0.3***,###
Vehicle/CO_2	9	21.0 ± 0.5	32.9 ± 0.4	31.8 ± 0.4**	−1.1 ± 0.4
PIP (800 mg/kg)/CO_2	9	21.6 ± 0.4	33.2 ± 0.2	28.3 ± 0.2**,###,!!!	−4.9 ± 0.3***,###,!

2.3.1. Intracerebroventricular injections

A midline incision was made in the skin overlaying the skull and the pup positioned between two ear bars of a stereotaxic frame (Leica Biosystems, Ontario, Canada) for neonatal rat surgery. A Hamilton syringe was used to inject either the TRPV1 ligands (piperine, capsaicin or AMG-9810) or vehicle into the right lateral ventricle using the following coordinates from bregma: 0.6 mm posterior, 2 mm lateral, 3.3 mm ventral (Baram and Schultz, 1991). The skin was closed with

Table 2

Baseline characteristics of postnatal day 10 TRPV1 KO and C57BL/6 mice subjected to experimental febrile seizure following i.p. injection of TRPV1 agonist, piperine (PIP). The data are presented as mean $\pm$ SEM. Compared to vehicle-treated controls, post-injection T_{body} was lower in PIP-treated controls, and in vehicle and PIP-treated TRPV1 KO mice (***$p < 0.001$; one way ANOVA followed by Holm-Sidak post hoc comparison).

	C57BL/6				TRPV1 KO	
	Vehicle	PIP − 30 min	PIP − 15 min	PIP − 5 min	Vehicle	PIP − 5 min
N	7	9	4	9	6	6
Body weight (g)	6.4 ± 0.4	6.7 ± 0.2	8.0 ± 0.4	6.6 ± 0.4	5.5 ± 0.3	5.6 ± 0.3
T_{body}: post-injection (°C)	37.1 ± 0.5	33.8 ± 0.7***	29.2 ± 0.2***	31.0 ± 0.2***	31.6 ± 0.4***	32.4 ± 0.3***

Table 3

Behavioral response of postnatal day 10 rats subjected to i.p. or i.c.v. administration of the TRPV1 agonists, piperine (PIP) or capsaicin (CAP) and/or antagonist, AMG-9810 (AMG). The data are presented as mean ± SEM. No difference in surface righting or negative geotaxis latencies between treatment groups. Significant difference in open field latency between pups treated i.c.v. with 100 µg AMG and controls (*p = 0.035); one way ANOVA followed by Holm-Sidak *post hoc* comparison.

		Time (sec)		
	N	Surface righting	Negative geotaxis	Open field
i.p. (mg/kg)				
25 PIP	6	1.50 ± 0.22	32.44 ± 8.11	53.33 ± 14.63
50 PIP	6	1.67 ± 0.27	26.61 ± 9.61	43.33 ± 11.05
100 PIP	6	1.83 ± 0.19	32.22 ± 8.61	30.00 ± 13.93
200 PIP	6	1.56 ± 0.27	43.44 ± 11.23	62.83 ± 17.26
400 PIP	6	1.89 ± 0.57	41.94 ± 13.16	61.83 ± 17.83
800 PIP	10	6.47 ± 4.47	49.93 ± 9.71	72.83 ± 8.25
100 AMG + 800 PIP	6	1.89 ± 0.14	60.83 ± 9.08	58.00 ± 15.44
200 AMG + 800 PIP	6	1.22 ± 0.11	34.56 ± 8.87	54.06 ± 3.43
Naïve control	6	1.33 ± 0.17	21.22 ± 6.38	41.50 ± 12.27
Vehicle-injected control	11	1.36 ± 0.11	21.33 ± 4.18	42.06 ± 11.29
Control (vehicle-injected + naïve)	17	1.33 ± 0.09	21.17 ± 3.62	40.56 ± 8.68
25 AMG	6	1.17 ± 0.07	19.00 ± 4.71	39.00 ± 17.15
100 AMG	6	1.50 ± 0.17	36.83 ± 12.63	51.83 ± 11.36
200 AMG	5	1.20 ± 0.13	44.13 ± 11.91	72.73 ± 13.36
300 AMG	6	1.33 ± 0.12	28.22 ± 6.89	44.17 ± 15.78
Naïve control	5	1.47 ± 0.08	17.53 ± 3.05	37.60 ± 7.19
Vehicle-injected control	7	1.62 ± 0.21	26.71 ± 6.33	46.38 ± 13.43
Control (vehicle-injected + naïve)	12	1.56 ± 0.13	22.89 ± 4.00	42.72 ± 8.18
i.c.v. (µg)				
1 PIP	6	4.17 ± 2.97	26.72 ± 4.65	64.50 ± 16.05
10 PIP	7	2.29 ± 0.45	41.14 ± 11.84	52.14 ± 15.74
25 PIP	7	1.71 ± 0.22	30.38 ± 6.27	31.86 ± 15.07
100 PIP	7	3.38 ± 1.22	32.43 ± 8.49	67.86 ± 11.01
1 AMG	6	1.44 ± 0.32	44.89 ± 9.19	65.33 ± 15.96
10 AMG	6	1.67 ± 0.26	29.28 ± 10.78	18.83 ± 14.28
25 AMG	7	2.38 ± 0.88	35.76 ± 9.70	40.57 ± 14.29
100 AMG	8	2.50 ± 0.73	50.99 ± 10.59	80.25 ± 9.75*
Naïve control	6	1.11 ± 0.07	25.33 ± 5.97	53.83 ± 11.65
Vehicle-injected control	10	2.30 ± 0.35	33.47 ± 10.58	30.80 ± 10.78
Control (vehicle-injected + naïve)	16	1.85 ± 0.26	30.42 ± 6.89	39.44 ± 8.30
25 CAP	7	2.05 ± 0.20	33.38 ± 8.41	54.57 ± 15.87
50 CAP	7	4.53 ± 2.54	55.05 ± 11.25	74.43 ± 11.00
Control (vehicle-injected)	6	1.56 ± 0.19	34.78 ± 7.19	39.67 ± 16.38

Table 4

Baseline characteristics of postnatal day 10 rats subjected to plethysmography following i.p. or i.c.v. administration of the TRPV1 agonist, piperine (PIP) and/or antagonist, AMG-9810 (AMG), and vagotomy. The data are presented as mean ± SEM. In the i.p. study, baseline rectal temperature (T_{body}) was lower in all treatment groups compared to controls (*p = 0.014, ***p < 0.001), and different between PIP and AMG/PIP treated animals (##p = 0.008); one way ANOVA followed by Holm-Sidak *post hoc* comparison. Baseline respiratory rate (RR), minute ventilation ($\dot{V}E$) and rate of expired CO_2 ($\dot{V}_{CO2}$) were lower in rats treated with PIP and AMG/PIP compared to controls (all ***p < 0.001) and baseline RR, tidal volume (V_T) and $\dot{V}E$ were different between PIP and AMG/PIP treated animals (###p < 0.001, #p = 0.04, #p = 0.017; respectively); one way ANOVA followed by Holm-Sidak *post hoc* comparison. In the i.c.v. study, baseline parameters were not different between treatment groups. In the vagotomy study, body weight was higher in vagotomised/PIP treated rats compared to sham/vehicle controls (**p = 0.01); one way ANOVA followed by Holm-Sidak *post hoc* comparison. Baseline RR was lower in all treatment groups compared to sham/vehicle controls (***p < 0.001) and different between sham/PIP and vagotomised/PIP treated animals (###p < 0.001); one way ANOVA followed by Holm-Sidak *post hoc* comparison. Baseline V_T was higher in vagotomised/PIP treated rats compared to sham/vehicle controls (***p < 0.001) and different between sham/PIP and vagotomised/PIP treated animals (##p = 0.003); Kruskal-Wallis one way ANOVA on ranks followed by Tukey *post hoc* comparison. Baseline $\dot{V}E$ and $\dot{V}_{CO2}$ were lower in sham/PIP treated rats compared to sham/vehicle controls (**p = 0.009 and *p = 0.021, respectively); one way ANOVA followed by Holm-Sidak *post hoc* comparison.

	N	Body weight (g)	T_{body} (°C)	RR (breaths/min)	V_T (mL/g)	$\dot{V}E$ (mL/min/g)	$\dot{V}_{CO2}$ (mL/min/g)
i.p. (mg/kg)							
800 PIP	19	20.4 ± 0.8	32.6 ± 0.2***,##	76.2 ± 4.0***,###	$5.8^{-3} ± 3.1^{-4}$	0.44 ± 0.03***,#	$0.018 ± 6.6^{-4}$***
200 AMG	11	19.2 ± 0.8	33.8 ± 0.2*	153.4 ± 3.9	$4.9^{-3} ± 1.2^{-4}$	0.74 ± 0.03	$0.030 ± 1.9^{-3}$
200 AMG + 800 PIP	12	19.0 ± 0.9	31.8 ± 0.2***	123.2 ± 6.1***	$4.6^{-3} ± 1.8^{-4}$#	0.58 ± 0.05***	$0.017 ± 1.1^{-3}$***
Control (vehicle)	11	20.5 ± 1.2	34.9 ± 0.3	156.1 ± 6.2	$5.5^{-3} ± 3.9^{-4}$	0.83 ± 0.05	$0.034 ± 2.8^{-3}$
i.c.v. (µg)							
25 Piperine	5	19.4 ± 1.2	35.1 ± 0.4	149.0 ± 13.7	$6.8^{-3} ± 4.7^{-4}$	1.01 ± 0.11	$0.034 ± 3.8^{-3}$
100 Piperine	5	20.4 ± 0.8	34.1 ± 0.4	121.0 ± 6.4	$5.4^{-3} ± 2.8^{-4}$	0.66 ± 0.06	$0.023 ± 2.4^{-3}$
25 AMG	5	19.8 ± 1.0	34.4 ± 0.3	117.9 ± 8.0	$5.8^{-3} ± 2.9^{-4}$	0.68 ± 0.07	$0.028 ± 3.7^{-3}$
100 AMG	5	19.8 ± 0.6	34.1 ± 0.4	127.9 ± 10.8	$6.4^{-3} ± 2.6^{-4}$	0.83 ± 0.08	$0.029 ± 2.9^{-3}$
Control (vehicle)	5	20.4 ± 0.7	34.2 ± 0.5	122.1 ± 8.7	$5.6^{-3} ± 3.9^{-4}$	0.69 ± 0.09	$0.031 ± 4.7^{-3}$
Sham + vehicle	8	19.3 ± 1.3	34.0 ± 0.3	155.7 ± 9.5	$4.3^{-3} ± 3.1^{-4}$	0.68 ± 0.08	$0.027 ± 2.3^{-3}$
Vagotomy + vehicle	8	21.5 ± 0.8	33.1 ± 0.4	65.4 ± 6.0***	$8.0^{-3} ± 1.2^{-3}$	0.49 ± 0.06	$0.022 ± 2.2^{-3}$
Sham + 400 mg/kg PIP	8	20.9 ± 0.4	32.9 ± 0.2	88.9 ± 8.1***	$4.7^{-3} ± 3.0^{-4}$	0.41 ± 0.04**	$0.018 ± 9.2^{-4}$*
Vagotomy + 400 mg/kg PIP	8	23.4 ± 0.6**	33.0 ± 0.3	41.6 ± 3.4***,###	$0.012 ± 9.6^{-4}$***,##	0.49 ± 0.04	$0.024 ± 2.0^{-3}$

Vetbond tissue adhesive and the pups were allowed to recover on a heating pad for 30 min. The pups were then transferred to room temperature for 30 min prior to EFS or plethysmography.

