A role for neurokinin 1 receptor expressing neurons in the paratrigeminal nucleus in bradykinin-evoked cough in guinea pigs.

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Competing interests

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Author contribution

AD conducted experiments and contributed to drafting and editing the manuscript. AM, RB and AAKM conducted experiments and contributed to manuscript editing. MF contributed to experimental design and manuscript editing. SM conceived and designed experiments and contributed to writing and editing the manuscript. All authors contributed to interpretation of data. Experiments were conducted in the research laboratory of Professor Mazzone at the University of Melbourne.

Author's approval

All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work presented in the manuscript. All persons designated as authors qualify for authorship and all those that qualify have been listed as authors.

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Key points

1. Airway projecting sensory neurons arising from the jugular vagal ganglia terminate centrally in the brainstem paratrigeminal nucleus, synapsing upon neurons expressing the neurokinin 1 receptor.

2. This study aimed to assess the involvement of paratrigeminal neurokinin 1 receptor neurons in the regulation of cough, breathing and airway defensive responses.

3. Lesioning neurokinin 1 receptor expressing paratrigeminal neurons significantly reduced cough evoked by inhaled bradykinin but not inhaled ATP or tracheal mechanical stimulation.

4. The reduction in bradykinin-evoked cough was not accompanied by changes in baseline or evoked respiratory variables (e.g. frequency, volume or timing), animal avoidance behaviours or the laryngeal apnoea reflex.

5. These findings warrant further investigations into targeting the jugular ganglia and paratrigeminal nucleus as a therapy for treating cough in disease.

Abstract

Jugular vagal ganglia sensory neurons innervate the large airways and are thought to mediate cough and associated perceptions of airway irritations to a range of chemical irritants. The central terminals of jugular sensory neurons lie within the brainstem paratrigeminal nucleus, where postsynaptic neurons can be differentiated based on the absence or presence of the neurokinin 1 (NK1) receptor. Therefore, in the present study, we set out to test the hypothesis that NK1 receptor expressing paratrigeminal neurons play a role in cough evoked by inhaled chemical irritants. To test this, we performed selective neurotoxin lesions of NK1 receptor expressing neurons in the paratrigeminal nucleus in guinea pigs using substance P conjugated to saporin (SSP-SAP). Sham lesion control or SSP-SAP lesion guinea pigs received nebulised challenges, with the pan-nociceptor stimulant bradykinin or the nodose ganglia specific stimulant adenosine 5'-triphosphate (ATP), in conscious whole-body plethysmography to study cough and associated behaviours. Laryngeal apnoea reflexes and cough evoked by mechanical stimulation of the trachea were additionally investigated in anaesthetised guinea pigs. SSP-SAP significantly and selectively reduced the number of NK1 receptor expressing neurons in the paratrigeminal nucleus. This was associated with a significant reduction in bradykinin-evoked cough, but not ATP-evoked cough, mechanical cough or laryngeal apnoeic responses. These data provide further evidence for a role of jugular vagal pathways in cough, and additionally suggest an involvement of NK1 receptor expressing neurons in the paratrigeminal nucleus. Therefore, this neural pathway may provide novel therapeutic opportunities to treat conditions of chronic cough.

Introduction

Coughing is a vital respiratory defence mechanism that protects the airways and lungs from potential damage caused by inhaled or locally produced irritants and aspirate. The presence of cough evoking stimuli in the airway can trigger reflex coughing to rapidly expel the offending irritant and protect airway patency. Alternatively, airway sensory inputs to the brain can result in the conscious perception of an airway irritation, often defined as the urge-to-cough, which can promote volitional or behavioural coughing in order to satiate the urge (Davenport et al., 2007; Farrell et al., 2012; Mazzone et al., 2013). In pathological conditions the neural circuits subserving both cough and the urge-to-cough become hypersensitive (Lundberg et al., 1991; Hilton et al., 2015; Ando et al., 2016; Zaccone et al., 2016), leading to abnormal pulmonary sensations and excessive coughing that no longer serves a physiological purpose (Chung et al., 2013; Morice, 2013; Irwin et al., 2018). This defines a clinical condition known as cough hypersensitivity syndrome (Chung, 2011; Morice, 2013), which currently remains a difficult-to treat component of pulmonary disease (Mazzone et al., 2018). Accordingly, a better understanding of the sensorimotor neural processes governing cough and their altered function is warranted.

The stimuli that induce cough do so by activating subsets of sensory neuron terminals that innervate the airways. Early studies of the innervation of the airways identified different vagal receptors capable of evoking or modulating cough because of their sensitivity to noxious and irritant stimuli in the airways (Keller and Loeser, 1929; Widdicombe, 1954; Sant'Ambrogio et al., 1984). More recent studies have defined these reflex pathways in some detail (Shannon et al., 1998; Canning et al., 2008; Chou et al., 2018). Heterogenous populations of airway projecting sensory neurons are derived from two vagal sensory origins, known as the nodose and jugular ganglia (Canning et al, 2004; Undem et al., 2004; McGovern et al., 2012). With respect to cough, both nodose and jugular sensory pathways have been shown to induce coughing when activated (Tsubone et al., 1991; Canning et al., 2004; Chou et al., 2018). Nodose cough-inducing neurons contribute myelinated, non-peptidergic nerve fibres to the submucosal tissues in the large airways (Riccio et al., 1996; Canning et al., 2004; Undem et al., 2004; Mazzone et al., 2009). These fibres are considered low threshold

mechanosensors because they are highly responsive to epithelial displacement (Canning et al., 2004; Mazzone et al., 2009). Their mechanosensitivity is therefore thought to allow for the detection of stimuli that could severely compromise airway patency such as inhaled particulate matters, aspirated substances or luminal mucus translocating along the epithelial lining. By contrast, jugular cough neurons contribute chemically-sensitive fibres to the mucosa of the airways (Riccio et al., 1996; Canning et al., 2004; Undem et al., 2004), many of which are unmyelinated and express the neuropeptide substance P and the transient receptor potential vanilloid 1 (TRPV1) ion channel for the irritant chemical capsaicin (Riccio et al., 1996; Canning et al., 2004; Undem et al., 2004; Driessen et al., 2015). Because they respond to a wide variety of chemical stimuli and inflammatory mediators (Chou et al., 2008; Mazzone and Undem, 2016) jugular sensory fibres may play a prominent role in the development of chronic cough in disease. Importantly, the combination of nodose and jugular pathways likely avail the airways protection against diverse stimulus modalities.

Despite two complimentary vagal pathways subserving cough, the nodose and jugular neural circuits are in fact quite distinct. Embryologically, nodose and jugular neurons are derived from different tissues (D'Autréaux et al., 2011) and this imparts important differences in their molecular profiles, the peripheral and central organisation of their fibres, and consequently the manner by which they evoke physiological responses (Undem et al., 2004; Kwong et al., 2008; McGovern et al., 2015a; McGovern et al., 2015b). Nodose vagal neurons are derived from the epibranchial placodes, while neurons of the jugular vagal ganglia are derived from the neural crest (D'Autréaux et al., 2011). One striking consequence of these distinct embryological lineages is the very different central nervous system circuits that they contribute to. Thus, while nodose sensory neurons project almost entirely to the nucleus of the solitary tract, a brainstem region well known for integrating a wide range of visceral sensory information, jugular ganglia neurons terminate in a remote and poorly described region of the brainstem known as the paratrigeminal nucleus (Driessen et al., 2015; McGovern et al., 2015b). This observation argues that nodose and jugular sensory representations in the brain are integrated separately, a notion that has yet to be investigated with respect to cough but may prove important for designing interventions that specifically target one pathway over the other.

We previously identified a population of paratrigeminal neurons that specifically express NK1 receptors (Driessen et al., 2018). Furthermore, NK1 receptor antagonists have long been of interest for the treatment of chronic cough (Chapman et al., 2004; El-hashim et al., 2004; Grobman and Reinero, 2016), perhaps acting via a mechanism in the brain (Bolser et al., 1997; Mazzone and Geraghty, 1999; Mutoh et al., 2000; Mutolo et al., 2008). Therefore, in the present study we set out to specifically test the hypothesis that NK1 receptor expressing neurons in the paratrigeminal nucleus are involved in the induction of cough and accompanying behaviours evoked by chemical stimulation of the airways in guinea pigs. For comparison, we also investigated the involvement of paratrigeminal NK1 receptor expressing neurons in the apnoeic response evoked by stimulating jugular afferent fibres innervating the laryngeal mucosa.

Methods

Ethical approval

The experiments were approved by the University of Melbourne, Faculty of Health Sciences Animal Research Ethics Committee, accredited under the Australian Code for the Care and Use of Animals (approval number 1613989). The investigators understand the ethical principles under which The Journal of Physiology operates, and our work complies with the animal ethics checklist as described by Grundy (Grundy, 2015). All steps were used to minimise the animals' pain and suffering, as mandated in the Australian Code. Experiments were conducted on adult *Dunkin Hartley* guinea pigs of either sex (*n=48*, weight range = 350-1000g, age 7-20 weeks) obtained from an institutional breeding colony. The animals were housed in an approved animal facility under a 12:12 hr light/dark cycle with controlled temperature and humidity, large open plan floor pens (up to 8 animals per pen) providing unrestricted access to guinea pig chow, fresh vegetables, water and environmental enrichment. In conducting these experiments, we noted that there were no statistical differences between the measured responses of male (*n=7*) and female (*n=13*) guinea pigs in initial experiments (no-intervention cohort), and in turn the data was pooled for all statistical comparisons. Animals were

randomly assigned to one of three cohorts as follows: the no intervention cohort, the sham lesion cohort or the NK1 receptor neuron lesion (SSP-SAP lesion) cohort.

