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Cardiovascular autonomic effects of transcutaneous auricular nerve stimulation via the tragus in the rat involve spinal cervical sensory afferent pathways.

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1. Introduction:

Non-invasive electrical stimulation of the external ear has generated increasing interest as a potential treatment for disorders ranging from heart failure (1-3), epilepsy (4, 5) autism (6), depression (7-9), and tinnitus (10, 11). Such stimulation has been applied to different areas of the external auricle, including the tragus (3, 12-17), the cavum concha (8, 9, 18, 19) and the cymba concha (20). Stimulation at any of these different auricular locations has often been termed transcutaneous vagal nerve stimulation (tVNS) on the presumption that the auricular branch of the vagus nerve (ABVN) has been activated.

The view that these stimulation sites activated the ABVN has mainly come from a single study which reported from human cadaveric dissections that the ABVN could supply innervation to the cymbae conchae, antihelix, cavity of concha, tragus, crus of helix and crura of helix. Most of these regions were also reported to be innervated by the greater auricular nerve (GAN) and/or the auriculotemporal nerve (ATN) (21). Further, bioimaging studies revealed that auricular stimulation in humans resulted in activation of brain regions associated with vagal input, including the region of vagal afferent innervation, the nucleus tractus solitarius, as well as upstream regions such as the locus coeruleus (13, 19, 20, 22). However, uncertainties regarding original anatomical descriptions of neural innervation of the auricle has resulted in calls for careful interpretation on vagal involvement on auricular stimulation (23, 24).

Given the difficulties in obtaining precise information on auricular afferent terminations from human subjects, animal models could shed some light on pathways underlying auricular stimulation. This study therefore utilised rats to examine the central nervous

sites of termination of primary afferent projections from the tragus, a stimulation site utilised in several studies in humans. The cardiovascular autonomic modulatory effects of stimulation of the tragus in rats were examined in an anaesthetic free Working Heart Brainstem Preparation (WHBP). Since tVNS is most often applied in people when awake whensympathetic activity is higher than asleep, the effect of time of day on autonomic activity in the WHBP was first examined to provide a suitable comparison.

2. Methods:

All procedures were conformed to the UK Animals (Scientific Procedures) Act of 1986 and received approval from local ethics. 54 male Wistar rats were used (Figure 1).

2.1 Neuronal tracing

Young male Wistar rats (65-85g; n=4) were deeply anaesthetised with a 4% mixture of isoflurane in O_2. Cholera Toxin B (CTB) was injected into the right tragus with a total of 1-2 µl of 20 mg/ml in 0.1 M phosphate buffer saline (PBS) using a glass microelectrode attached to a 10 µl Hamilton syringe (Sigma Aldrich, UK). The animals were allowed to recover for 3-4 days.

2.2 Tissue sampling and Immunohistochemistry

Animals were deeply anaesthetized with 60-80 mg/kg of intraperitoneal sodium pentobarbitone (Euthatal, Merial UK) and transcardially perfused with 4% paraformaldehyde (PFA) as described previously (25). The brainstem and spinal cord were dissected and post-fixed overnight in PFA. Tissue was then sectioned transversely at 50 µm from the C4 level of cervical spinal cord through to the rostral

brainstem, washed three times in phosphate buffer solution (PBS) before incubation in rabbit anti-CTB (Sigma, C3062). Some sections were also incubated in Goat anti-Choline acetyl transferase (ChAT) (1:500 dilution; Chemicon; AB144P) in PBS with 0.3% Triton X-100 (Row & Haas, UK). Sections were left on a shaker at 4^{o}C overnight (12 - 18h). Following overnight incubation (~3 nights for ChAT), sections were rinsed 3 times with PBS. The sections were incubated in secondary antibody; Alexafluor^{488} conjugated donkey anti-goat (1:1000 in PBS, Invitrogen, Paisley UK); Alexafluor^{555} conjugated donkey anti-rabbit (1:1000 in PBS; Invitrogen, Paisley, UK) for ~ 2 hours at room temperature. Tissue was then rinsed for 3 times for 10 minutes before being air dried and mounted on glass slides with Vectashield mounting medium (Vector Laboratories, Peterborough, UK) and sealed with nail varnish.