2.3.2. CSN denervation and vagotomy in P10 rats

A midline incision (2 cm long) was made on the ventral surface of the neck, from the sternum to the submandibular glands. The fascia was cut and the sternomastoid and sternocleidomastoid muscles carefully retracted. For the CSN denervation (n = 29), the carotid bifurcation was exposed, and the occipital artery retracted in order to visualize the carotid body and then the CSN were sectioned bilaterally. For the vagus nerve dissections (n = 31), the vagus trunk was separated from the common carotid artery and the sympathetic chain, using a fine glass micropipette and a 1–2 mm segment of the nerve was removed bilaterally. Sham-operated rats underwent the same surgical procedure to expose the CSN (n = 24) or vagus nerves (n = 30), but they were left intact. Vetbond was used to close the skin and the pups allowed to recover on a heating pad for 2 h prior to EFS or plethysmography.

2.3.3. AAV virus injections

Using a NanojectII microinjector (Drummond Scientific Company, PA, USA), neonatal rats (P1) were injected bilaterally in the nodose ganglia of the vagus nerves with 1 μL of a combination of 2×10^{10} genomic copies/μL of AAV2/9 pAAV promTRPV1-Cre-GFP Lot AAV281 (Cre recombinase) and 5.9×10^{10} genomic copies/μL of AAV2/9 pAAV-hSYN-DIO-hM4D(Gi)-mCherry Lot AAV202 (inhibitory DREADD; n = 15) or 5.3×10^{10} genomic copies/μL of AAV2/9 pAAV-syn-DIO-mCherry Lot AAV295 (control; n = 13), obtained from the Canadian Neurophotonics Platform Viral Vector Core at the University of Laval (Quebec, Canada). The rats were allowed to recover on a heating pad and then returned to the dam. At P10, pups were administered 10 mg/kg of Clozapine N-oxide (CNO; Cayman Chemical, MI, USA) i.p., 1 h prior to EFS to activate the DREADD.

2.4. Behavioral tests

In the dose response experiments, rat pups treated i.p. or i.c.v. with the TRPV1 ligands were subjected to the following behavioral tests, just prior to EFS, to assess drug toxicity: surface righting, negative geotaxis and open field activity, as previously described (Scantlebury et al., 2010).

2.5. Induction of EFS

At P10, EFS was induced using the modified heated dry air model, as previously described (Scantlebury et al., 2004). Prior to heat exposure, a fine thermocouple was inserted into the rectum for continuous measurement of T_{body}. Pups were placed at the bottom of a Plexiglas box (height: 32 cm × width: 30 cm) which was at an ambient temperature of 30 °C. Warm dry air (45–50 °C) was circulated throughout the box by a standard 2-speed hair-dryer (Conair, Ontario, Canada) that was fitted 27 cm from the bottom of the box. The pups remained in the box until a generalized convulsion (tonic-clonic movements of all four limbs, with loss of righting reflex (Scantlebury et al., 2004)) occurred, at which time the pups were placed on a surface at room temperature and recovery monitored for 20 min. The start T_{body}, seizure threshold T_{body}, ΔT_{body} and latency were recorded and the rate of rise of T_{body} determined by plotting the ΔT_{body} from the start of hyperthermia until seizure onset.

In a subset of vehicle or piperine-treated rats (n = 9 per group), 5% CO_2 was continuously delivered to the hyperthermic chamber prior to and during EFS induction to prevent the development of hyperthermia-induced respiratory alkalosis. The CO_2 concentration inside the chamber was monitored continuously by a CO_2 analyzer (Oxzilla™ II,

CA-2A; Sable Systems International, Inc., NV, USA). Piperine-treated rats were removed from the hyperthermic chamber and deemed to have not had a seizure if no seizures were observed after 2 standard deviations above the ΔT_{body} (6 °C) or seizure latency (> 8 min) determined previously in piperine-treated rats that were not exposed to 5% CO_2.

The effects of piperine on EFS expression were also assessed in P10 TRPV1 KO and C57BL/6 mice to confirm that the piperine effects observed in the rats were TRPV1-mediated and not due to off-target effects of piperine (Schoffmann et al., 2014; Hu et al., 2015; Kumar et al., 2015). Since metabolism is known to be different in rats and mice (Kreiling et al., 1986; Richardson et al., 1999; Radermacher and Haouzi, 2013), we did a time response study in C57BL/6 mice, to determine the optimal drug exposure period that would produce the most robust and reproducible effect. Piperine was injected i.p. in C57BL/6 mice (n = 4–9 per group) at 5, 15 or 30 min prior to EFS. The optimal drug exposure time was determined to be 5 min, and so TRPV1 KO mice were injected i.p. with either vehicle or piperine (n = 6 per group), 5 min prior to inducing EFS.

2.6. Plethysmography

Head-out plethysmography was used, as described previously (Barrett et al., 2016b), to evaluate the effects of the TRPV1 ligands on ventilation and the rate of expired CO_2 in response to hyperthermia. Each pup was placed in the plethysmograph and allowed to acclimatize at an ambient temperature (T_{amb}) of 30 °C for 30 min while room air was pulled through the head chamber at a rate of ~400 mL/min by a downstream pump (PP-2, Sable Systems International, Inc., NV, USA). Air exiting the head chamber was passed through a desiccant/membrane dryer (DM-110-24; Perma Pure LLC, NJ, USA) before it was sampled by a CO_2 analyzer (Oxzilla™ II, CA-2A; Sable Systems International, Inc., NV, USA). After acclimatization, breathing was recorded for 5 min at 30 °C (baseline), and at 50 °C (hyperthermia) for 15 min, which led to an increase in the T_{body} to an average value of 41.4 ± 0.2 °C. Inspiratory and expiratory airflow were detected by a pneumotach attached to a differential pressure transducer (Omega Engineering, Inc., Quebec, Canada) and connected to the open end of the head chamber. Respiratory signals were amplified (Amplifier 440, Brownlee Precision Co., CA, USA) and recorded using the Axon data acquisition system (Digidata 1322A, Axon Instruments Inc., CA, USA), and the data analyzed with LabChart8 Reader (AD Instruments Inc., Colorado Springs, CO). The respiratory rate (RR; breaths/min), tidal volume (V_T; mL/g), minute ventilation ($\dot{V}E$; mL/min/g) and rate of expired CO_2 ($\dot{V}_{CO2}$; mL/min/g) were calculated during quiet breathing at baseline and during hyperthermia. The RR was obtained directly from the traces, whereas integration of the airflow traces was used to calculate V_T. $\dot{V}E$ was calculated as the product of the RR and V_T. $\dot{V}_{CO2}$ was calculated as the product of the flow rate and the difference between the fractional concentration of CO_2 present in the inspired and expired air, normalized to body weight [*i.e.* flow rate (mL/min)·(FeCO$_2$-FiCO$_2$) / body weight (g)] (Barrett et al., 2016b). We used the $\dot{V}_{CO2}$ as an indicator of the status of the pH in the blood, as prior studies have shown that changes in the rate of expired CO_2 are proportional to changes in arterial P_{CO2} and [H$^+$] which is also tightly linked to changes in T_{body} (Smith et al., 1983; Schuchmann et al., 2006). Rectal T_{body} was measured continuously throughout the procedure.

2.7. In vitro vagus nerve recording

To assess the functionality of the inhibitory DREADD expressed in the nodose ganglion, we developed the superfused *in vitro* vagus nerve/nodose ganglion preparation. P21 rats were injected with the inhibitory or control DREADD as described above and allowed 9 days for DREADD

expression, as this was the time period allowed for DREADD expression in the P10 rats used in the EFS studies. DREADD-expressing and control rats (P30–P40) were heavily anesthetized with ketamine/xylazine and then decapitated at the lower cervical level. Older rats were used for this portion of the study as the nodose ganglion was larger, making it much less difficult to manipulate at this age *ex vivo*. The vagus nerve including the nodose ganglion was quickly dissected and transferred into a dissection dish containing artificial cerebrospinal fluid (aCSF; in mM: 115 NaCl, 4 KCl, 24 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 2 CaCl$_2$, 10 D-glucose, 12 sucrose) and equilibrated with carbogen (95% O$_2$–5% CO$_2$). After 10 min, the tissue was transferred to a recording chamber with a built-in heating circuit. A peristaltic pump was used to set the superfusion rate of aCSF at ~20 mL/min. Throughout the experiment, the superfusate was equilibrated with a computer-controlled gas mixture of 100 Torr P$_{O_2}$ and 36 Torr P$_{CO_2}$ balanced with N$_2$, and monitored using CO$_2$ and O$_2$ gas analyzers (models CA-2A and PA1B, respectively, Sable Systems, Las Vegas, NV, USA). Before reaching the recording chamber, the superfusate was passed through a bubble trapper and heat exchanger. The temperature of the superfusate was measured continuously and the effluent from the chamber was recirculated.

The vagus nerve on both the central and peripheral ends of the nodose ganglion was desheathed and the fibres from the central end were connected to a glass suction electrode to record the neural activity extracellularly. A reference electrode was placed close to the preparation. Neural activity was monitored using a differential AC amplifier (model 1700, AM Systems, WA, USA). The neural activity was amplified, filtered (300-Hz low cut-off, 1-kHz high cut-off), displayed on an oscilloscope, rectified, integrated (200-ms time constant) and stored on a computer using an analog-to-digital board (Digidata 1322A, Axon Instruments Inc., CA, USA) and data acquisition software (Axoscope 9.0, Axon Instruments Inc., CA, USA). The preparation was exposed to a brief temperature challenge (37 °C to 43 °C) to assess viability. To achieve the temperature change, two aCSF solutions (100 mL each) were placed separately in a dual chambered water bath; one maintained at 37 °C and the other at 43 °C (Sheldon Manufacturing Inc., Portland, Oregon, USA). At the temperature change, the superfusate was briefly switched from the 37 °C aCSF solution to the 43 °C aCSF solution. The temperature of the superfusate was continuously monitored with a digital thermometer (Omega 871A, USA) attached to the recording chamber. The superfusate was re-circulated at a flow rate of 15 mL/min which took precisely 1 min to attain the required temperature change. Preparations that failed to show a definitive increase in activity during the temperature challenge were discarded. After the temperature challenge, the preparation was left undisturbed for 30 min to stabilize before the start of the experimental protocol.

The following protocol was used to assess the effect of inhibitory DREADD activation on the temperature sensitivity of the vagus nerve: 1) the preparation was superfused with aCSF for 10 min at 37 °C to determine baseline neural activity; 2) the preparation was subjected to 2 brief (1 min) temperature increases to 43 °C as described above, separated by a 5 minute baseline interval; 3) CNO (100 μM) was then added to the bath and recirculated for 60 min at 37 °C to activate the inhibitory DREADD; 4) the preparation was subjected to another 1 minute temperature challenge; 5) CNO was washed out for 15 min at 37 °C; 6) the preparation was subjected to another 1 minute temperature challenge.

The data was analyzed offline using custom software (written by R.J.A. Wilson). Vagus neural activity was divided into 2 s time bins, and the activity in each bin was rectified and summed (and expressed as integrated neural discharge). The neural activity in response to the temperature challenge was normalized to the baseline temperature. The normalized peak neural activity in response to the temperature challenges were compared between rats expressing the inhibitory DREADD (n = 4) and rats expressing the control DREADD (n = 3).

2.8. Immunohistochemistry

Immunohistochemistry was performed as previously described (Barrett et al., 2016b). Following EFS, DREADD-treated and control pups were anesthetized with ketamine/xylazine, and the nodose ganglia dissected and incubated overnight in 4% PFA fixative solution (in 1 M PBS) at 4 °C. The tissue was then incubated in 30% sucrose solution (sucrose in 1 M PBS) for 72 h at 4 °C. The nodose ganglia were frozen in tissue freezing medium (VWR, PA, USA) before sectioned (20 μm) with a cryostat (Thermo Scientific, MA, USA). The tissue sections were collected directly onto superfrost plus microscope slides (VWR, PA, USA) and stored at −80 °C until processed.