Chemical lesions of the paratrigeminal nucleus

Targeted toxin lesions of the paratrigeminal nucleus were conducted using Saporin conjugated to substance P (SSP-SAP, Advanced Targeting Systems, IT-11) following protocols described for other brain nuclei (Wilkinson et al., 2011; Fu et al., 2017). SSP-SAP is a ribosomal inactivating protein that binds specifically to NK1 receptors, upon which it is internalised resulting in the selective cell death of NK1 receptor expressing neurons (Riley et al., 2002; Abdala et al., 2006). Guinea pigs were prepared for recovery surgery with subcutaneous injections of the muscarinic antagonist, atropine (0.2mg kg^{-1}, atropine sulfate salt monohydrate, Sigma Aldrich, A0257) to decrease oral and airway secretions. 30-minutes post atropine treatment animals were anaesthetised with isoflurane (4% induction and 2.5% maintenance, in medical oxygen) via a nose cone and their heads were placed into a stereotaxic frame at a 45 degree angle. The adequacy of anaesthesia was monitored by routinely assessing limb withdrawal and palpebral reflexes and minor adjustments made to the isoflurane as needed to ensure a stable anaesthetic plane during surgery. Body temperature was measured via a rectal probe and maintained at ~37 degrees Celsius via a feedback regulated thermostatically controlled heating mat. A midline incision was made through the skin, posterior neck muscle and dura mater to expose the medulla at the level between the occipital bone and C1 vertebra. Using Calamus Scriptorius as a reference point (~0.5mm caudal to Obex at Bregma -17.3mm) bilateral microinjections of 0.1ng nl^{-1} SSP-SAP (SSP-SAP lesion cohort, *n=14*) or the toxin diluent, 0.9% sterile saline (sham lesion control cohort, *n=14*) were made across three sites of the paratrigeminal nucleus (200nl per injection site), to cover the rostro-caudal extent of this nucleus, as previously described in Driessen et al., 2018; anterior-posterior: +0.1mm caudal, +0.5mm central and +1.3mm rostral, medial-lateral: +/- 2.5mm and dorsal-ventral (relative to the surface of the brainstem): 1.3mm Microinjections were performed using pulled glass micropipettes (tip diameter ~20μm) connected to a

microprocessor controlled picopump (model PV820, World Precision Instruments, Sarasota USA). We previously reported the area of spread of injectate in guinea pigs using comparable volumes of injected dye, confirming that injectate should remain in the area of the paratrigeminal nucleus and bordering spinal trigeminal nucleus (Driessen et al., 2018). Incisions were sutured and animals received subcutaneous injections of 1ml kg^{-1} of 0.5mg ml^{-1} Meloxicam (Boehringer Ingelheim), 1mg kg^{-1} of 5mg ml^{-1} Endofloxacin (Bayer Health Care) and 3ml of 0.9% sterile saline. Animals were allowed to recover for three-weeks before entering conscious cough challenge studies.

Conscious cough studies

Conscious unrestrained guinea pigs were exposed to aerosolised irritants in a four site Buxco whole body plethysmography system, calibrated before every recording against known injected volumes of room air as described in the manufacturer's instructions. Initially, the no-intervention cohort was investigated to assess irritant-evoked cough and associated changes in animal behaviour. Subsequently, experiments were repeated on the sham lesion and SSP-SAP lesion cohorts. The experimental design consisted of an 11-day protocol (Figure 1) whereby animals were first acclimatised to the plethysmography chambers without challenge (days one to six). During the acclimatisation, animals were individually placed into the chambers for 20-minutes in the morning of each day. The animals were then challenged on day seven with increasing doses of bradykinin (bradykinin acetate salt, Sigma Aldrich, B3259; *n=20* no-intervention, *n=14* sham lesion and *n=14* SSP-SAP lesion), a nodose and jugular C-fibre stimulant (Kaufman et al., 1980; Hewitt et al., 2016). This was followed by a rest day (day eight) and a further two days of acclimatisation (days nine and ten) before a final challenge on day 11 with increasing doses of adenosine 5'-triphosphate (ATP, Sigma Aldrich, A7699; *n=9* no-intervention, *n=12* sham lesion and *n=10* SSP-SAP lesion), a nodose selective stimulant (Kwong et al., 2008; Muroi et al., 2013). Digital video cameras (Axis communications network cameras model M1054) were positioned correctly for each chamber to record (via Media Recorder 3 operating on a PC) each animal's behaviour for the duration of the experiment. Note the difference in animal number between bradykinin and ATP challenges relates to

some animals being sacrificed after the bradykinin challenge to allow for an initial histological characterisation of the lesion. Bradykinin was selected over the commonly used nodose/ jugular nociceptor stimulant capsaicin to avoid potentially confounding effects of acute bronchospasm and inadvertent activation of nodose mechanoreceptor pathways, which occurs with capsaicin but not bradykinin (Canning et al., 2001).

On challenge days, animals were placed in the chambers for 20-minutes before receiving an initial 0.9% saline (vehicle) challenge followed by either bradykinin (0.3, 1 and 3 mg ml^{-1}) or ATP (1.1, 11 ,110 mg ml^{-1}). Bradykinin doses were chosen comparable to previous studies in the guinea pig (Mazzone et al., 2005; Hewitt et al., 2016), while ATP doses were chosen at concentrations used in human studies (Basoglu et al., 2015; Fowles et al., 2017). In addition, all doses were confirmed as appropriate in preliminary experiments on animals in the no-intervention group. Challenges were nebulised (Buxco Aerosol Distribution Unit 10LPM, Aerogen nebuliser unit AG-AL1000 with filter cap of 3.1µm particle size) as one-millilitre (1mL) solutions over five-minutes (i.e. 0.2 ml min^{-1}), achieved with an air flow velocity of five litres min^{-1} and at 60% duty cycle. Airway irritants were nebulised for five-minutes and this was followed by an additional five-minute response period. A further five-minutes was allowed between each challenge dose to allow respiratory parameters and behaviours to return to baseline.

Laryngeal stimulation-evoked respiratory reflexes in anaesthetised guinea pigs

Three days after the conclusion of conscious cough challenge studies, guinea pigs from the sham lesion control (*n=9*) and SSP-SAP lesion *(n=9*) groups were prepared for assessing laryngeal stimulation-evoked respiratory reflexes, as previously described in Driessen et al., 2015 and Driessen et al., 2018. Briefly, urethane (1.5g kg^{-1}, i.p. given as two doses of 0.75g kg^{-1}, approximately 30 minutes apart) anaesthetised animals were placed supine on a feedback controlled thermostatic heating mat to maintain body temperature at ~ 37 degrees Celsius. This dose of urethane is sufficient to maintain a stable plane of anaesthesia for the entire experiment but was assessed intermittently

using limb withdrawal and palpebral reflexes. A midline incision was made along the ventral surface of the neck to expose the larynx, trachea and underlying nerves and blood vessels. Bilateral recurrent laryngeal nerve transection was performed to investigate the jugular-paratrigeminal afferent pathway without the confounding influence of the nodose innervation to the larynx (Canning et al., 2004; Driessen et al., 2015; Driessen et al., 2018). The left carotid artery was cannulated using polyethylene tubing (internal diameter=0.5mm and outer diameter=0.9mm) attached to a pressure transducer filled with heparinised saline (50 U ml^{-1}, Sigma Aldrich, H3393) to measure arterial blood pressure (ABP) and heart rate (HR). The distal extra-thoracic trachea was cannulated and connected via a side port to a pressure transducer to measure the tracheal pressure (TP) and changes associated with spontaneous respiration. Output from pressure transducers was filtered and amplified (Neurolog Systems, Digitimer, Hertfordshire, UK), digitised and recorded (Micro1401 A-D converter operated by spike II software, CED, Cambridge UK). A midline incision was made through the larynx and a platinum bipolar stimulating electrode was inserted onto the mucosal surface. Guinea pigs were allowed to stabilise for 20-minutes and then the larynx was stimulated at a voltage optimal for inducing reflex changes in respiration (Driessen et al., 2018), over a 10-second train duration (1msec pulses) with sequentially increasing stimulation frequencies (1-32 Hz).

Tissue harvest and histological analyses

Following completion of the cough or physiological studies, sham lesion control and SSP-SAP lesion guinea pigs were overdosed with sodium pentobarbital (100mg kg^{-1} i.p.) and transcardially perfused with 150ml of 5% sucrose in 0.1M phosphate buffered saline (PBS, pH7.4) and 200ml of 4% paraformaldehyde (PFA) in 0.1M PBS. Brainstems were dissected and postfixed overnight in 4% PFA before being cryoprotected in 20% sucrose at 4 degrees Celsius. Tissues were frozen in OCT embedding compound and 50µm cryostat cut sections were collected serially in 0.1M PBS. Sections were blocked in 10% goat serum in 0.1M PBS for one hour at room temperature. Tissue sections were then incubated for 48-hours in primary antibodies for the NK1 receptor (1:5000, Rabbit anti-substance

P receptor, Merck Millipore, ab5060) to determine the extent and specificity of the lesion, and adjacent sections were stained for the calcium binding protein Calbindin (1:1000, Rabbit anti-Calbindin D-28k, Swant CB-38a) as a marker of paratrigeminal nucleus neurons that do not express NK1 receptors (Driessen et al., 2018). All antibodies were diluted in antibody diluent (2% goat serum and 0.3% Triton X-100 in 0.1M PBS). After the required incubation, sections were washed three times with 0.1M PBS and then Calbindin immunostained sections were incubated in goat anti-rabbit conjugated to Alexa Fluor 488 (1:500; Invitrogen, A-11008) secondary antibody for one hour, while NK1 receptor stained sections were processed using tyramide signal amplification which required the following additional steps. These sections were incubated with a goat anti-rabbit biotinylated secondary antibody (1:500, Invitrogen, A-31820). They were then washed before being incubated in horse radish peroxidase conjugated to streptavidin for 30-minutes. A tyramide 488 flurophore was made up in reaction buffer according to the tyramide signal amplification protocol (Tyramide Superboost Kit, Thermor Fischer, B40932) and incubated on tissue sections for eight-minutes. All immunostained brainstems were mounted onto gelatin-coated slides and coverslipped with an anti-fade mounting media (Fluoroshield with DAPI, Sigma Aldrich, F6057 or Fluoroshield, Sigma Aldrich, F6057). All tissues were visualised under the appropriate filter cube using a Leica DM6B LED microscope and images were captured using a Leica DFC7000T camera. Representative photomicrographs were assembled in Adobe Photoshop CS6.