2.3 Image capture, manipulation and examination

Images were visualized with an epifluorescence microscope (Eclipse E600; Nikon, UK). Confocal images were captured by a Zeiss LSM 880, exported in TIFF format and final image editing (eg: brightness, contrast and intensity) was processed with CorelDRAW® software (X8 Edition).

2.3 Circadian study

Daytime animals (n=10, age 16-21 days) were caged in normal lighting condition (lights on 7 am and off 7 pm; subsequently referred to as day time animals). Another group of animals (n=20) were caged in a reversed lighting cabinet (lights on 7 pm and off 7 am) and henceforth referred to as night time animals. The reversed lighting group were acclimatised in this regime for 7 days before being used in the experiment. During transfer handling, light exposure of the night time animals was minimised by using a dark box. During statistical analysis, animals were divided according to specific time

of preparation. The preparations under light phase were done either at 1030 or 1430, while the recordings were conducted at dark phase were equivalent at 2130 or 0230.

2.4 Working Heart Brainstem Preparation (WHBP)

Procedure for the WHBP has been described previously (26, 27). In brief, 18 day old male rats were deeply anaesthetized through 4% isoflurane inhalation, bisected sub diaphragmatically, and decerebrated in ice cold modified artificial cerebrospinal fluid (aCSF) solution. The preparation was then skinned and the phrenic nerve, descending aorta, and lumbar sympathetic chain were carefully isolated (n = 40). The descending aorta was cannulated and retrogradely perfused with modified aCSF containing, in mM: NaCl, 125; $NaHCO_3$, 25; KCl, 4; $CaCl_2.2H_2O$, 2.5; $MgSO_4$, 1.25; KH_2PO_4 1.25 and D-Glucose, 10. Polyethylene glycol (2.5 mg per 200 ml perfusate). Vecuronium bromide (2-4 µg/ml, Organon Teknica, Cambridge, UK) was added during initial stages of the preparations to block neuromuscular transmission (Potts et al., 2000).

Perfusion flow rate was altered to obtain a eupnic-like pattern in the phrenic nerve discharge. Baseline heart rate (HR), perfusion pressure (PP), sympathetic nerve discharge (SND) and phrenic nerve discharge (PND) were recorded following at least 30 min of eupnoeic breathing.

2.5 Recordings

Multiple unit nerve discharge was recorded from the distal ends of the phrenic and lumbar sympathetic nerves via glass suction electrodes connected to a pre-amplifier head stage (Digitimer, NL 100) and fed into a Neurolog amplifier (x 1000 amplification; Digitimer, NL 900D). 50/60 Hz noise and harmonics were eliminated using a Humbug (Quest Scientific, Canada). Signals were also bandpass filtered between 50 Hz and 4

kHz and further digitised with a sampling frequency of 8 kHz and saved on computer using an interface (CED 1401, UK) for analysis on Spike 2 software offline.

2.6 Neurectomy

All nerve sections were performed ipsilateral to the site of ear stimulation, with the dissection process initiated after animal decerebration and prior to the preparations being taken into the recording chamber.

In one group of animals, the dorsal roots of the upper cervical cord were sectioned (n=10). In brief, posterior neck muscles that covered the upper cervical cord axis were dissected to expose vertebrae. The spinous processes were carefully laminectomized and the dorsal roots from the first to the third cervical spinal nerves were cut using springbow dissecting scissors.

In another group, the cervical vagus nerve was sectioned (n=10). Dissection was initiated by removal of the right superficial muscle, the sternohyoid muscle, exposing the carotid bifurcation. At this point, the vagus nerve is visible lying next to the common carotid artery surrounded by a layer of sheath. Careful identification was required as the superior cervical sympathetic ganglion also lies nearby. The vagus nerve was carefully isolated from the sheath that attached to the common carotid artery and a thread was tied around the vagus nerve trunk. This thread was used to pull the nerve gently away during the WHBP preparation while iris scissors were utilised to cut the nerve without touching the surrounding tissues.