The tissue sections were incubated for 2 h at room temperature in a blocking solution consisting of 1% normal donkey serum in PBS with 0.3% Triton-X (PBST). The sections were then incubated overnight in a humidity chamber at 4 °C in blocking solution with the primary antibodies, chicken anti-GFP (1:1000; Aves Labs, OR, USA), mouse anti-mCherry (1:1000; Abcam, Cambridge, UK) and rabbit anti-TRPV1 (1:2000; Alomone, Jerusalem, Israel). The tissue sections were then incubated for 2 h at room temperature with the secondary antibodies, Alexa Fluor 488 goat anti-chicken (1:1000; Invitrogen, CA, USA), Alexa Fluor 594 donkey anti-mouse (1:1000; Invitrogen, CA, USA) and donkey anti-rabbit Cy5 (1:1000; Jackson Immuno Research Labs, PA, USA). The sections were also stained with the nuclear stain, 4′diamidino-2-phenylindole (DAPI, 1 μg/mL; Sigma-Aldrich, MO, USA). The slides were coverslipped with fluoromount-G (SouthernBiotech, AL, USA) and Z-stacked photomicrographs of immunofluorescence-labeled nodose ganglia sections were imaged using the 40× objective on a Nikon A1R confocal microscope (Nikon Corporation, Tokyo, Japan). Confocal microscopic Z-stacked images were processed as maximum intensity projections using the NIS Elements software (Nikon Instruments, Tokyo, Japan), and co-localization of GFP and mCherry (for Cre-recombinase and inhibitory DREADD, respectively) or GFP and cy5 (for Cre-recombinase and TRPV1, respectively) noted.

2.9. Statistics

The results of both male and female pups were combined, as in our hands there were no sex differences in the thresholds of EFS, and epidemiological data from clinical studies have not shown significant gender differences in the incidence and prevalence of FS in humans (Stafstrom, 2002). We estimated the approximate sample sizes for this study as previously described (Whitley and Ball, 2002), using data from our preliminary studies and assuming a power of 80% and a significance level of 0.05. The data are presented as mean ± SEM. A one way ANOVA was used to compare body weight, start T$_{body}$, seizure threshold T$_{body}$, ΔT$_{body}$, seizure latency, behavioral responses and baseline breathing parameters between treatment groups. In the dose-response experiments (Table 1), there were no differences between naïve and vehicle-treated controls, and so both groups were combined into one control group. All control groups in subsequent experiments were vehicle-treated controls. Holm-Sidak *post hoc* comparisons were performed when significant effects were found. When the data were not normally distributed, the Kruskal-Wallis one way ANOVA followed by Tukey *post hoc* comparison was used to assess differences between treatment groups. The effects of drug treatment on T$_{body}$ pre- and post-injection, as well as on the respiratory response to hyperthermia were assessed by two way repeated measures ANOVA followed by Holm-Sidak *post hoc* comparison when significant effects were found. Only the first 6 min of the respiratory response to hyperthermia were included in the analysis, as the animals in all the treatment groups are expected to be in a pre-seizure state. Ventilation and the rate of expired CO$_2$ in piperine-treated animals are expected to be significantly depressed following their seizure at approximately 6 min, and so comparison with

the other treatment groups after 6 min would have been inappropriate. A student *t*-test was used to assess differences in the *in vitro* vagus neural activity in response to the temperature challenge between rats expressing the inhibitory and control DREADD. Differences were considered significant at $p < 0.05$.

3. Results

### 3.1. Peripheral, but not central, TRPV1 activation has pro-convulsant effects

To investigate the potential role of TRPV1 in FS genesis and whether its effects are centrally or peripherally mediated, we injected P10 rats with the TRPV1 agonists, piperine or capsaicin, and/or antagonist, AMG-9810, and assessed their effects on the seizure threshold temperature (T_{body}) and latency. Baseline characteristics of the rats are outlined in Table 1. Since post-injection T_{body} was different between control and treatment groups (Table 1), we also expressed the seizure threshold T_{body} as the change in T_{body} from the start of hyperthermia to seizure onset (seizure threshold ΔT_{body}) for each group, to correct for this difference in baseline T_{body}. However, it is unlikely that the variability in the baseline temperatures seen predominantly post-injection, would have an effect on the EFS thresholds as it has been elegantly shown by Millichap (1959) that significant differences in baseline temperatures (18.3–37.8 °C), alterations in the rate of rise of temperature and intensity of the heat current had no effect on EFS thresholds. Piperine, administered i.p. significantly reduced the thresholds of EFS in a dose-dependent manner (Fig. 2A–C). Lower doses of piperine (25–100 mg/kg) had no effect on seizure threshold T_{body}, ΔT_{body} or latency, but higher doses (200–800 mg/kg) reduced seizure threshold T_{body} by 4.3–7.2 °C and seizure latency by 2.8–5.3 min. Since i.p. administration of 800 mg/kg piperine produced the most robust

pro-convulsant effect on both the seizure threshold T_{body} and latency, this dose of piperine was used in subsequent experiments. Pre-treatment with 100 mg/kg AMG-9810 did not alter the pro-convulsant effect of piperine on seizure threshold T_{body}, but partially reversed the effect on seizure threshold ΔT_{body} and completely reversed the effect on seizure latency. Pre-treatment with 200 mg/kg AMG-9810 completely reversed the pro-convulsant effect of piperine, which indicates that this pro-convulsant effect of piperine is TRPV1-mediated. Interestingly, when AMG-9810 was administered i.p. by itself (Fig. 2D–F), it had no effect on the seizure thresholds. This suggests that if TRPV1 receptors are to play a role in FS genesis, they must first be sensitized prior to the febrile event.

Piperine is known to have off-target effects (Schoffmann et al., 2014; Hu et al., 2015; Kumar et al., 2015). Therefore, to confirm that its pro-convulsant effects were entirely TRPV1-mediated and were not due to these off-target effects, we assessed the effect of piperine on the seizure thresholds in P10 TRPV1 KO mice. Baseline characteristics of the mice are presented in Table 2. We first performed a time-response study on C57BL/6 mice (Fig. 3A–C) since metabolism is known to be different in rats and mice (Kreiling et al., 1986; Richardson et al., 1999; Radermacher and Haouzi, 2013), and found that at 5 minute post-injection, piperine had the most robust pro-convulsant effect on seizure threshold T_{body}, ΔT_{body} and latency. This pro-convulsant effect of piperine was completely abolished in TRPV1 KO mice (Fig. 3D–F), confirming that the pro-convulsant effect of piperine was entirely TRPV1-mediated and not due to off-target effects. The lower absolute seizure threshold T_{body} observed in vehicle-treated TRPV1 KO mice compared to vehicle-treated controls (Fig. 3D) is likely due to the lower baseline T_{body} in these animals (Table 2). This is supported by the finding that despite the lower seizure threshold T_{body}, the same ΔT_{body} as controls was required for seizure onset in the TRPV1 KO mice (Fig. 3E), similar to what we reported previously in naïve TRPV1 KO and control mice at
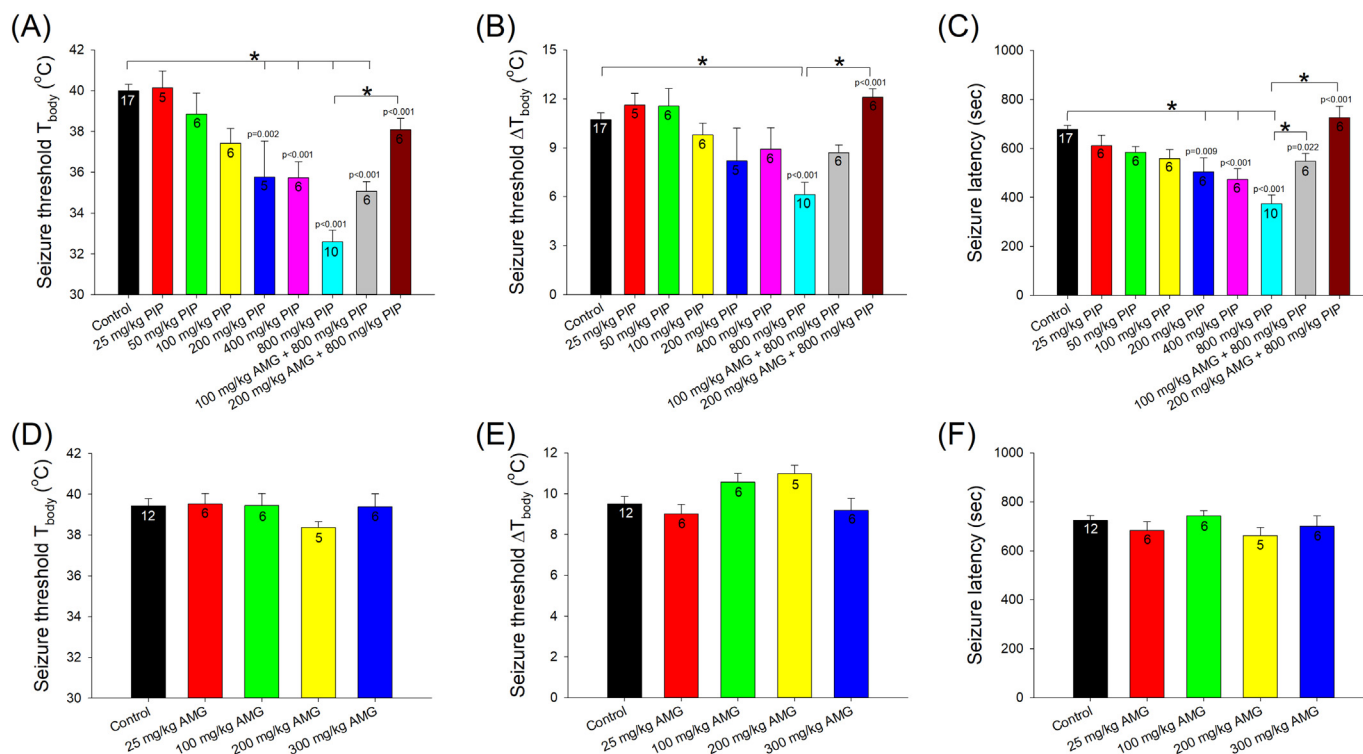
Fig. 2. Piperine (PIP) administered i.p. decreases experimental febrile seizure thresholds in a dose-dependent fashion that is reversed by the TRPV1 antagonist, AMG-9810 (AMG), in postnatal day 10 rats. The data are presented as mean ± SEM. PIP administered i.p. decrease (A) seizure threshold temperature (T_{body}) at 200, 400 and 800 mg/kg, (B) seizure threshold ΔT_{body} at 800 mg/kg, and (C) seizure latency at 200, 400 and 800 mg/kg. Pre-treatment with 100 or 200 mg/kg AMG dose-dependently reversed the pro-convulsant effects of 800 mg/kg PIP, with complete reversal with 200 mg/kg. AMG-9810 administered i.p. by itself had no effect on (D) seizure threshold T_{body}, (E) seizure threshold ΔT_{body} or (F) seizure latency. One Way ANOVA followed by Holm-Sidak *post hoc* comparison.
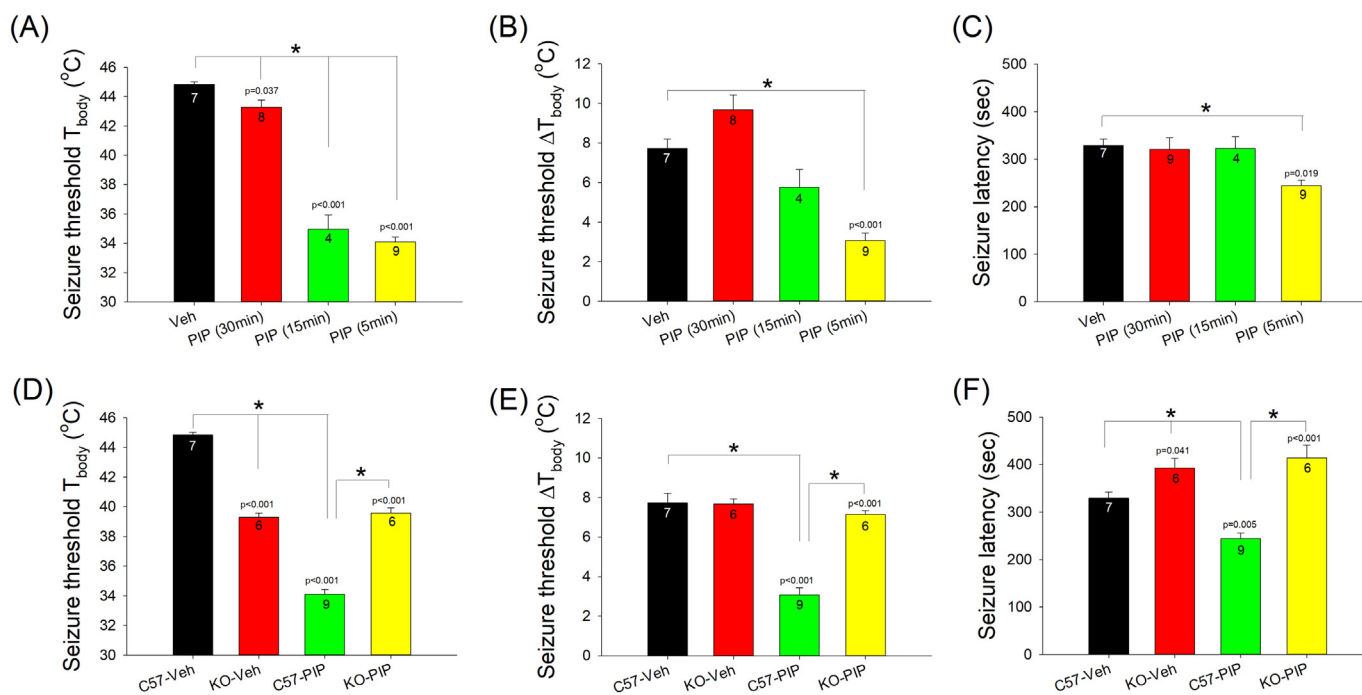
Fig. 3. The pro-convulsant effects of piperine (PIP) administered i.p. is abolished in postnatal day 10 TRPV1 KO mice. The data are presented as mean ± SEM. PIP (800 mg/kg) decreased (A) seizure threshold temperature (T_{body}), (B) seizure threshold ΔT_{body} and (C) seizure latency in a time-dependent fashion in C57BL/6 control mice, but had no effect on the seizure thresholds in TRPV1 KO mice (D–F). The data for the vehicle- and PIP-treated C57BL/6 mice in panels D–F were re-drawn from panels A–C. One-way ANOVA followed by Holm-Sidak *post hoc* comparison.