Data analyses

The effect of SSP-SAP on the neuronal composition in the paratrigeminal nucleus was quantified by manually counting NK1 receptor and Calbindin immunopositive neurons in sham lesion control and SSP-SAP lesion animals, under a Leica DM6B LED microscope at 40x objective by identifying NK1 receptor labelled neuronal profiles encompassing the nuclear marker DAPI. Based on our previous characterisation of the rostral-caudal extent of the guinea-pig paratrigeminal nucleus (Driessen et al., 2018), counts were pooled across four levels of the brainstem relative to Calamus

Scriptorius; most caudal (-0.1mm), caudal (+0.1mm), central (+0.5mm) and rostral (+1.3mm).To assess the spatial specificity of the lesion, NK1 receptor counts were performed in the nucleus of the solitary tract at the same rostro-caudal medullary levels. In addition, tile-scans of the entire brainstem section at each of the four rostro-caudal levels were acquired and positive neurons selected using the region of interest (ROI) tool on Leica X acquisition software. ROI's were then transposed to traces of the brainstem and the four regions were collapsed to show a representation of neuronal density in sham lesion and SSP-SAP lesion animals. These counts are also presented as the mean region-pooled total count of neurons expressing the NK1 receptor or Calbindin ± SD. A two-tailed unpaired Students' t-test was conducted to statistically compare the number of neurons counted in sham lesion control and SSP-SAP lesion animals for both the paratrigeminal nucleus and the nucleus of the solitary tract.

In conscious cough studies, respiratory frequency (breaths min^{-1}), tidal volume (mL kg^{-1}) and inspiratory and expiratory time (seconds) were automatically sampled every 2 seconds from the calibrated Buxco flow trace, corrected for animal body weight and ambient chamber temperature, and calculated in real time by the plethysmography software (Buxco Finepointe Software Version 2.1.0.9). Real time data collected every 2 seconds were later (offline) used to calculate the mean per minute ± SD for each cohort at baseline and during each nebulised challenge. Individual coughs were counted manually at the end of the experiment by assessing the Buxco chart traces and video recordings to confirm the characteristic airflow and behavioural 'cough stance' posture (Figure 1; Tsubone et al., 1991; Fox et al., 1996). For this study a coughing event was defined as any large expiratory outflow with a preceding inspiration occurring in rapid succession without an interspersing breath (Figure 1). In addition, video files were imported into a behavioural analysis software (Observer XT12) to code for the duration of avoidance-like behaviours that accompanied nebulised challenges, in an attempt to quantify the perception of airway irritation. Under single blinded conditions, behaviours were categorised as oral (i.e. chewing and licking that involved mastication of themselves or their environment), locomotor (i.e. notable movement around the chamber and 'dog' shakes) or coughing-associated (i.e. time spent coughing, 'cough stance' and yawn). The durations of

behaviours were normalised to baseline behavioural profiles to account for inter-animal variability. Both cough and behavioural data are presented as the mean per minute ± SD and as the cumulative total (sum across doses) ± SD. D'Agostino-Pearson normality tests were employed, and cumulative values were statistically compared using an ordinary two-way ANOVA with a Tukey's multiple comparisons tests where appropriate. Finally, Pearson's r-correlation co-efficient was used to investigate the relationship between the histological extent of the SSP-SAP lesion and the physiological effect size, as well as the relationships between number of coughs and associated ancillary behaviours. Statistical significance was set at $p<0.05$ for all comparisons.

In anaesthetised physiological studies, respiratory rate, blood pressure and heart rate were calculated from the raw chart recordings over a 10-second period before any stimulation (baseline) and then again during each stimulation train (evoked response) and multiplied by six to determine the equivalent per minute values. Data were normalised to baseline values for each animal and then plotted as the mean ± SD for both sham lesion control and SSP-SAP lesion animals. Normalisation was performed to remove the confounding influences of inter-animal variability in baseline parameters. D'Agostino-Pearson normality test was used and then stimulus response curve data were compared using an ordinary two-way ANOVA with a Tukey's multiple comparisons test where appropriate, while a two-tailed unpaired Students' t-test was employed to compare the maximal reduction in respiratory rate (E_{max}) and the electrical frequency required to elicit 50% of this maximum response (EF_{50}). Statistical significance was set at $p<0.05$. In addition, mechanical induced cough was assessed in each animal by probing the tracheal mucosa for five-seconds with the tip of a luer stub (Canning et al., 2004; Undem et al., 2004; Mazzone et al., 2009). The proportion of animals, from each experimental cohort, coughing in response to mechanical stimulation (i.e. yes or no) during the five-second period was recorded.

Results

Targeted toxin lesions specifically reduced NK1 receptor expressing neurons in the paratrigeminal nucleus

Microinjections of SSP-SAP into the paratrigeminal nucleus significantly reduced the number of NK1 receptor expressing neurons (46.4±19.2 in sham lesion control animals *n=14* vs. 19.4±13.4 in SSP-SAP lesion animals *n=14*, two-tailed unpaired Students' t-test *p=0.0002*, Figure 2), but did not significantly reduce the number of non-NK1 receptor expressing neurons (44.8±12.5 vs 38.0±23.3 Calbindin expressing neurons in sham lesion controls *n=6* and SSP-SAP lesion animals *n=10*, respectively, *p=0.52,* Figure 2). Furthermore, within the nucleus of the solitary tract there was no change in the number of NK1 receptor expressing neurons between sham lesion control and SSP-SAP lesion animals (69.8±15.3 vs. 71.1±27.0 in sham lesion control *n=14* and SSP-SAP lesion *n=14* animals respectively, *p=0.87,* Figure 2).

Lesioning NK1 receptor expressing paratrigeminal neurons differentially affected cough evoked by nebulised challenges with bradykinin and ATP

Aerosolised bradykinin evoked cough in all cohorts displaying a significant dose and cohort interaction effect (p=0.006). In the no-intervention (*n=20*) and sham lesion control cohorts (*n=14*), bradykinin reliably evoked cough at the 1 and 3mg ml^{-1} doses (Figure 3A). Alternately, SSP-SAP lesion (*n=14*) of the paratrigeminal nucleus produced notable alterations in the bradykinin-evoked cough (Figure 3A). Thus, lesion animals universally failed to cough at the 1mg ml^{-1} dose of bradykinin (Figure 3), and demonstrated a significant reduction in the cumulative total number of coughs at 3mg ml^{-1} compared to no-intervention and sham lesion control animals (, *p<0.0001 and p=0.006 respectively*, Figure 3B). In addition, the proportion of animals that demonstrated any cough to bradykinin (i.e. the number of responders) was lower in SSP-SAP lesion (50%) versus sham lesion control and no-intervention (80%) cohorts (Figure 3B). Similarly, the number of coughing events differed, which was typically confined to a single bout in the lesion animals compared to multiple

distinct bouts in the no-intervention and sham lesion control animals respectively (2.4±2.0 vs. 2.3±1.8 vs. 1.0±1.2 coughing events for no-intervention, sham lesion control and lesion animals respectively, *p=0.005,* Table 1). There was no significant relationship (r=0.003, *p=0.99)* observed between the lesion size (i.e. the number of NK1 receptor expressing paratrigeminal neurons; Figure 2) and the cough reduction (Figure 3) .

Aerosolised ATP evoked cough in all cohorts but did not display a significant dose and cohort interaction effect (p=0.31), despite an overall significant ATP dose effect ($p<0.0001$) related to cough events occurring exclusively at the highest dose of ATP tested. In addition, lesioning NK1 receptor expressing neurons in the paratrigeminal nucleus had no effect on ATP-evoked cough (*n=9* no-intervention, *n=12* sham lesion control and *n=10* SSP-SAP lesion, Figure 3). .

Lesioning NK1 receptor expressing paratrigeminal neurons did not alter behavioural responses evoked by nebulised challenges with bradykinin and ATP

Aerosol challenge with both bradykinin and ATP evoked a range of ancillary behaviours. We analysed both the sum of all behaviours (Figure 4) and each individual behaviour (Tables 1 and 2) across dose and cohort. For the sum of all behaviours, neither aerosolised bradykinin (*n=20* no-intervention, *n=14* sham lesion control and *n=14* SSP-SAP lesion) nor ATP (*n=9* no-intervention, *n=12* sham lesion control and *n=10* SSP-SAP lesion) displayed a significant dose and cohort interaction effect (p=0.99 and p= 0.82), despite overall significant dose effects for both bradykinin ($p<0.0001$) and ATP ($p<0.0001$). For individual behaviours, interaction effects were seen for bradykinin-evoked behaviours (summarized in Table 1) but not for ATP (summarized in Table 2). Thus, further level statistical analyses were conducted for bradykinin data only.

SSP-SAP lesion animals showed no differences to no-intervention and sham lesion controls in the sum of all behaviours evoked by bradykinin (Figure 4). Of the individual behaviours, a significant reduction in the time spent in cough stance was noted between no intervention and both the sham lesion (p=0.03) and SSP-SAP lesion ($p<0.0001$) cohorts during bradykinin challenge (Table 1). In

addition, both the time spent coughing and the number of coughing events was significantly different between no intervention and SSP-SAP lesion cohorts ($p<0.0001$ and $p<0.0001$, respectively), while the number of coughing events also differed between sham lesion control and SSP-SAP lesion cohorts ($p=0.002$; Table 1). Neither grooming nor movement differed between any cohort (Table 1). Notably, observed coughing and behavioural effects were not confounded by any effect on breathing per se as SSP-SAP lesion animals did not demonstrate modified respiratory rate, tidal volume or inspiratory/expiratory time parameters measured either under baseline conditions (i.e. before the beginning of cough challenge testing) or associated with either of the inhaled tussive challenges when compared to no-intervention and sham lesion controls (Table 3 and 4). Of all ancillary behaviours measured, only time spent in cough stance, time spent coughing and the number of cough events showed relationship with actual cough counts, and this was true regardless of whether bradykinin or ATP was the stimulus (time in cough stance, $p=0.02$, *r=0.41* and *p≤0.0001, r=0.81* bradykinin and ATP respectively; time spent coughing, $p=0.0002$, *r=0.59* and *p≤0.0001, r=0.96* bradykinin and ATP respectively and the number of cough events, $p=0.02$, *r=0.59* and *p≤0.0001, r=0.82* bradykinin and ATP respectively).