the same age (Barrett et al., 2016a).

Piperine administered i.c.v. (Fig. 4A–C) had no significant effect on either the seizure threshold T_{body}, ΔT_{body} or latency. Although capsaicin is more potent than piperine at the TRPV1 receptor (McNamara et al., 2005), it too did not alter the seizure thresholds when administered i.c.v. (Fig. 4D–F). In line with previous studies (Jancsó-Gábor et al., 1970), i.c.v. administration of 50 μg of capsaicin caused hypothermia (Table 1), which indicates that the dose and delivery of the drug was adequate, and that the pro-convulsant effects of piperine were peripherally as opposed to centrally mediated. Similarly, i.c.v. administration of AMG-9810 (Fig. 4G–I) had no effect on the seizure thresholds.

We used isoflurane to anesthetize the rats during the i.c.v. injections, however, it can also sensitize TRPV1 receptors and reduce the threshold to heat activation (Cornett et al., 2008), and has an elimination half-life of 7–9 min in young rats (Chen et al., 1992). Therefore, following the i.c.v. injections, we allowed 1 h before inducing EFS to ensure complete elimination of isoflurane from the system, compared to seizure induction 30 minute post-i.p. injection. To determine whether the absence of an effect of i.c.v. injection of piperine on the seizure thresholds was due to the effect of piperine wearing off during the 1 h from i.c.v. injection to seizure induction, we assessed whether piperine still had pro-convulsant effects if EFS was induced 1 hour post-i.p. injection of piperine. We found that piperine administered i.p. 1 h prior to EFS induction had a similarly robust pro-convulsant effect, indicated by a significant decrease in seizure threshold T_{body} (32.2 ± 0.6 *vs* 38.2 ± 0.4 °C in controls; $p < 0.001$), ΔT_{body} (6.6 ± 0.3 *vs* 9.3 ± 0.4 °C in controls; $p < 0.005$) and latency (499.8 ± 42.0 *vs* 709.5 ± 31.2 s in controls; $p < 0.01$). This indicates that the absence of a pro-convulsant effect of i.c.v. administration of piperine is not due to wearing off of the drug.

There were no significant effects of i.p. or i.c.v. administration of the TRPV1 ligands on behavior (Table 3), except for i.c.v. administration of 100 μg of AMG-9810, which caused the pups to stay in the open field longer than controls ($p = 0.035$). This suggests that the pro-convulsant effects of piperine administered i.p. were not due to adverse behavioral side effects of the ligands.

3.2. The rate of rise of body temperature cannot account for the pro-convulsant effects of piperine

Since some studies suggest an association between FS genesis and a rapid rate of rise of T_{body} (Berg, 1993), we asked whether this could account for the pro-convulsant effects of higher, as opposed to lower doses of piperine that we observed in the P10 rats. We found that the higher doses of piperine (100–800 mg/kg), some of which produced pro-convulsant effects, had no effect on the rate of rise of T_{body}, while the lower doses (25–50 mg/kg) which had no effect on the seizure thresholds, caused the rate of rise of T_{body} to increase more rapidly than controls (Fig. 5). This suggests that the rate of rise of body temperature cannot account for the pro-convulsant effects of piperine.

3.3. The pro-convulsant effect of piperine is associated with an exacerbated thermal hyperpnea

To determine whether the pro-convulsant effects of piperine were associated with thermal hyperpnea, we assessed ventilation and the rate of expired CO_2 in response to hyperthermia in P10 rats following i.p. or i.c.v. administration of vehicle, piperine and/or AMG-9810. We used 200 mg/kg AMG-9810 in the i.p. experiments as this dose was the most effective in completely reversing the pro-convulsant effects of piperine in the EFS studies. Baseline body weight, T_{body} and breathing characteristics are outlined in Table 4. Notably, i.p. injection of piperine reduced baseline RR, $\dot{V}E$ and $\dot{V}_{CO_2}$ by 51% ($p < 0.001$), 47% ($p < 0.001$) and 47% ($p < 0.001$), respectively, in line with its hypothermic effect. For this reason, we expressed the respiratory response to hyperthermia as the percent change from baseline.

Typical examples of the breathing traces at baseline and during early and late hyperthermia following i.p. injections for each treatment group are shown in Fig. 6A. Note the slower baseline (30 °C) breathing frequency in rat pups following i.p. administration of PIP compared to pups treated with AMG-9810 and control pups. By 3–4 min of hyperthermic exposure, there was a marked increase in breathing frequency in PIP-treated
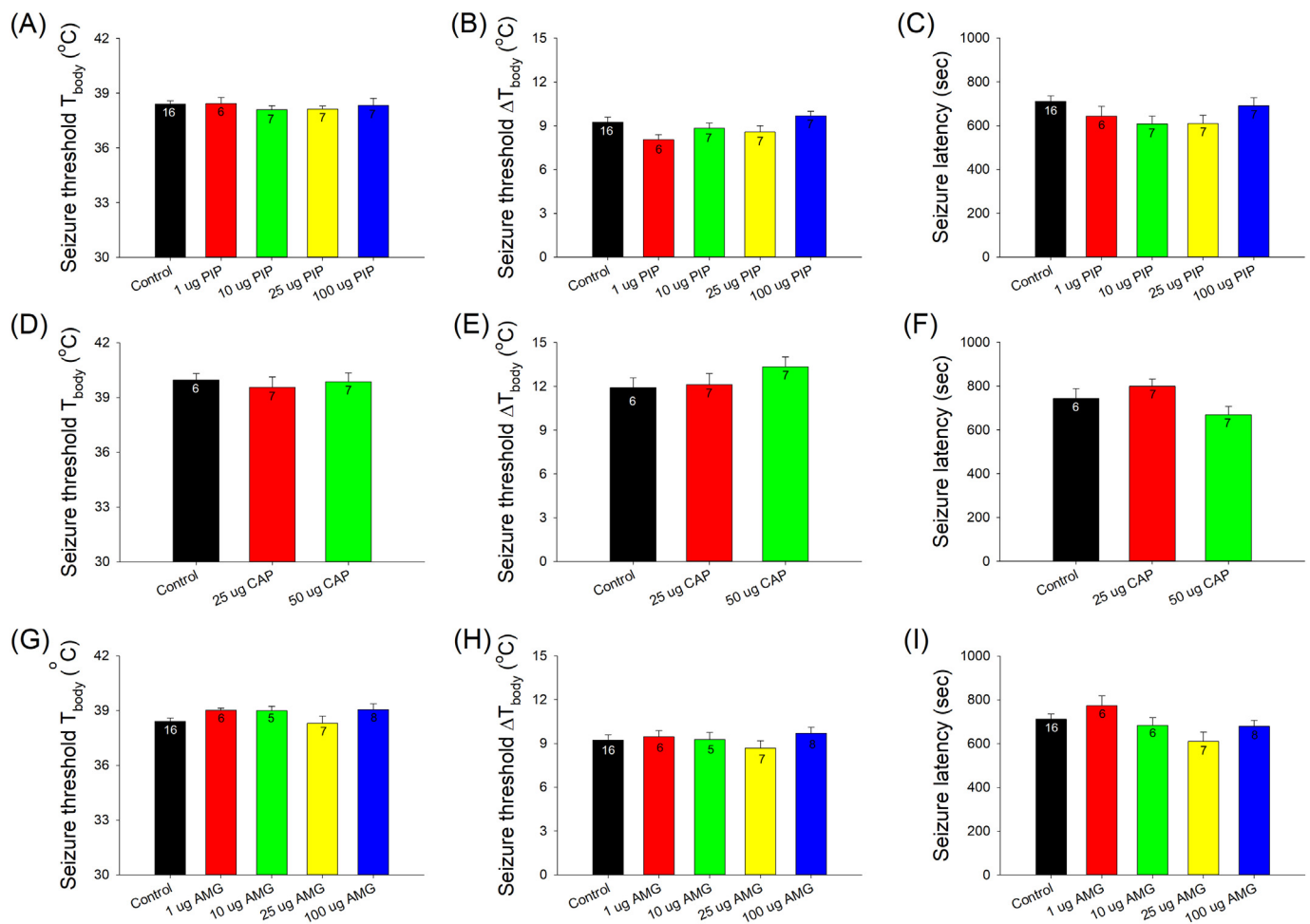
Fig. 4. No effect of i.c.v. administration of TRPV1 agonists, piperine (PIP) and capsaicin (CAP) or antagonist, AMG-9810 (AMG), on experimental febrile seizure thresholds in postnatal day 10 rats. The data are presented as mean ± SEM. Seizure thresholds were unaffected following i.c.v. injections of PIP (A–C), CAP (D–F) or AMG (G–I). One Way ANOVA.

animals. A similar increase in breathing frequency was observed much later in PIP-treated pups that were pre-treated with AMG-9810, while no such increase was observed in control pups or pups treated with only AMG-9810. In response to hyperthermia (Fig. 6B–E), i.p. administration of piperine induced a rapid, early thermal hyperpneic response, characterized by significant increases in RR, V_T, $\dot{V}E$ and $\dot{V}_{CO2}$. The peak of this thermal hyperpneic response was within 3–4 min of hyperthermia, which is just prior to the latency of seizure onset (6.2 ± 0.6 min) observed with this treatment in the EFS studies (we could not reliably score the seizures while the pups were in the plethysmograph). The early increase in $\dot{V}E$ and $\dot{V}_{CO2}$ suggests a link between thermal hyperpnea and the early seizure onset seen in piperine-treated animals. Pre-treatment with AMG-9810 significantly attenuated these effects of piperine, pushing the peak of the thermal hyperpneic response from 3 to 4 min to later during the hyperthermic exposure (14–15 min), which is later than the latency of seizure onset (12.1 ± 0.8 min) observed with this treatment. Similarly, the peak thermal hyperpneic response in rats treated with vehicle or AMG-9810 only, was also observed later during the hyperthermic exposure (14–15 min), also later than the latency of seizure onset (vehicle:11.3 ± 0.3 min; 200 mg/kg AMG: 11.0 ± 0.6 min) observed with these same treatments. I.c.v. administration of the TRPV1 ligands (Fig. 6F–I) had no effect on the thermal hyperpneic response. Overall, these results indicate that peripheral as opposed to central TRPV1 receptor activation induces a rapid, early onset thermal hyperpnea, which is known to lead to respiratory alkalosis (White, 2006), and might account for the rapid, early onset EFS expression observed in piperine-treated rats.