Lesioning NK1 receptor expressing paratrigeminal neurons produced minimal effects on laryngeal-evoked respiratory reflexes in anaesthetised guinea pigs

In sham lesion control animals (*n=9*), electrical stimulation of the larynx produced a stimulation frequency-dependent reduction in breathing frequency (maximum reduction (Emax) of 44.7±19.2 breaths min^{-1}; *p<0.0001,* Figure 5) and blood pressure (maximum reduction of 11.0±5.5 mmHg; *p<0.0001, data not shown*), culminating in an apnoea (Figure 5A), while only a small effect was seen on heart rate (maximum reduction of 6.2±8.0 beats min^{-1}; $p=0.25$, *data not shown*) as previously reported (Driessen et al., 2018). Evoked respiratory responses in animals from the SSP-SAP lesion cohort (*n=9*) demonstrated a small, albeit statistically insignificant reduction in the reflex sensitivity (EF_{50}) and magnitude (E_{max}) during laryngeal stimulations compared to the sham control

cohort (Figure 5C). Indeed, the characteristics of this reduction in responsivity was comparable to what we previously reported after microinjections of neurokinin receptor antagonists into the paratrigeminal nucleus (Driessen et al., 2018). Additionally, cough evoked by mechanical probing of the tracheal mucosa, known to be mediated by nodose mechanosensors (Canning et al., 2004), was not different between sham lesion control and SSP-SAP lesion animals with 6/8 and 8/9 responders respectively (data not shown).

Discussion

The laryngeal, tracheal and mainstem bronchial airways are vitally important reflexogenic sites for airway defence and serve as a primary site for the induction of coughing. It is acknowledged that heterogenous sensory neural mechanisms subserve cough evoked from these airway locations, involving sensory neurons derived from both the nodose and jugular vagal ganglia. However, little is known about the brainstem processes that differentiate these sensory neural inputs. Our study has begun to address this gap in knowledge by revealing that selective lesions of neurons expressing the NK1 receptor within the paratrigeminal nucleus, significantly reduce cough evoked by inhalation of the nociceptor stimulus bradykinin, but not the putative nodose selective stimulus ATP. We demonstrate that cough reduction is not accompanied by general changes in baseline or evoked breathing frequency, timing or volume, or animal avoidance behaviours accompanying inhaled challenges, and that NK1 receptor expressing neurons at this location only contribute a minor component to the laryngeal-evoked apnoeic reflex and play no apparent role in mechanically-evoked coughing. Collectively these data suggest an involvement of NK1 receptor expressing paratrigeminal nucleus neurons in bradykinin evoked cough responses in guinea pigs. These novel findings support the hypothesis that the NK1 receptor expressing neurons of the jugular-paratrigeminal system may be involved in cough mediated by jugular sensory neurons and highlight a potential of targeting this neural circuit for treating dysfunctional cough in disease.

Nodose and jugular vagal sensory pathways mediating cough

Two well described cough evoking pathways exist in the large airways. The first is best described as a low threshold mechanosensitive and acid sensitive pathway that arises from neurons in the nodose ganglia (Tsubone et al., 1991; Widdicombe, 1996; Canning et al., 2004), and is extremely efficient at mediating rapid and robust reflex coughing to protect the airways from aspirations and obstructions that could fatally compromise ventilation. This pathway is readily activated by mechanical or acid stimulation of the large airways, even in the anaesthetised or decerebrate animal (Canning et al., 2004), allowing for detailed mechanistic studies to be performed. Accordingly, previous studies have described the central neural mechanisms important for cough evoked by nodose mechanosensitive airway afferents, and as predicted by neuronal tracing studies and consistent with that observed with a wide range of other nodose sensory mediated reflexes, nodose cough is inhibited by injection of glutamatergic antagonists into the nucleus of the solitary tract (Ezure et al., 1999; Mutolo et al., 2007; Mutolo et al., 2009; Canning and Mori, 2011).

A second cough-evoking pathway is chemosensitive and thought to be derived from jugular ganglia nociceptive neurons. Although the nodose ganglia also give rise to airway projecting chemosensitive nociceptors, very few of these reach the large airways where cough is usually triggered, and the available functional data indicate nodose nociceptors to be poor at evoking cough, and in fact may even be acutely inhibitory to cough in some settings (Chou et al., 2018). In electrophysiological studies, jugular neurons are like many nociceptors in the body and are activated by a wide variety of chemicals including bradykinin and the chilli extract capsaicin (Canning et al., 2004; Mazzone et al., 2005; Hewitt et al., 2016). However, jugular neurons are largely unresponsive to ATP which activates nodose neurons readily through P2X2/3 heteromeric ion channels (Kwong et al., 2008; Nassenstein et al., 2010; Potenzieri et al., 2012). The results of our current study align with this collective published literature as the nodose specific stimulant ATP, was a poor tussigen in comparison to the pan-nociceptor stimulant bradykinin which dose dependently evoked cough in our conscious unrestrained guinea pigs. However, because cough evoked by chemosensitive afferent stimuli is highly sensitive to the inhibitory effects of anaesthesia, substantially less is known about the

neurophysiology of this putative cough pathway. Nevertheless, chronic cough patients present with an increased Arnold's nerve reflex, representative of hypersensitive vagal afferents in the auricular branch of which the sensory afferents are derived from the jugular vagal ganglia (Dicpinigaitis et al., 2019). Furthermore, blocking the superior laryngeal nerve, which in the guinea pig predominately carries jugular vagal afferents (Canning et al., 2004), in chronic cough patients results in a 50% reduction in their cough (Simpson et al., 2018). Together these data clearly argue for a role of jugular airway afferent processing in the manifestation of cough.

Evidence for the paratrigeminal nucleus as a novel sensory integration site regulating pulmonary defence: role of NK1 receptor expressing neurons

Unlike nodose neurons which terminate centrally in the nucleus of the solitary tract, jugular neurons project to the medullary paratrigeminal nucleus (Driessen et al., 2015; McGovern et al., 2015b). The paratrigeminal nucleus is best known for processing of somatic nociception and for a role in the generation of pain (Bon et al., 1997, Ma et al., 2005; Koepp et al., 2006). We recently reported that laryngeal-evoked respiratory depression in anaesthetised guinea pigs is dependent upon jugular ganglia afferent inputs and glutamatergic neurotransmission within the paratrigeminal nucleus (Driessen et al., 2018). In that study, reflex responses to electrical or chemical stimulation of the guinea pig larynx were largely intact following blockade of neurokinin receptors in the paratrigeminal nucleus, suggesting that action potential-dependent neurotransmission between primary afferent terminals and paratrigeminal neurons may not ordinarily involve neuropeptides (Driessen et al., 2018). Indeed, peptidergic vesicle release from primary afferent terminals often requires calcium responses in the nerve terminal that are distinct to those that accompany conventional action potential-dependent depolarization (Huang et al., 2008; Medvedeva et al., 2008; Wang et al., 2017). Conversely, we showed that the release of neuropeptides following capsaicin microinjections in the paratrigeminal nucleus, which is presumably not action potential dependent, increases respiratory drive through neurokinin receptor dependent mechanisms (Driessen et al., 2018). These differential respiratory effects are particularly interesting because two distinct populations of laryngeal projecting jugular ganglia nociceptors exist, defined by their conduction velocity and the absence or presence of

peptide transmitters such as substance P (Riccio et al., 1996; Canning et al., 2004; Undem et al., 2004; Driessen et al., 2015). Furthermore, we recently identified two populations of paratrigeminal neurons, one expressing Calbindin and a second expressing the NK1 receptor (Driessen et al., 2018). Thus, parallel non-convergent A and C fibre processing pathways may exist for afferent traffic through the paratrigeminal nucleus, similar to that described in the nucleus of the solitary tract (Bailey et al., 2002; McDougall and Andresen, 2013).

Because our previous study suggested that the non-peptidergic jugular pathway from the airways mediates apnoeic reflexes, perhaps via Calbindin expressing paratrigeminal neurons, (Driessen et al., 2018), in the present study we speculated that the peptide-expressing afferent pathway, and hence the NK1 receptor expressing population of paratrigeminal neurons, might be involved in the modulation of cough mediated by jugular ganglia sensory neurons. SSP-SAP injections reduced the NK1 receptor expressing neuron population in the paratrigeminal nucleus by approximately 50% which resulted in a comparable reduction in cough. The effects of NK1 receptor neuron lesion occurred without modifying basal breathing consistent with no known role of the paratrigeminal nucleus in respiratory rhythmogenesis (Driessen et al., 2018). Furthermore, neither the cough or coughing behaviour evoked by ATP in conscious animals, nor mechanically-evoked cough in anaesthetised animals were different in lesioned animals. This is consistent with these stimuli activating nodose circuits that terminate in the nucleus of the solitary tract (Canning et al., 2004; Canning and Mori, 2011).