3.4. 5% CO₂ attenuates the pro-convulsant effect of piperine

We found that exposure to 5% CO_2 prevented behavioral seizures in 4/9 (44%) piperine-treated rats. In the remaining piperine-treated animals (5/9), exposure to 5% CO_2 did not alter the pro-convulsant effect of piperine on seizure threshold T_{body} (Fig. 7A), but significantly increased the seizure threshold ΔT_{body} (Fig. 7B) and prolonged the latency (Fig. 7C). Interestingly, exposure to 5% CO_2 had no effect on the seizure thresholds in vehicle-treated animals, contrary to what has been previously reported (Morimoto et al., 1996; Schuchmann et al., 2006; Tolner et al., 2011).

3.5. Bilateral vagotomy, but not CSN denervation, attenuated the pro-convulsant effects of piperine

Since the results indicate that the effects of piperine on EFS expression are peripherally as opposed to centrally mediated, and likely involves thermal hyperpnea and possibly respiratory alkalosis, we hypothesized that TRPV1-expressing neurons in the vagus nerves and/or carotid bodies might be involved in the mechanism, as these structures are thought to play a role in the respiratory response to hyperthermia (Richards, 1968; Gleeson and Brackenbury, 1984; Roy et al., 2012). To investigate this possibility, we performed bilateral vagotomy or CSN denervation, and assessed the effect on EFS expression in piperine-treated P10 rats. Bilateral vagotomy significantly attenuated the pro-convulsant effect of piperine on seizure threshold T_{body} (Fig. 8A), and
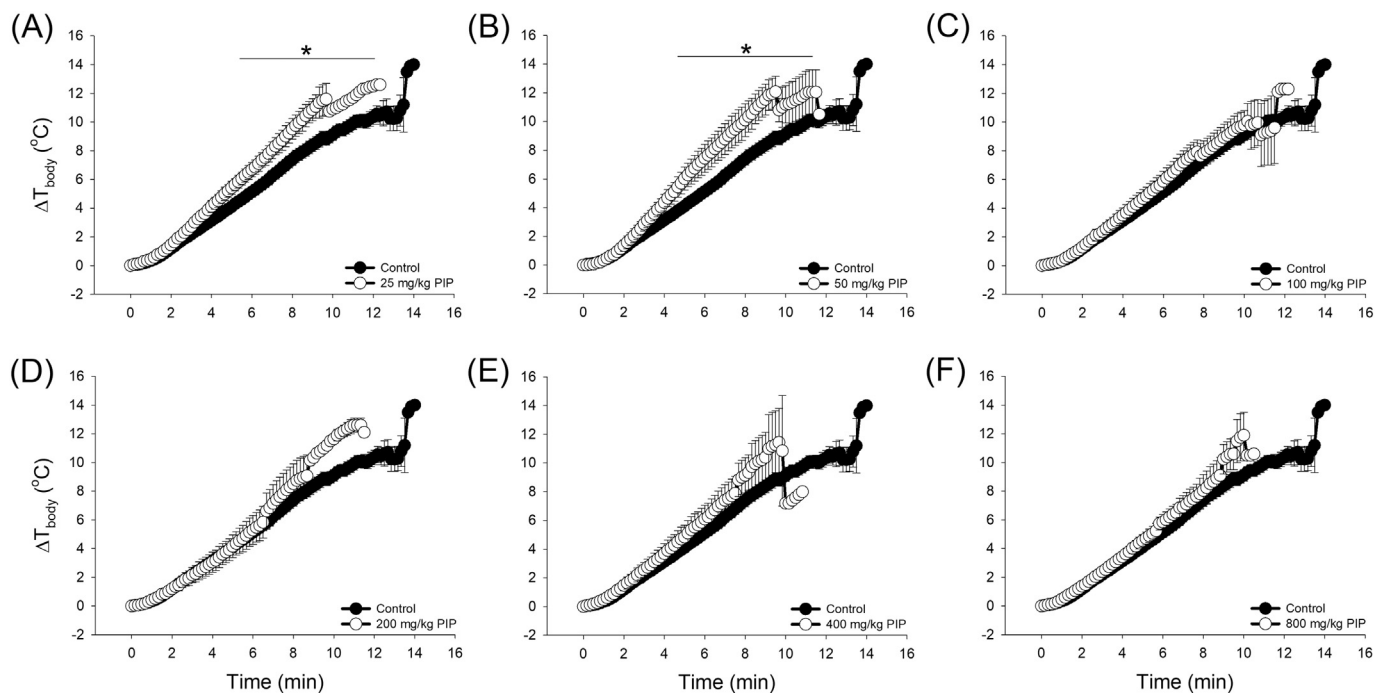
Fig. 5. The rate of rise of body temperature (ΔT_{body}) is faster with low doses of piperine (PIP) but not with high doses in P10 rats. The data are presented as mean ± SEM. Peripheral administration of PIP caused a faster rate of rise of ΔT_{body} at (A) 25 mg/kg (n = 5; *$p < 0.001$–0.048) and at (B) 50 mg/kg (n = 5; *$p < 0.001$–0.046) compared to controls (n = 17), but not at 100 mg/kg (n = 6), 200 mg/kg (n = 5), 400 mg/kg (n = 6) or 800 mg/kg (n = 10). Two-way repeated measures ANOVA, followed by Holm-Sidak *post hoc* comparison.

latency (Fig. 8C), but completely reversed the effect on seizure threshold ΔT_{body} (Fig. 8B). Bilateral CSN denervation, on the other hand, had no effect on the piperine-induced decrease in the seizure thresholds (Fig. 8D–F). This suggests that TRPV1 receptors localized in the vagus nerves but not in the carotid bodies are involved in mediating the pro-convulsant effects of piperine.

3.6. Bilateral vagotomy abolished the effect of piperine on thermal hyperpnea

To determine whether the effects of piperine on thermal hyperpnea are mediated by TRPV1 receptors in the vagus nerves, we administered piperine i.p. and assessed its effect on ventilation and the rate of expired CO_2 in response to hyperthermia in bilaterally vagotomised and sham-operated P10 rats. Baseline body weight, T_{body} and breathing characteristics of vagotomised and sham-operated rats are outlined in Table 4. In response to hyperthermia, vagotomy had no effect on the piperine-induced increase in *RR* (Fig. 9A), but completely abolished the early onset, piperine-induced increases in V_T (Fig. 9B), $\dot{V}E$ (Fig. 9C) and $\dot{V}_{CO2}$ (Fig. 9D). This suggests that activation of vagal TRPV1 receptors induces a rapid, early onset thermal hyperpnea, which is likely associated with the rapid, early onset EFS observed in piperine-treated rats.

3.7. Vagal nodose ganglia TRPV1-expressing neurons are involved in mediating the pro-convulsant effects of piperine on EFS expression

To tie TRPV1-expressing neurons in the vagus nerves to the piperine-induced seizure responses, we used DREADD technology to specifically inhibit TRPV1-expressing cells in the vagus nodose ganglia bilaterally (Fig. 10A,B). In line with previous studies showing that ~80% of nodose ganglia cells express TRPV1 throughout development (Korobkin et al., 2013), we observed variable, but high DREADD expression in the nodose ganglia of P10 rats. This DREADD expression

was restricted to TRPV1-containing cells as confirmed with confocal microscopy (Fig. 10C–H). Using the vagus nerve/nodose ganglion preparation to confirm functionality of the inhibitory DREADD (Fig. 10I–K), we found that in response to a temperature challenge, CNO-mediated activation of the inhibitory DREADD significantly blunted the nerve activity compared to controls. However, prior to CNO exposure and after CNO washout, the vagus nerve activity in response to the temperature challenge was not different between DREADD-expressing and control rats, thus confirming that the inhibitory DREADD expressed in the nodose ganglia is functional. We then assessed EFS thresholds in DREADD-expressing and control rats treated with or without piperine to confirm that TRPV1-expressing cells in the vagal nodose ganglia are involved in mediating the pro-convulsant effects of piperine (Fig. 10L–N). We found that CNO-mediated activation of the inhibitory DREADD significantly attenuated the pro-convulsant effect of piperine on the seizure threshold T_{body}, and completely reversed the effects on seizure threshold ΔT_{body} and latency. In rats treated with the control DREADD, CNO administration had no effect on the piperine-induced decreases in the seizure thresholds. This finding provides strong support that vagal TRPV1-containing cells are involved in mediating the pro-convulsant effects of piperine on EFS genesis in immature rats.