Previous studies have demonstrated a role for central NK1 receptors, including in the nucleus of the solitary tract, in cough in some species and this warrants discussion in light of the present findings (Bolser et al., 1997; Bolser et al., 1999; Mutoh et al., 2000; Joad et al., 2004; Mutolo et al.,2008). In rabbits and cats, NK1 receptor antagonists delivered either ICV or to the nucleus of the solitary tract suppress cough mechanically-evoked from the large airways (Bolser et al., 1997; Mutolo et al., 2008), presumably mediated by nodose mechanosensors (Canning et al., 2008). In guinea pigs, microinjection of NK1 receptor antagonists into the nucleus of the solitary tract has no effect on mechanical cough (Mazzone et al., 2005), but modestly reduces citric acid coughing in healthy

animals (Joad et al., 2004). After cigarette smoke exposure, cough hypersensitivity in guinea pigs is reversed by NK1 receptor antagonism in the nucleus of the solitary tract (Joad et al., 2004), whereas hypersensitivity evoked by concomitant mechanoreceptor and nociceptor activation in guinea pigs is not reversed by neurokinin receptor blockade in the nucleus of the solitary tract (Mazzone et al., 2005). These data suggest that in the nucleus of the solitary tract the involvement of NK1 receptors in cough maybe context and species dependent, highlighting the importance of confirming the specificity of the NK1 receptor expressing neuron lesions in the present study. Importantly, Calbindin expressing neurons within the paratrigeminal nucleus and NK1 receptor expressing neurons outside the paratrigeminal nucleus (e.g. in the nucleus of the solitary tract) were unaltered by SSP-SAP, suggesting that the reduction in cough observed was not due to poorly localised lesions. The specificity of our NK1 receptor expressing neuron lesion may also explain the absence of effect of paratrigeminal SSP-SAP on laryngeal reflex evoked respiratory slowing in anaesthetised animals, which we believe to be predominately mediated by non-peptidergic laryngeal projecting jugular vagal afferents and paratrigeminal Calbindin expressing neurons (Driessen et al., 2018). Taken together, our findings represent the first evidence that jugular-nociceptor evoked cough involves a different central neural circuit to that shown previously to mediate nodose cough and other protective reflexes mediated by jugular vagal afferents.

In humans, chemical irritations of the airways are accompanied by conscious awareness of the irritation, resulting in a feeling of desire (or urge) to cough. The urge-to-cough is an important sensorineural component of coughing but has proven difficult to model in animals because it requires an appropriate behavioural assay that specifically reflects the perception of airway irritation. We reasoned that avoidance behaviours in guinea pigs may reflect their level of urge-to-cough. Both nebulised bradykinin and ATP resulted in periods of increased locomotor activity, chewing and grooming, consistent with a perception of noxious irritation. Interestingly, avoidance behaviours associated with bradykinin inhalation displayed a lower threshold for induction compared to that for evoked cough. This is comparable to the urge-to-cough perception described in human cough challenge studies (Davenport et al., 2007; Farrell et al., 2012). On the other hand, ATP associated

behaviours were only significantly elevated above saline at the same dose that evoked coughing. These data may suggest that nodose and jugular vagal afferents differentially contribute to airway sensory experiences. However, even though we noted an increase in avoidance-like behaviours during both bradykinin and ATP challenges, these were not impacted by paratrigeminal NK1 receptor neuron lesions. It is conceivable that the lower order sensory neural processes governing the urge-to-cough are different to those that evoke the motor act of coughing and or that this sensory experience is related to processing via an alternate neuronal population such as those that express Calbindin. However, it is equally possible that the behaviours monitored were not specifically related to airway irritation but may also be driven by nebulised agents irritating ocular, nasal and additional orofacial tissues. Although the measured behaviours are likely reflective of noxious irritation, our data do not allow us to definitively conclude that these measures adequately model the perception of the urge-to-cough.

Limitations of the study

Cough is challenging to study because of the profound inhibitory effects of anaesthesia. This limits studies often to conscious animals which makes acute interventions to restricted regions of the central nervous system, and especially the brainstem, challenging. Here we utilised prior selective chemical lesions of the paratrigeminal nucleus to test our hypothesis. It is conceivable that in the post-lesion period, prior to cough challenge testing, some reorganisation of the neural circuitry occurs (Lin et al., 2012). However, the selective effects of the lesion on cough evoked by bradykinin in the present study, without any effect on the laryngeal evoked apnoea responses which is also mediated by the paratrigeminal nucleus (Driessen et al., 2018), suggests an absence of significant changes in synaptic organisation in the paratrigeminal nucleus after lesions. Targeted NK1 receptor lesions have also been used extensively in other studies, including to investigate pontomedullary respiratory circuits, without reports of extensive network reorganisations (Souza et al., 2018).

In our study we took advantage of the putative selectivity of ATP to activate nodose and not jugular neurons. However, we cannot exclude the possibility that ATP may activate jugular-derived Aδ fibres as there is no published data reporting the ATP sensitivity of this population (although unpublished data do confirm their insensitivity; B.J. Undem, *personal communication*). Nevertheless, even if Aδ jugular neurons were responsive to ATP, then our interpretation of the involvement of paratrigeminal NK1 receptor expressing neurons in bradykinin cough would not differ nor would our hypothesis that jugular Aδ and jugular C-fibre pathways do not converge onto common neurons in the paratrigeminal nucleus (Driessen et al., 2018).

Studying cough is complicated by the fact that experiments using local administration of neurokinin receptor antagonists to the paratrigeminal for acute inhibition are unlikely to be efficacious because (as described above) we have previously reported minimal involvement of central NK1 receptors in responses to peripheral jugular vagal afferent stimulation (Driessen et al., 2018) and central nervous system penetrant NK1 receptor antagonists given systemically do not block bradykinin cough in guinea pigs (Hewitt et al., 2016). As such, whilst we can propose a role for paratrigeminal NK1 receptor expressing neurons in bradykinin-evoked cough, this is not synonymous for a role of NK1 receptors. Notably, all vagal sensory neurons express the glutamate transporter vGlut2 (Kupari et al., 2019; Nassenstein et al 2010), and as such glutamateric neurotransmission likely mediates rapid signalling from peptidergic as well as non-peptidergic jugular neurons in the paratrigeminal nucleus.

Physiological significance and conclusion.

Chronic cough is a difficult to treat symptom in many patients with respiratory disease and drives increased morbidity associated with these conditions (Polley et al., 2008; Chung, 2011; Morice, 2013; Hilton et al., 2015). This in part relates to the apparent heterogeneity in the mechanisms precipitating cough (Mazzone et al., 2018; McGovern et al., 2018; Mazzone and Farrell, 2019) as well as a poor understanding of the organisation of the neural circuits that are responsible for cough induction. It is further complicated by the fact that reflexive cough is an essential protective

mechanism for airway clearance and therapeutics that diminish protective cough may negatively compromise respiratory function and leave patients vulnerable to aspiration pneumonia. The findings of the present study provide functional evidence to suggest that nodose and jugular airway afferent pathways regulate cough in part through different central neural circuits. Furthermore, we have identified a distinct subtype (NK1 receptor expressing) of paratrigeminal neurons that is important for processing jugular vagal afferent evoked cough, while potentially differentiating the function of this jugular afferent neural pathway from that regulating important defensive apnoeic reflexes evoked from the larynx. In this regard, it is interesting that recent clinical trial data has shown promising anti-tussive potential of the centrally penetrating NK1 receptor antagonist, Orvepitant, in chronic refractory cough (Smith et al.,2019). Our current data would argue that a mechanistic site of action for Orvepitant may be in part at the level of jugular afferent processing within the paratrigeminal nucleus. As neuropeptides probably contribute only a neuromodulatory role to afferent transmission in the paratrigeminal nucleus under normal conditions (Driessen et al., 2018), a better understanding about the impact of disease on the contribution of NK1 receptors to chronic cough will be important. Furthermore, understanding the phenotype of paratrigeminal NK1 receptor expressing neurons may help to identify novel pharmacological targets that are effective at cough suppression.

Figure legends

Figure 1: Schematic representation of the experimental timeline. Cough studies were conducted in conscious unrestrained guinea pigs using whole body plethysmography over an 11-day protocol. Animals were allowed 20 minutes acclimatization (-15 to 5 minutes), followed by. five-minute periods for baseline (blue), nebulised challenge (green) and response time (white). An initial challenge of 0.9% Saline (Sal) was followed by each dose of the stimulus. An example of the corresponding changes in the buxco flow trace during a coughing event (grey dashed square) evoked by 3mg ml^{-1} of bradykinin is shown. The still image demonstrates the characteristic cough stance that consists of the animal raising on their forelimbs in a hunched posture (Tsubone et al., 1991; Fox et al., 1996). Artefact = reflection from video recording camera.

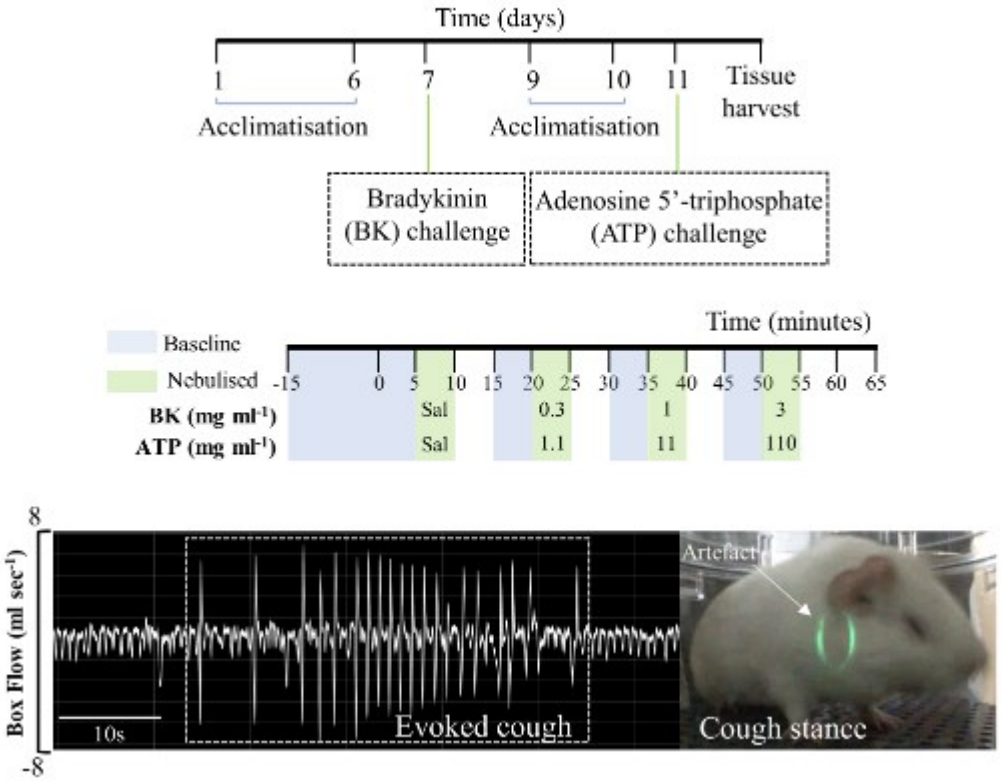


Figure 2: Characterisation of substance P-Saporin (SSP-SAP) lesions of the paratrigeminal nucleus (Pa5) in the guinea pig. (A) Calbindin (yellow triangles) and neurokinin 1 receptor (NK1R; green circles) positive neurons in a representative sham lesion and SSP-SAP lesion animal transposed onto schematic traces of the dorsal medulla at four rostro-caudal levels. The lateral and medial dashed boxes show the the Pa5 and nucleus of the solitary tract (nTS). (B) Example photomicrographs of NK1R immunostaining in the Pa5 and nTS (~0.5mm rostral to Calamus Scriptorius) in sham lesion and SSP-SAP lesion guinea pigs. Scale bars represent 150µm. Insets show high magnification photomicrographs (NK1R, green and DAPI, blue; scale bars represent 40µm). Mean cell counts ± SD of NK1R (n=14) and Calbindin (n=6-10) positive Pa5 or nTS neurons in the sham lesion and SSP-SAP lesion animals are represented in the histograms. ***, p=0.0002 (unpaired two-tailed Student's t-test).

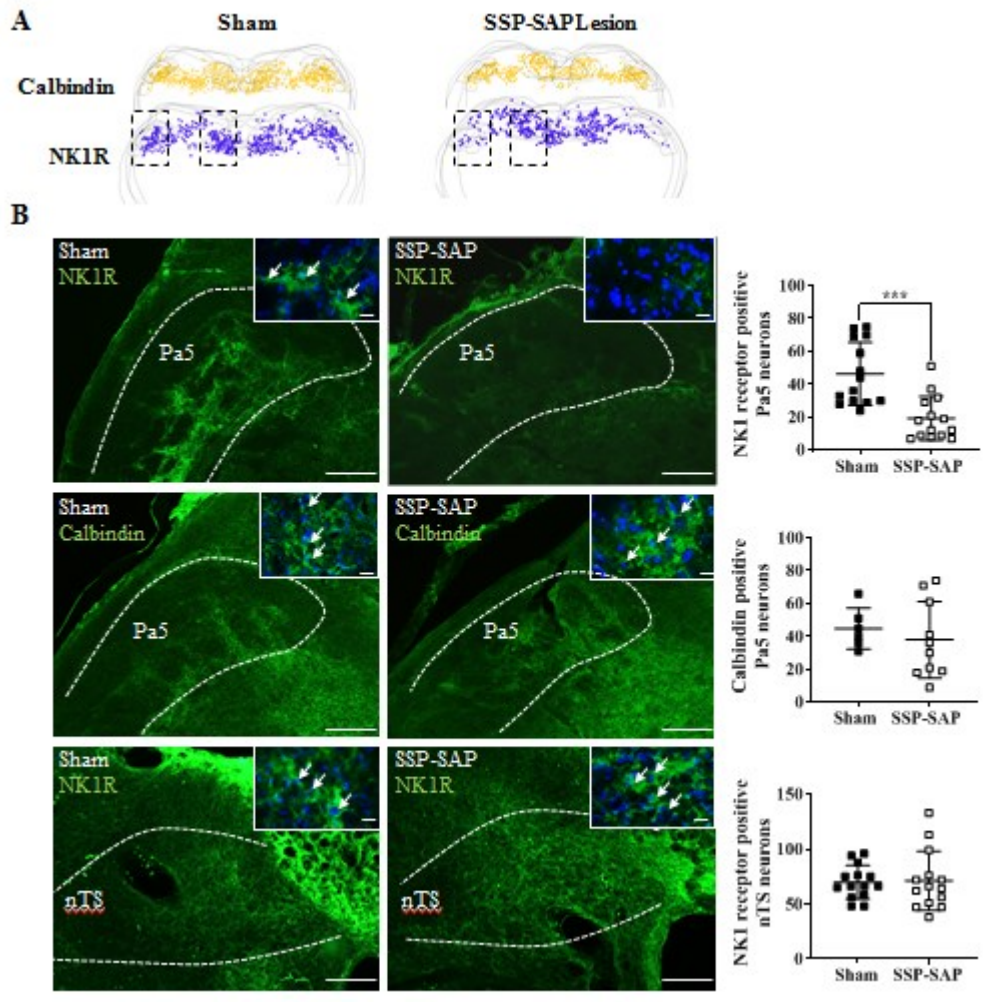


Figure 3: Substance P-Saporin (SSP-SAP) lesions of the paratrigeminal nucleus reduces bradykinin-evoked cough in conscious guinea pigs but does not alter adenosine 5'-triphosphate (ATP)-evoked cough. A) The mean number of coughs per minute over the course of the entire bradykinin and ATP challenge (0-65 minutes) for no-intervention (NoI, grey circles, *n=20* for bradykinin and *n=9* for ATP), sham lesion control (black squares, *n=14* for bradykinin and *n=12* for ATP) and SSP-SAP lesion (open squares, *n=14* for bradykinin and *n=10* for ATP) animals. Blue bars represent baseline (Base) recordings and green bars represent periods of nebulised challenge (with challenge doses shown above in mg ml^{-1}). B) The cumulative total coughs presented as the mean ± SD. **p=0.01*, ***p=0.006* and *****p<0.0001 (ordinary two-way ANOVA with Tukey's multiple comparisons test)*.

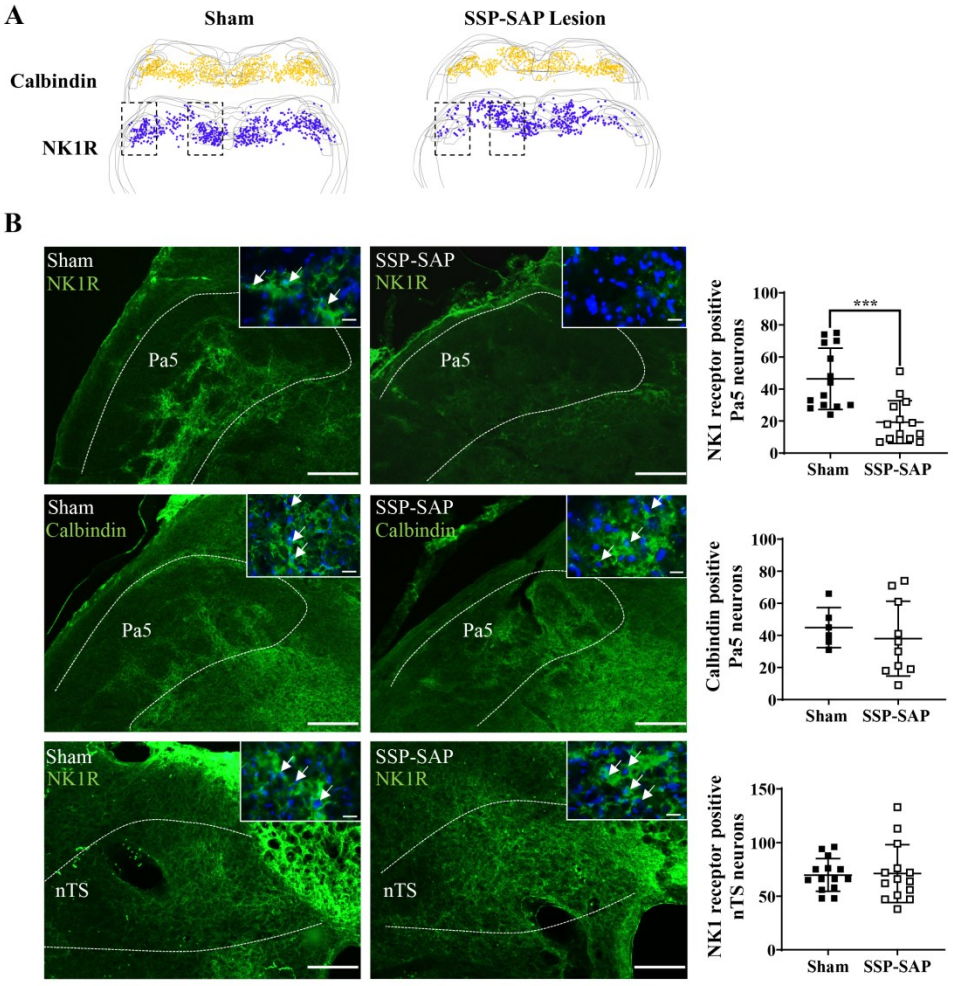


Figure 4: Substance P-Saporin (SSP-SAP) lesions of the paratrigeminal nucleus do not alter behaviours evoked by nebulised bradykinin or adenosine 5'-triphosphate (ATP) in conscious guinea pigs. A) Mean duration (seconds) of all behaviours combined per minute over the course of the entire bradykinin and ATP experimental challenges for no-intervention (NoI, grey circles, *n=20* for bradykinin and *n=9* for ATP), sham lesion control (black squares, *n=14* for bradykinin and *n=12* for ATP) and SSP-SAP lesion (open squares, *n=14* for bradykinin and *n=10* for ATP) animals. Blue bars represent baseline (Base) recordings and green bars represent periods of nebulised challenge (with challenge doses shown above in mg ml^{-1}) (B) The cumulative total behaviour duration presented as the mean ± SD.