4. Discussion

4.1. Main findings

The major findings of this study are that the TRPV1 agonist, piperine, has pro-convulsant effects due to an exacerbation of thermal hyperpnea linked to peripheral activation of the TRPV1 receptors in the vagus nerve; thus implicating TRPV1 in the pathogenesis of FS. We also observed that the pro-convulsant effects of piperine were abolished in TRPV1 KO animals and reversed with 5% CO_2 supporting TRPV1-mediated respiratory mechanisms in FS genesis.
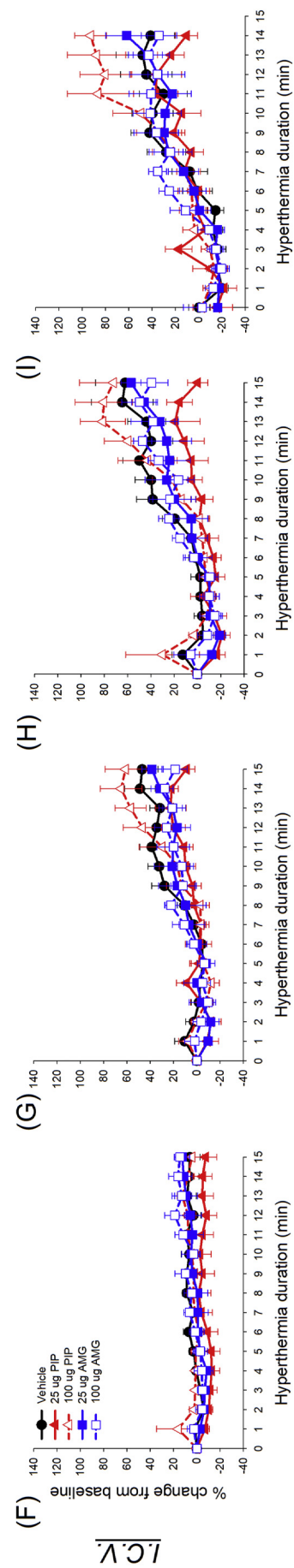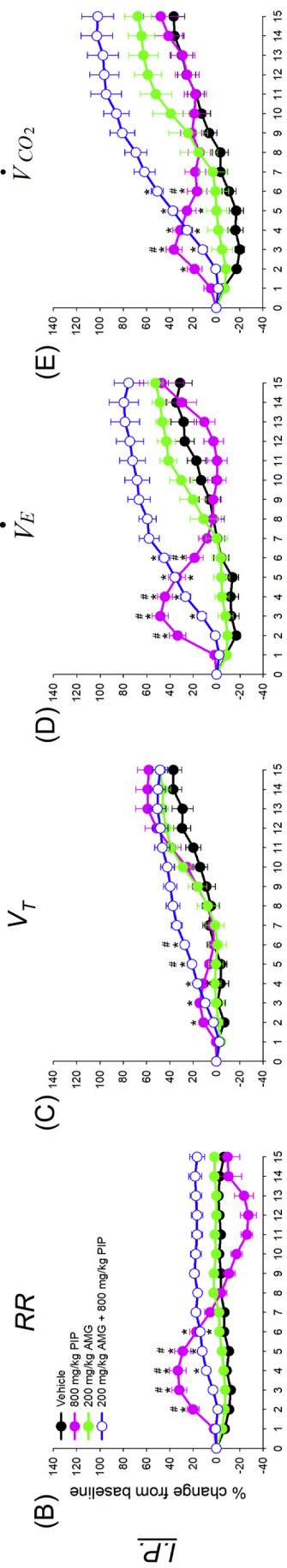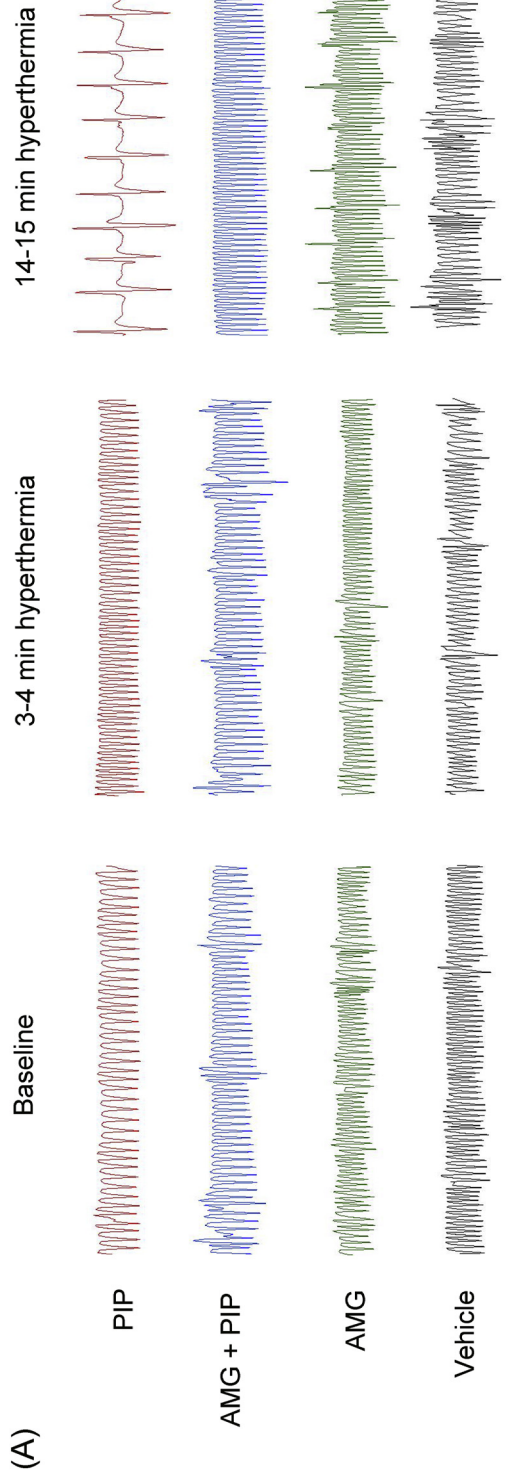
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Fig. 6. Peripheral (i.p.) administration of piperine (PIP) exacerbates thermal hyperpnea which is attenuated by the TRPV1 antagonist, AMG-9810 (AMG), in postnatal day 10 rats. The data are presented as mean ± SEM. (A) Representative breathing tracings from rats treated i.p. with the TRPV1 ligands. Vehicle (n = 11), PIP (n = 19), AMG (n = 11) and AMG/PIP (n = 12). Scale: vertical bar (0.05 V/s); horizontal bar (5 s). Compared to controls, PIP potentiated the (B) respiratory rate (RR) (*$p < 0.001$ at 2, 3, 4, 5, 6 min), (C) tidal volume (V_T) (*$p = 0.006$, *$p = 0.025$, *$p = 0.029$ at 2, 3, 4 min, respectively), (D) minute ventilation ($\dot{V}E$) (*$p < 0.001$ at 2, 3, 4, 5 min, *$p = 0.009$ at 6 min) and (E) rate of expired CO_2 ($\dot{V}_{CO2}$) (*$p < 0.001$ at 2, 3, 4, 5 min, *$p = 0.005$ at 6 min) in response to hyperthermia. Pre-treatment with AMG-9810 significantly attenuated these effects of PIP. [#]Significant differences between PIP and AMG/PIP treated rats at the times specified (RR: [#]$p < 0.001$ at 2, 3, 4 min, [#]$p = 0.01$, at 5 min; V_T: [#]$p = 0.017$, [#]$p < 0.001$ at 5 and 6 min, respectively; $\dot{V}E$: [#]$p < 0.001$ at 2, 3 min, [#]$p = 0.036$, [#]$p = 0.002$ at 4 and 6 min, respectively; $\dot{V}_{CO2}$: [#]$p = 0.011$ at 3 min, [#]$p < 0.001$ at 6 min). When administered by itself, AMG had no effect on ventilation or the rate of expired CO_2 compared to controls. (F–I) Neither PIP nor AMG had any effect on ventilation or the rate of expired CO_2 compared to controls (vehicle; n = 5) when injected i.c.v. at the following doses, PIP: 25 μg (n = 5) or 100 μg (n = 5); AMG: 25 μg (n = 5) or 100 μg (n = 5). Two way repeated measures ANOVA followed by Holm-Sidak *post-hoc* comparison.
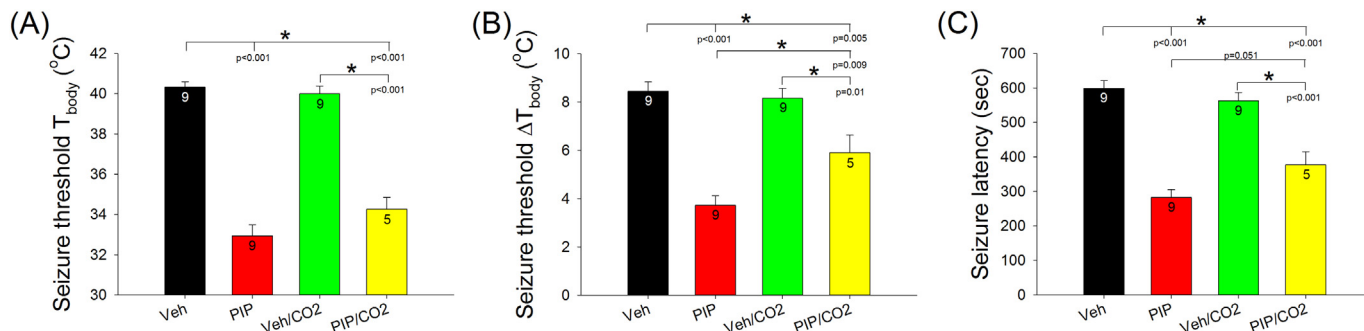
Fig. 7. Exposure to 5% CO_2 attenuates the pro-convulsant effects of i.p. administration of piperine (PIP) in postnatal day 10 rats. The data are presented as mean ± SEM. Exposure to 5% CO_2 during hyperthermia had no effect on (A) seizure threshold temperature (T_{body}), but significantly attenuated the effect of 800 mg/kg PIP on (B) seizure threshold ΔT_{body} and (C) seizure latency, and had no effect on seizure expression in vehicle-treated pups. One way ANOVA followed by Holm-Sidak *post hoc* comparison.
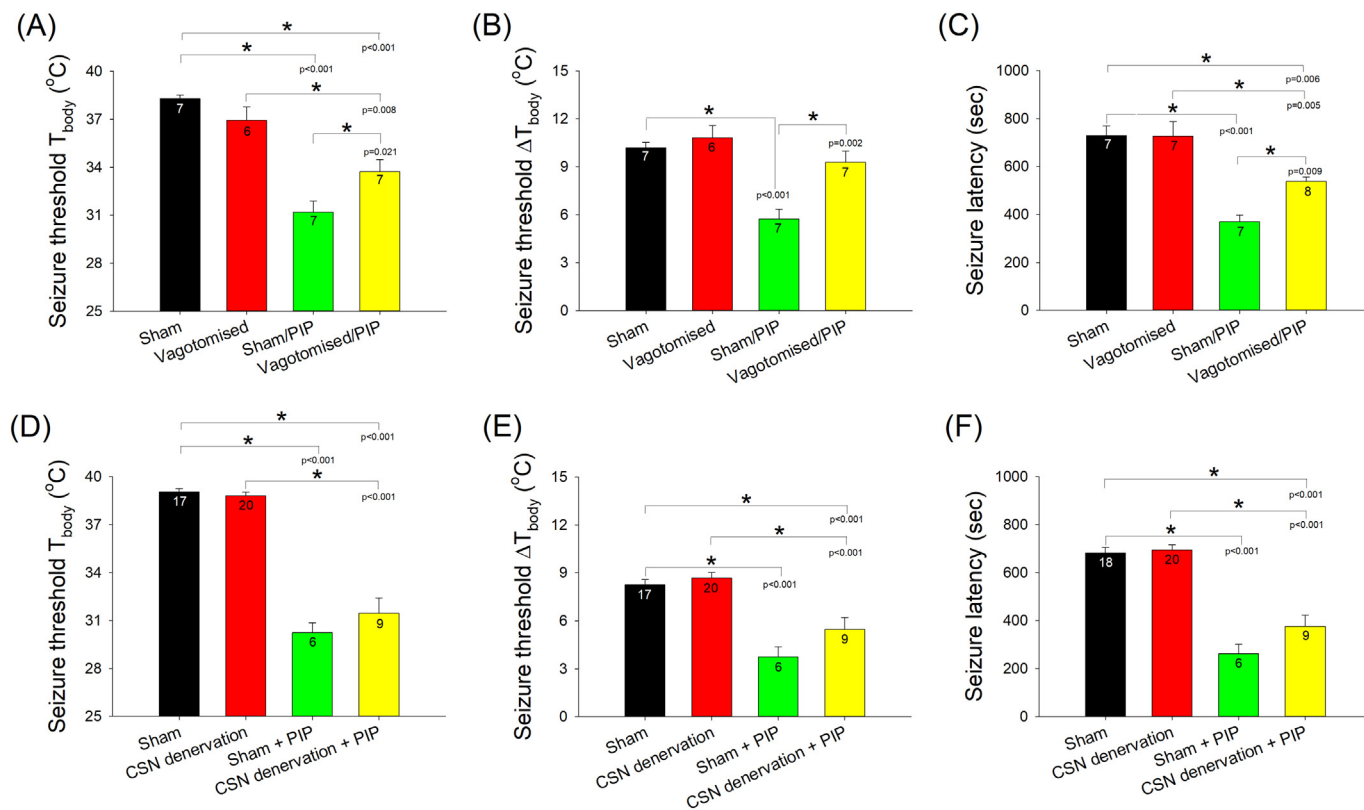
Fig. 8. Vagotomy, but not carotid sinus nerve (CSN) denervation, attenuates the pro-convulsant effects of i.p. administration of piperine (PIP) in postnatal day 10 rats. The data are presented as mean ± SEM. Bilateral vagotomy significantly attenuated the pro-convulsant effect of 400 mg/kg PIP on (A) seizure threshold temperature (T_{body}), and (C) seizure latency and completely reversed the effect on (B) seizure threshold ΔT_{body} (one way ANOVA followed by Holm-Sidak *post hoc* comparison). Bilateral CSN denervation did not alter the pro-convulsant effect of PIP on (D) seizure threshold T_{body}, (E) seizure threshold ΔT_{body} or (F) seizure latency.
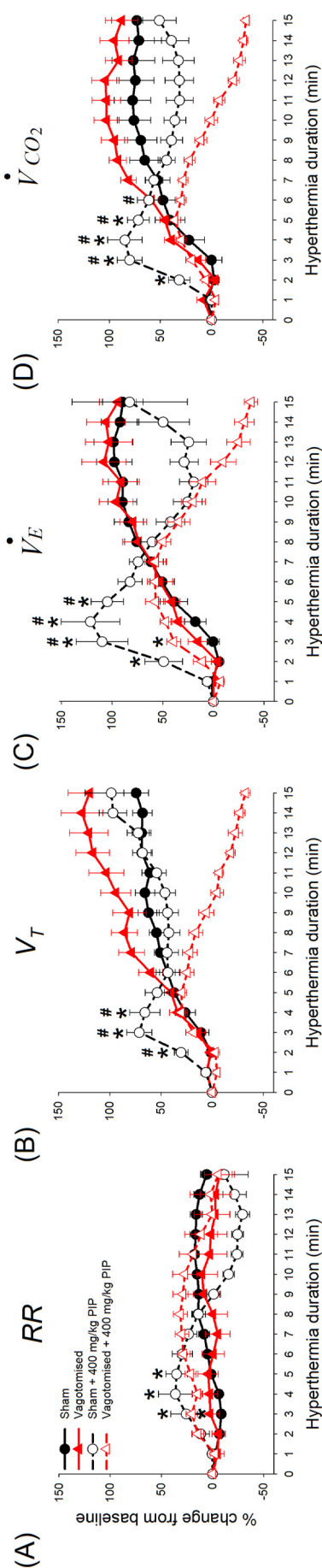
Fig. 9. Vagotomy abolishes the piperine (PIP)-induced early exacerbation of thermal hyperpnea in postnatal day 10 rats. The data are presented as mean ± SEM. PIP enhanced (A) respiratory rate (RR: $*p = 0.007$, $*p < 0.001$, $*p = 0.009$ at 3, 4, 5 min, respectively), (B) tidal volume (V_T; $*p = 0.04$, $*p < 0.001$, $*p = 0.003$ at 2, 3, 4 min, respectively), (C) minute ventilation ($\dot{V}_E$; $*p = 0.05$ at 2 min, $*p < 0.001$ at 3, 4, 5 min) and (D) rate of expired CO_2 ($\dot{V}_{CO_2}$: $*p = 0.025$ at 2 min, $*p < 0.001$ at 3, 4 min, $*p = 0.044$ at 5 min) in response to hyperthermia in sham/PIP-treated rats (n = 8) compared to sham/vehicle controls (n = 8). Bilateral vagotomy by itself (n = 8) had no effect on breathing or the rate of expired CO_2, but reversed the effects of PIP on V_T ($^#p = 0.015$, $^#p < 0.001$, $^#p = 0.007$ at 2, 3, 4 min, respectively), $\dot{V}_E$ ($^#p < 0.001$ at 3, 4 min, $^#p = 0.018$, $^#p = 0.046$ at 5, 6 min, respectively) in response to hyperthermia (n = 8). Two way repeated measures ANOVA followed by Holm-Sidak *post-hoc* comparison.

4.2. Activation of TRPV1 receptors produced pro-convulsant effects in a rodent model of EFS

The observation that peripheral and not central activation of TRPV1 receptors with piperine leads to pro-convulsant effects uniquely supports a peripheral based mechanism for the pathogenesis of EFS. The absence of a central effect of piperine is not due to an absence of TRPV1 receptors, as TRPV1 is ubiquitously expressed in the P10 rat brain (Khatibi et al., 2011), but suggests that sensitization of TRPV1 receptors in the brain is likely not sufficient to increase susceptibility to EFS. However, as piperine can cross the blood-brain barrier (Liu et al., 2013), it is plausible that activation of central TRPV1 receptors will render the brain more susceptible to other pro-convulsant factors such as that which can occur as a result of piperine-induced exacerbation of thermal hyperpnea, as observed in this study. Although it has been reported that piperine causes significantly less pain than the prototypical TRPV1 agonist, capsaicin (Ursu et al., 2010), we cannot rule out the possibility that pain induced by piperine treatment could have contributed to the responses observed in these animals, as we did not specifically test the animals for pain signals. However, we did not observe any pain-related behavioral differences in terms of vocalizations, rubbing, scratching or biting of the injection site in the animals treated with piperine compared to the other groups. Also, a typical sign of pain is increased ventilation (Jafari et al., 2017), but ventilation was decreased in piperine-treated pups 30 minute post-injection (Table 4), which suggests that piperine injection did not cause significant and enduring pain and discomfort sufficient enough to affect the interpretation of our results. This also supports that piperine treatment did not cause and likely did not contribute to the increased seizure susceptibility in this group of pups.

Although piperine is not specific for TRPV1 receptors, the fact that the pro-convulsant effect of piperine was reversed by the TRPV1 antagonist, AMG-9810, and in TRPV1 KO animals, supports that the pro-convulsant effect of piperine was TRPV1 mediated. Similarly, 5–40 mg/kg of capsaicin, the prototypical and more specific TRPV1 agonist, has been shown to have pro-convulsant effects (Jia et al., 2015). Given that capsaicin is > 100 times more potent than piperine (McNamara et al., 2005), it follows that the dose of piperine required to produce a similar TRPV1-mediated pro-convulsant effect would be between 500 and 4000 mg/kg, which is in line with our results. However, much lower doses of piperine has been shown in prior studies to have anti-convulsant effects (Chen et al., 2013; Khom et al., 2013). In our hands, we have not observed anti-convulsant effects of piperine at any of the doses tested. This may be due to the method of seizure induction and the age of animals tested. The mechanisms for the anti-convulsant effects of piperine are thought to involve sodium channel inhibition and modulation of GABAergic, serotonergic and/or opioid neurotransmission (Mori et al., 1985; Bukhari et al., 2013; da Cruz et al., 2013; Khom et al., 2013; Mishra et al., 2015). Piperine also inhibits calcium channels at low doses, which may also contribute to its observed anti-convulsant effects (Taqvi et al., 2008). These off-target effects of piperine may also account for why higher doses of this drug were needed to produce a pro-convulsant effect.

TRPV1 is well known to be activated by heat (Pingle et al., 2007; Romanovsky et al., 2009; Trevisani and Gatti, 2013). However, it has not been thought to be important in FS as its activation threshold is ~43 °C (Pingle et al., 2007; Trevisani and Gatti, 2013) and therefore our important finding that the TRPV1 antagonist, AMG-9810, TRPV1 deletion or inhibitory DREADD did not alter seizure susceptibility, was not surprising. Even during fever, temperatures seldom rise above 40 °C, thus to implicate TRPV1 receptors in FS would require temperatures more elevated than are found in either experimental or clinical fevers. Previous studies involving FS models requires elevated temperatures of around 41–42 °C to induce seizures (Baram et al., 1997; Scantlebury et al., 2004), whereas FS for babies generally occur at 38–39 °C (Graves et al., 2012), which is well below the TRPV1
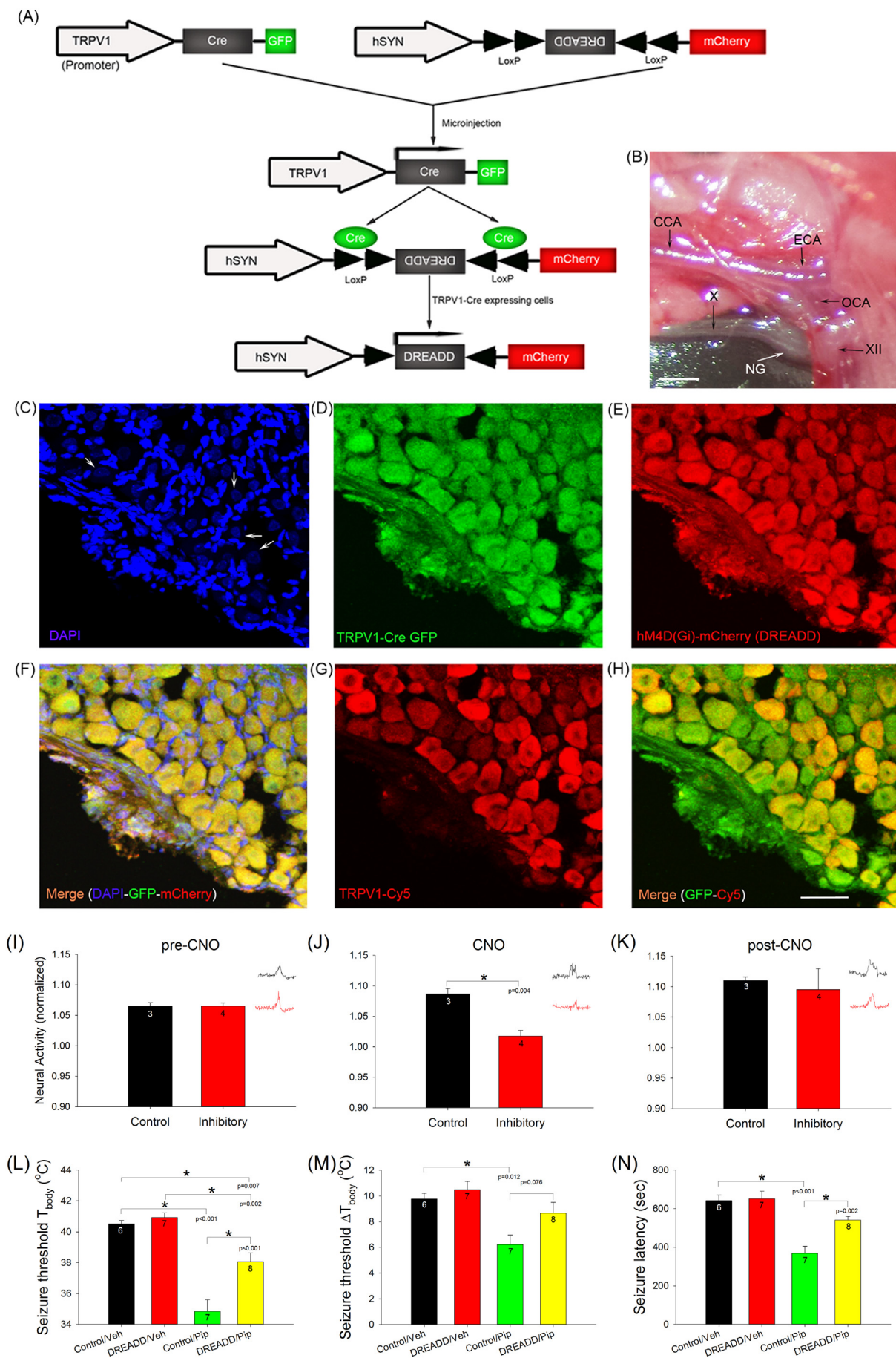
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Fig. 10. DREADD-mediated inhibition of TRPV1-containing cells in the vagal nodose ganglia reverses the pro-convulsant effects of piperine in postnatal day (P) 10 rats. (A) Schematic of the DREADD system employed in this study. A virus containing the Cre recombinase gene sequence whose expression is under the control of the TRPV1 promoter (TRPV1-Cre-GFP) and a virus containing the double-floxed inverted inhibitory DREADD (hM4D(Gi)) whose expression is dependent on Cre expression (hSYN-DREADD-mCherry) are microinjected bilaterally into (B) the nodose ganglia of the vagus nerves in P1 rats. Following Cre-mediated recombination, the DREADD is flipped into the correct orientation, leading to expression specifically in Cre-expressing cells. Scale bar: 0.5 mm. Representative confocal microscopic Z-stacked images showing (C) 4′diamidino-2-phenylindole (DAPI) - nuclear stain (blue); the arrows indicate the more faintly stained nuclei of the TRPV1-expressing cells, (D) Cre recombinase-GFP (green) and (E) hM4D(Gi)-mCherry (inhibitory DREADD; red) expression in the nodose ganglia, with (F) complete co-localization of Cre and DREADD expressing cells. (G) Cy5-labeled TRPV1-containing cells (far red) co-localize with nodose ganglia cells expressing TRPV1-Cre-GFP (H). Scale bar: 50 μm. Summary graphs showing *in vitro* vagus nerve activity (I) before, (J) during and (K) after clozapine N-oxide (CNO; 100 μM) application in response to a temperature challenge from 39 °C to 43 °C using the vagus nerve/nodose ganglion preparation from rats expressing either the control or inhibitory DREADD. Insets are representative traces from vagus nerve expressing the control (black) or inhibitory DREADD (red). Summary bars show that CNO activation of the inhibitory DREADD reduces the temperature response compared to the control DREADD (student *t*-test). CNO (10 mg/kg given i.p.) activation of the inhibitory DREADD significantly attenuated the pro-convulsant effect of PIP on (L) seizure threshold temperature (T_{body}) and completely reversed the pro-convulsant effect of piperine on (M) seizure threshold ΔT_{body} and (N) latency in DREADD-expressing P10 rats. One way ANOVA followed by Holm-Sidak *post-hoc* comparison. Abbreviations: CCA – common carotid artery; ECA – external carotid artery; OCA – occipital artery; XII – hypoglossal nerve; NG – nodose ganglion; X – vagus nerve. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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threshold. So how can the conundrum for these differential thresholds be addressed? The answer may be approached from the stand-point that infectious fevers are associated with the presence of inflammation, both peripherally and within the brain. Indeed inflammation is now thought to play an important role in seizure generation (Vezzani et al., 2013). Does this inflammation also play a role specifically in FS, and if so, how? The solution may lie in the observation that TRPV1 receptors can be activated at significantly lower temperatures, even as low as room temperature, if they are previously sensitized by various stimuli, including several pro-inflammatory cytokines such as IL-1β, TNF-α and bradykinin (Cesare and McNaughton, 1996; Premkumar and Ahern, 2000; Liang et al., 2001; Trevisani and Gatti, 2013). This ability of inflammatory cytokines/mediators to decrease the activation threshold of TRPV1 is probably critical to the pathogenesis of FS, and from a basic science stand-point may explain the dichotomy between the threshold temperature for FS in babies with infections (~39 °C) and that in current rodent models without concurrent infection (~42 °C). Of note, inflammation has been shown to increase TRPV1 receptor expression (Shieh et al., 2010; Homma et al., 2013; Simonic-Kocijan et al., 2013), which is another mechanism by which inflammation can lead to decreased FS threshold in relation to TRPV1. Based on the results of this study and a review of the literature, it is therefore reasonable to speculate that FS are linked to inflammatory sensitization followed by fever-induced activation of TRPV1 receptors (Sarnat and Scantlebury, 2017). Additionally, to our knowledge, we report here for the first time on the effects of TRPV1 ligands on the basal body temperature in neonatal animals. Not surprisingly, we have not noted hyperthermia following the injections with AMG-9810, which is opposite to that reported in adult animals. We suspect that this is due to immaturity of neonatal thermoregulatory networks (Schmidt et al., 1987). This important finding strengthens support for exploring the potential role of TRPV1 antagonists in the management of febrile seizures, as the side effect of hyperthermia which has limited its usefulness for other conditions in adult patients is not seen in baby animals, at least in this study.