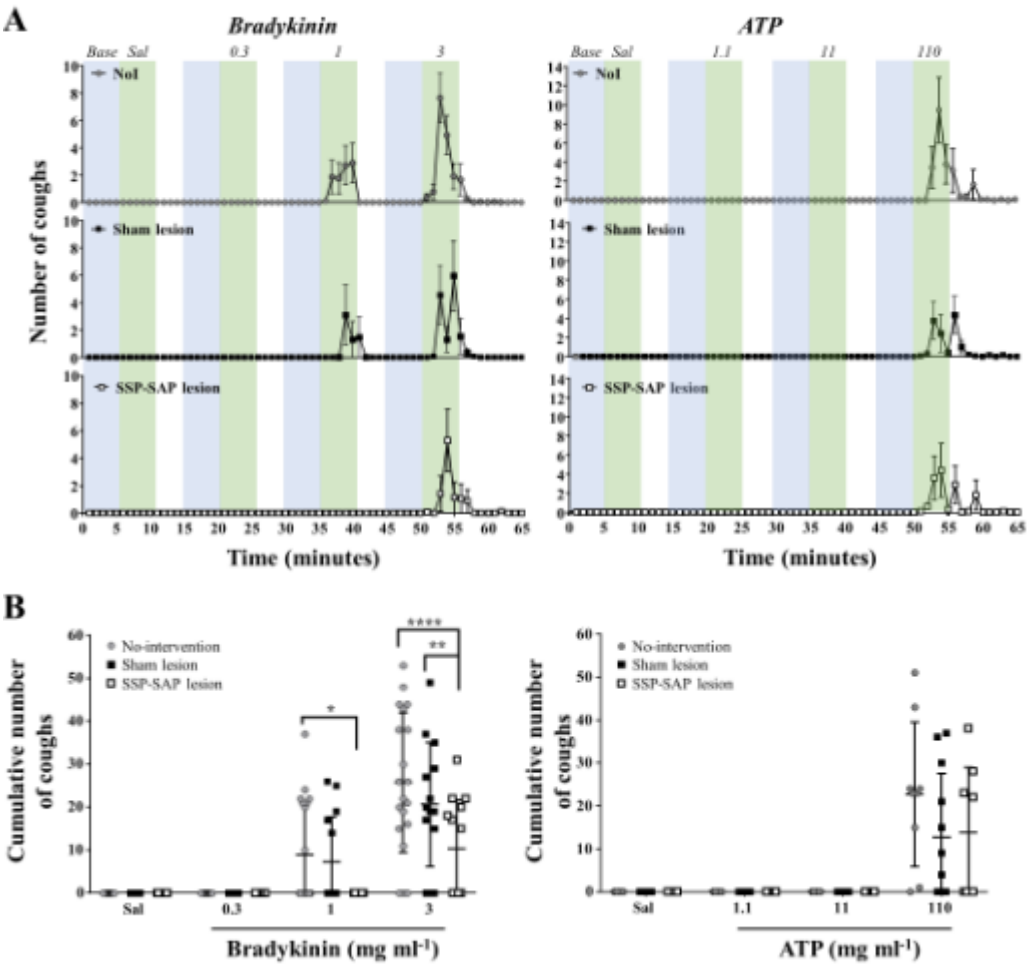
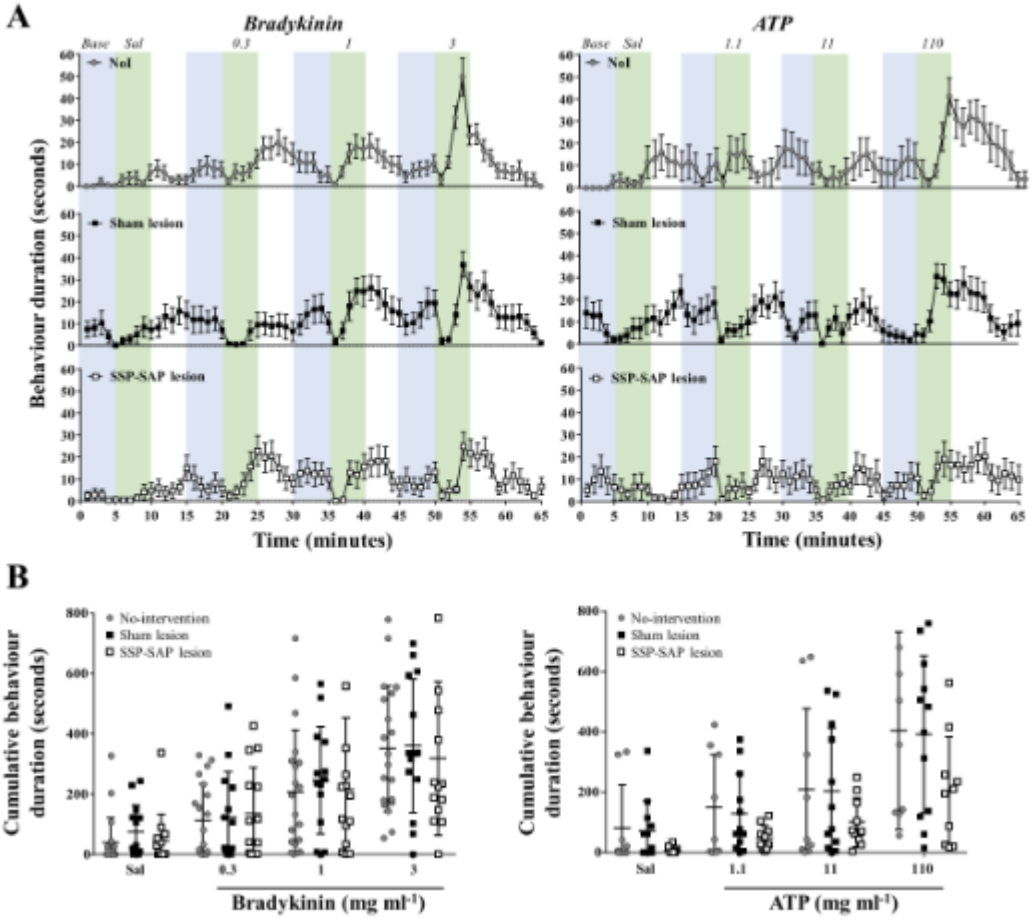
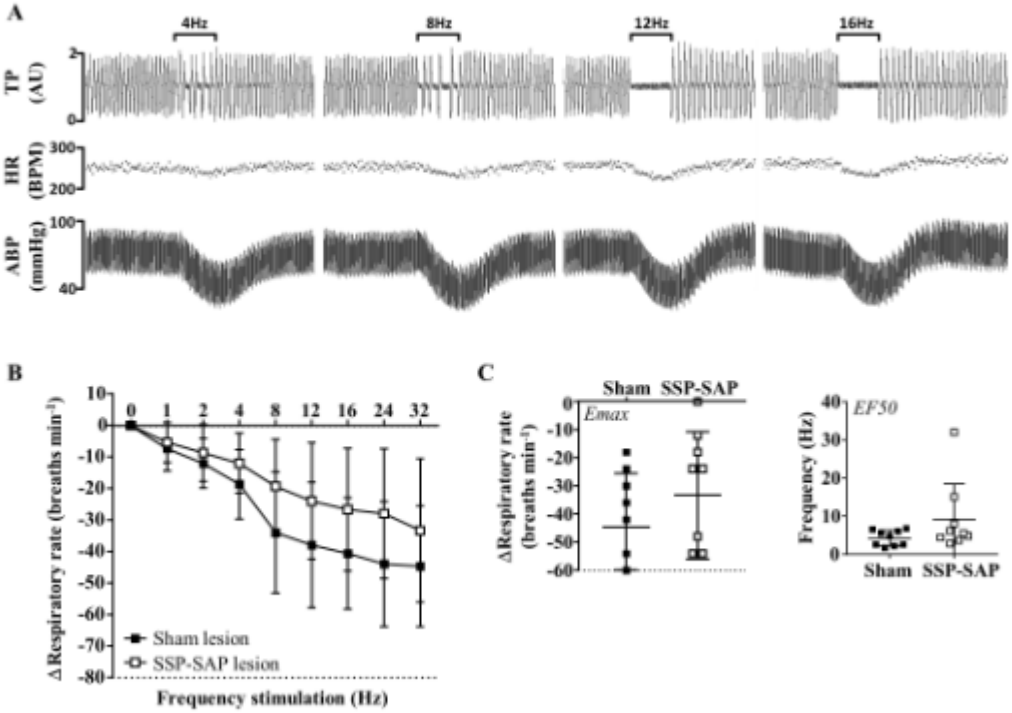


Figure 5: Effect of lesioning neurokinin 1 (NK1) receptor expressing neurons in the paratrigeminal nucleus (Pa5) on the laryngeal-evoked apnoeic reflex in anaesthetised guinea pigs. A) Representative traces of the tracheal pressure (TP, arbitrary units (AU)), heart rate (HR, beats per minute (BPM)) and arterial blood pressure (ABP, milligrams of mercury (mmHg)) during electrical stimulation of the larynx at increasing frequencies. B) Mean data showing sham lesion control (black squares; *n=9*) and SSP-SAP lesion (open squares; *n=9*) animals. C) Comparison of the effect size (maximum respiratory reduction, E_{max}) and sensitivity (50% of the maximum effect, EF_{50}) between the sham lesion control and SSP-SAP lesion animals.





Tables

Table 1: Cumulative duration of ancillary behaviours associated with aerosolised bradykinin in the conscious unrestrained guinea pig

Behaviour	**Saline (mg ml^{-1})**			**0.3 (mg ml^{-1})**			**1 (mg ml^{-1})**			**3 (mg ml^{-1})**		
Cohort	***1***	**2**	**3**	***1***	***2***	***3***	***1***	***2***	***3***	***1***	***2***	***3***
Time spent in cough stance (seconds) *[dose and cohort interaction p=0.01]*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.1 ± 0.4	0 ± 0	16.7 ± 21.9	11.9 ± 17.9	0 ± 0	52.1 ± 53.5	33.3 ± 27.6 *p= 0.03	16.2 ± 16.8 **** $p\leq0.0001$
Time spent coughing (seconds) *[dose and cohort interaction*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	11.1 ± 13.2	8.5 ± 12.4	0 ± 0	33.4± 25.4	22.7 ± 17.4	10.8 ± 14.3 **** $p\leq0.00$

p=0.002]												*01*
Number of coughing events *[dose and cohort interaction p=0.005]*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1.0 ± 1.3	0.6 ± 0.9	0 ± 0	3.3 ± 2.8	2.9 ± 2.4	1.0 ± 1.2 **** *p≤0.0001* ## *p=0.002*
Time spent chewing (seconds) *[no statistical interaction]*	29.4 ± 73.5	73.6 ± 86.4	37.1 ± 87.5	105.3± 110.0	113.9 ± 148.2	121.4 ± 132.0	176.5 ± 172.5	202.9 ± 162.8	195.4 ± 200.6	270.0 ± 180.0	289.3 ± 197.5	276.3 ± 219.6
Time spent grooming (seconds) *[no statistical interaction]*	3.0 ± 10.0	4.9 ± 10.7	3.4 ± 7.7	7.2 ± 14.2	6.0 ± 11.1	12.6 ± 27.9	11.8± 23.3	12.8 ± 13.4	17.5 ± 35.3	19.9 ± 28.1	21.9 ± 26.1	22.0 ± 43.7
Time spent locomotor (seconds) *[no statistical interaction]*	5.3 ± 12.5	0.7 ± 1.4	4.7 ± 13.9	6.9 ± 15.2	1.4 ± 2.1	7.6 ± 24.8	8.5 ± 16.2	2.3 ± 3.2	9.2 ± 25.4	12.6 ± 19.1	3.6 ± 4.2	10.4 ± 25.1

*NB: 1 = No-intervention cohort (n=20), 2= Sham lesion control cohort (n=14) and 3= SSP-SAP lesion cohort (n=14), presented as the mean ± SD. Two-way ANOVA with Tukey's multiple comparisons were completed where appropriate. Statistical interactions are noted in the table. Statistical comparisons between cohorts within a dose have been identified in the table using the following key * significantly different to behaviour of cohort 1 (no-intervention) and # significantly different to behaviour of cohort 2 (sham lesion control).*

Table 2: Cumulative duration of ancillary behaviours associated with aerosolised adenosine 5'-triphosphate (ATP) in the conscious unrestrained guinea pig

Behaviour	**Saline (mg ml^{-1})**			**1.1 (mg ml^{-1})**			**11 (mg ml^{-1})**			**110 (mg ml^{-1})**		
Cohort	***1***	***2***	***3***	***1***	***2***	***3***	***1***	***2***	***3***	***1***	***2***	***3***
Time spent in cough stance (seconds) *[no statistical interaction]*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	3.9 ± 3.9	0 ± 0	38.4 ± 12.5	21.7 ± 39.9	19.3 ± 21.8
Time spent coughing (seconds) *[no statistical interaction]*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	22.6 ± 16.7	15.3 ± 19.5	14.4 ± 17.0
Number of coughing events *[no statistical interaction]*	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2.9 ± 2.8	3.5 ± 3.2	2.4 ± 3.0
Time spent chewing (seconds) *[no statistical interaction]*	75.4 ± 130.9	61.2 ± 98.8	28 ± 58.3	154.6± 180.8	114.1 ± 129.7	59.0 ± 78.4	213.61± 259.2	175.9 ± 202.5	108.7 ± 108.7	355.3 ± 309.3	322.4 ± 250.1	179.7 ± 181.3
Time spent grooming (seconds) *[no statistical interaction]*	1.8 ± 3.6	4.3 ± 6.1	1.5 ± 2.5	3.1 ± 4.91	10.3 ± 10.6	7.4 ± 11.6	3.8 ± 4.8	18.7 ± 20.4	8.3 ± 11.3	8.4 ± 9.9	10.4 ± 27.0	14.3 ± 16.0
Time spent locomotor (seconds) *[no statistical interaction]*	0 ± 0	1.2 ± 1.9	0.9 ± 2.9	0.4 ± 1.2	4.5 ± 6.0	1.4 ± 2.9	0.9 ± 1.8	6.4 ± 9.9	2.7 ± 4.6	3.6 ± 5.9	10.4 ± 15.2	3.3 ± 5.2

NB: 1 = No-intervention cohort (n=9), 2= Sham lesion control cohort (n=12) and 3= SSP-SAP lesion cohort (n=10), presented as the mean ± SD. Two-way ANOVA with Tukey's multiple comparisons were completed where appropriate. No statistical interactions were found.

Table 3: Respiratory measures associated with nebulised bradykinin in the conscious unrestrained guinea pig

Bradykinin challenge (mg ml^{-1})	**No-intervention (*n=20*)**				**Sham lesion (*n=14*)**				**SSP-SAP lesion (*n=14*)**			
	Freq	***Tvb***	***Ti***	***Te***	***Freq***	***Tvb***	***Ti***	***Te***	***Freq***	***Tvb***	***Ti***	***Te***
Baseline	119.30 ± 32.97	2.56 ± 1.17	0.31 ± 0.49	0.46 ± 0.46	118.80 ± 27.03	2.57 ± 4.48	0.24 ± 0.13	0.39 ± 0.14	118.20 ± 15.73	2.38 ± 0.56	0.20 ± 0.03	0.34 ± 0.06
Saline	125.60 ± 34.58	2.36 ± 1.06	0.28 ± 0.39	0.43 ± 0.36	119.60 ± 24.42	2.41 ± 0.42	0.21 ± 0.05	0.35 ± 0.08	119.40 ± 20.70	2.30 ± 0.51	0.20 ± 0.03	0.34 ± 0.07
0.3	122.80 ± 31.79	2.40 ± 1.03	0.29 ± 0.42	0.44 ± 0.39	116.00 ± 24.16	2.31 ± 0.43	0.22 ± 0.05	0.36 ± 0.08	119.40 ± 16.60	2.24 ± 0.44	0.20 ± 0.03	0.34 ± 0.06
1	122.80 ± 31.79	2.43 ± 1.08	0.29 ± 0.39	0.45 ± 0.37	117.30 ± 26.87	2.33 ± 0.39	0.22 ± 0.06	0.37 ± 0.11	126.00 ± 21.31	2.12 ± 0.43	0.19 ± 0.03	0.34 ± 0.07
3	124.20 ± 34.92	2.44 ± 1.13	0.28 ± 0.37	0.47 ± 0.37	121.90 ± 30.90	2.25 ± 0.35	0.22 ± 0.06	0.37 ± 0.15	121.60 ± 19.39	2.13 ± 0.31	0.20 ± 0.04	0.36 ± 0.09

NB: Frequency (freq), breaths min^{-1}; Tidal volume (Tvb), ml kg^{-1}; Inspiratory/Expiratory time (Ti/Te), seconds, presented as the mean per minute ± SD. Two-way ANOVA with Tukey's multiple comparisons were completed where appropriate, and no statistically significant differences were observed.

Table 4: Respiratory measures associated with nebulised adenosine 5'-triphosphate (ATP) in the conscious unrestrained guinea pig

ATP challenge (mg ml^{-1})	**No-intervention (*n=8*)**				**Sham lesion (*n=12*)**				**SSP-SAP lesion (*n=10*)**			
	Freq	***Tvb***	***Ti***	***Te***	***Freq***	***Tvb***	***Ti***	***Te***	***Freq***	***Tvb***	***Ti***	***Te***
Baseline	92.21 ± 16.23	2.69 ± 0.94	0.24 ± 0.04	0.45 ± 0.08	116.70 ± 24.53	2.66 ± 0.47	0.21 ± 0.06	0.35± 0.10	128.70 ± 22.79	2.51 ± 0.44	0.18 ± 0.02	0.32 ± 0.06
Saline	97.50 ± 14.32	2.58 ± 0.89	0.23 ± 0.03	0.43 ± 0.07	118.7 ± 23.63	2.57 ± 0.35	0.22 ± 0.08	0.36 ± 0.14	128.10 ± 38.03	2.33 ± 0.40	0.18 ± 0.04	0.33 ± 0.09
1.1	96.26 ± 12.11	2.52 ± 0.87	0.24 ± 0.03	0.44 ± 0.05	118.60 ± 27.52	2.48 ± 0.37	0.23 ± 0.05	0.36 ± 0.10	127.80 ± 29.24	2.26 ± 0.39	0.18 ± 0.04	0.32 ± 0.08
11	92.73 ± 13.38	2.49 ± 0.87	0.24 ± 0.05	0.46 ± 0.05	114.50 ± 24.36	2.39 ± 0.37	0.22 ± 0.05	0.37 ± 0.10	121.70 ± 22.24	2.22 ± 0.38	0.19 ± 0.04	0.34 ± 0.07
110	95.63 ± 13.73	2.74 ± 1.15	0.25 ± 0.05	0.46 ± 0.07	116.50 ± 20.80	2.41 ± 0.37	0.22 ± 0.04	0.35 ± 0.06	111.70 ± 13.45	2.33 ± 0.38	0.22 ± 0.04	0.48 ± 0.06

NB: Frequency (freq), breaths min^{-1}; Tidal volume (Tvb), ml kg^{-1}; Inspiratory/Expiratory time (Ti/Te), seconds, presented as the mean per minute ± SD. Two-way ANOVA with Tukey's multiple comparisons were completed where appropriate, and no statistically significant differences were observed.

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