In a prior study we observed that TRPV1 deletion had pro-convulsant effects, but this was in older animals and was thought to be due to abnormal thermoregulation (Barrett et al., 2016a). It is interesting that following injections of the vehicle in this study, TRPV1 KO animals had a significantly lower baseline temperature. It has been reported that saline injections and mild stress induced by measuring rectal temperature has a hyperthermic effect in mice (Bouwknecht et al., 2000). We postulate that this effect may be blunted in TRPV1 KO animals given its role in thermoregulation and responses to inflammation, accounting for the lower post-injection rectal temperature (Romanovsky et al., 2009; Fernandes et al., 2012). Unfortunately, pre-injection body temperatures were not measured for this part of the study. Although there is the possibility that the hypothermic profile may be protective against the effects of piperine on EFS, it is unlikely given the strong data supporting that wide ranging alterations in

baseline temperature has no effect on EFS thresholds in rodents (Millichap, 1959).

4.3. Association between the pro-convulsant effects of piperine and thermal hyperpnea

Elevated temperatures, as can occur with fever, are a strong stimulus to ventilation (O'Dempsey et al., 1993; Gadomski et al., 1994; Taylor et al., 1995). Two patterns of breathing are observed upon exposure to hyperthermia, which are thermal panting (increase in respiratory rate) and thermal hyperpnea (normal or increased respiratory rate associated with an increased tidal volume), the latter occurring once the body temperature rises (White, 2006). Thermal hyperpnea has been tightly linked to respiratory alkalosis (White, 2006), and respiratory alkalosis, occurring as a result of thermal hyperpnea, has been linked to EFS in rodent models and probably also contributes to the pathogenesis of FS in humans (Morimoto et al., 1996; Schuchmann et al., 2006; Schuchmann et al., 2011). The cause of thermal hyperpnea is very complex and have been hypothesized to be multifactorial involving interactions between the chemical modulators of pulmonary ventilation such as blood gases, pH, tissue temperature (Q_{10} effect), and/or independent additive effects of temperature induced changes on pulmonary ventilation (White, 2006). Thermal hyperpnea occurs with as little as a 1 °C increase in core body temperature in humans (White, 2006). Interestingly, for every 1–1.5 °C rise in body temperature during a fever or whole body hyperthermia, there is a 12–23% increase in metabolic rate (Del Bene, 1990; Nunneley et al., 2002), thus explaining why hyperventilation ensues during a fever. Activation of thermal sensors such as TRPV1 in the carotid bodies and vagus nerves are also thought to play a major role in driving the thermal hyperpneic response (Ni et al., 2006; Roy et al., 2012).

In this study, we found that the pro-convulsant effect of piperine was associated with a rapid, early onset, augmented ventilation and rate of expired CO_2 in response to hyperthermia, reminiscent of thermal hyperpnea. The piperine-induced exacerbation of thermal hyperpnea occurred just prior to seizure onset observed in the EFS experiments. This supports an association between thermal hyperpnea and seizure genesis. We postulate that the association between the piperine-induced reduction in seizure thresholds and thermal hyperpnea is most likely due to respiratory alkalosis. We find this explanation reasonable given the well-established link between thermal hyperpnea and respiratory alkalosis in mammals including rodents and humans (White, 2006). The pro-convulsant effects of respiratory alkalosis on seizures in general are well recognized and evidence in febrile seizures is mounting. The observation that AMG-9810 did not completely reverse the effects of piperine on breathing may suggest that the off-target effects of piperine contribute to the breathing response. However, since the pro-convulsant effects of piperine were completely reversed in TRPV1 KO animals, it suggests that a higher dose of AMG-9810 may be required to

achieve complete reversal of the piperine-induced thermal hyperpnea. Also, although the effect of 5% CO_2 was mild in piperine-treated animals that did have a seizure, 5% CO_2 completely blocked expression of behavioral seizures in 44% of piperine-treated animals, which we consider a significant effect. We are not sure why 5% CO_2 was only effective in some animals and not in others, but we speculate that exposure to a higher CO_2 content may have a more robust effect. Nonetheless, this finding supports that respiratory alkalosis is likely involved mechanistically. Despite the finding suggesting that the pro-convulsant effects of piperine may be due to TRPV1-mediated hyperthermia-induced respiratory alkalosis, we cannot definitively link the TRPV1-mediated reduction in seizure thresholds to alkalosis without measuring blood gases. Unfortunately, we were unable to confirm respiratory alkalosis with blood pH measurements as this would require obtaining repeated samples from anesthetized, catheterized small animals which would not accurately reflect processes occurring during FS in the awake, unrestrained state. Notwithstanding this limitation, the rate of expired CO_2 has been used as a good approximation of the status of the pH in the blood, given prior studies showing that changes in the rate of expired CO_2 is proportional to changes in arterial PCO_2 and $[H+]$ (Smith et al., 1983).

It is interesting that 5% CO_2 did not alter the seizure threshold in control animals, which was in contrast to that reported by Schuchmann et al. (2006). The reason for this variance is likely related to methods used to induce EFS which differed significantly between that and our studies. In our study, we subjected rodents to a short period of hyperthermia (~6–12 min) *versus* a prolonged exposure (> 30 min) in the Schuchmann study. Studies have shown that prolonged exposure to hyperthermia will result in inflammation (Neville and Sauder, 1988; Huang et al., 1996), and inflammation has been critically linked to the pathogenesis of hyperthermic seizures (Dube et al., 2005; Eun et al., 2015; Patterson et al., 2015). Exactly how inflammation induced by prolonged exposure to hyperthermia contributes to hyperthermic seizures is unknown. One possibility is through the direct effects inflammation has on brain excitability (Galic et al., 2012). It is also reasonable to speculate that inflammation may exacerbate respiratory responses to hyperthermia, perhaps through sensitization followed by heat-induced activation of temperature sensitive receptors located in the vagus nerve as supported by the data derived from our studies. Shorter exposures to hyperthermia may not activate inflammatory cascades to the same degree, and therefore the possibility exists that the role of respiratory alkalosis in the pathogenesis of hyperthermic seizures becomes less critical. Further studies are required to fully investigate the reason for the differences in the response to 5% CO_2 between the two models.

4.4. TRPV1-induced thermal hyperpnea and subsequent FS are peripherally mediated

Finally, the vagus nerves play an important role in cardiorespiratory control, thermoregulation, integration of inflammatory stimuli leading to fever and the respiratory response to hyperthermia (Richards, 1968; Gleeson and Brackenbury, 1984; Romanovsky et al., 1997; Thayer et al., 2011). The vast majority of vagal peripheral sensory afferents have cell bodies localized in the nodose ganglia of the vagus. Interestingly, TRPV1 receptors are highly expressed in the nodose (> 80%) from birth and remains high throughout development (Korobkin et al., 2013). Activation of these receptors is involved in the thermal sensitivity of the vagus in response to heat (Ni et al., 2006). As mentioned earlier, the vagus nerve is a key driver of thermal hyperpnea linked to respiratory alkalosis (Richards, 1968; Gleeson and Brackenbury, 1984). Our results support that the pro-convulsant effect of piperine are mediated by TRPV1 in the vagus as the effect of piperine was reversed with bilateral vagotomy and DREADD-mediated inhibition of TRPV1-containing cells in the vagal nodose ganglia. This finding has important clinical significance, as it has identified a novel peripheral anatomical

and molecular target for the development of more effective treatments for FS that could possibly eliminate the challenges associated with developing therapeutics that need to cross the blood-brain-barrier. We did not observe any effects of CSN denervation on EFS expression, but there was a trend towards reversal of the piperine-induced pro-convulsant effects. The absence of a significant effect of CSN denervation may be due to the functional immaturity of the carotid bodies at P10 (Donnelly, 2000). We also noted that CSN denervation in the immature rat led to a persistent hyperthermia, which may be a confounding factor in these experiments (data not shown). Nevertheless, we speculate that injection of the DREADD into both the carotid bodies and vagal nodose ganglia would have a more robust, cumulative effect on reversing the pro-convulsant effects of piperine. Due to the small size of these neonatal animals and the extreme difficulty associated with simultaneous DREADD injection into the carotid bodies and nodose ganglia, we were not able to address this speculation.

5. Conclusions

In conclusion, our data suggest that peripheral, but not central, activation of TRPV1 receptors prior to heat exposure, enhances the ventilatory response to hyperthermia, and increases the susceptibility to EFS. These effects are likely mediated by TRPV1-containing cells localized specifically in the vagal nodose ganglia, providing a novel peripherally-based anatomical and molecular target that should be taken into consideration when developing therapeutics for FS.

Competing interests

The authors declare no competing financial interests.

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