2.7 Analysis

SND was recorded as described in section 2.5, integrated and rectified in Spike 2 software prior to calculation of the amplitude of integrated SND from the area under

the curve. Heart rate was derived from ECG signals from phrenic nerve recordings and R-R intervals converted into a tachogram. Mean heart rate was averaged within one minute segments. In all preparations, any arrhythmic or irregular heartbeats were excluded.

2.9 Data presentation

All numerical data are presented as mean ± standard error of the mean. N refers to the number of animals. All statistical analyses were conducted using GraphPad Prism, Ver7. Differences were considered significant when $p < 0.05$. The normality of each dataset was analysed with Shapiro-Wilk test. Normally distributed datasets were analysed with repeated measures ANOVA and confirmed using Tukey's or Dunnett's post-hoc tests for multiple comparisons. The non-normally distributed data were analysed with Friedman's test followed by Dunn's post-hoc tests for multiple comparisons. To examine statistical significance between two groups, an independent samples t-test was performed for normally distributed data or for non-normally distributed data a Mann-Whitney U-test was performed.

3. Results:

3.1 Central afferent labelling from the tragus

Rat brains were examined starting from the level of the 4th ventricle (Bregma -10.04 mm) to upper cervical cord in C4. The areas labelled with CTB within the CNS were ipsilateral to the injected tragus and were identical for each of the 4 animals (Figure 2). In the rostral brainstem there was labelling within the paratrigeminal nucleus (Pa5). In the lower brainstem level a larger area of afferent termination was observed in the spinal trigeminal tract (Sp5) extending towards the cuneate nucleus (Cu). In the most

caudal brainstem, labelled afferents were observed coursing from Cu and extending ventromedially to the NTS covering the lateral, dorsomedial, and also medial area of the NTS**.** There were few close appositions between CTB-labelled afferents and ChAT immunofluorescent neurones.

Traced afferents within the upper cervical cord (C1-C3) showed substantial CTB labelling in varying laminae (Figure 3). In C1, little but noticeable staining could be observed in laminae III and IV. In C2, a large area of labelling covered laminae I, III, and IV but not lamina II**.** The main termination of CTB labelled from the tragus was detected in the section of C3 covering laminae III and IV. No CTB staining was observed caudal to the rostral portion of C3. In lamina IV, some of the CTB labelled afferent fibres were in close apposition to ChAT-immunofluorescent neurons that were presumable cholinergic interneurons.

3.2 Circadian study

Cardiorespiratory activity was recorded from the WHBP of rats at different times of day (Figure 4). In night time animals, the tonic discharge of the lumbar sympathetic chain was significantly higher (n= 17, 4.7± 0.5 AUC) than in day time animals (n=8, 3.0 ± 0.6 AUC, Mann-Whitney U test; $p < 0.05$). Respiratory rate, measured as phrenic nerve discharge frequency, was higher in night time (15.7 $\pm$ 0.6 breaths /min) compared to day time animals (12.9 $\pm$ 1.1 breaths/min; $p < 0.05$, Mann-Whitney U test). There were no statistical differences in heart rate or perfusion pressure. Since SND was higher in night time animals and tVNS was found to decrease SND in humans when SND is highest (in the morning in humans), night time animals were used to further examine the effects of tragus stimulation on SND.

3.3 Tragus Stimulation

Right tragus stimulation was performed using a DS3 Constant Current Isolated Stimulator (Digitimer Ltd, UK) connected to a modified metal ear clip (Figure 5). The stimulation was applied for 5 minutes at 100 Hz, 2.5 mA with any current leakage grounded. Comparing pre-stimulation with measurements at 1 minute and 10 minutes after electrical stimulation revealed that there were no significant changes in heart rate and perfusion pressure. In contrast, direct measurement of the lumbar sympathetic nerve activity (baseline= 4.87 ± 0.52 AUC) indicated significant sympathoinhibition at 1 minute post-stimulation (3.70 ± 0.36 AUC) and also at 10 minute post stimulation (3.17 ± 1.18 AUC; ANOVA, post-hoc Dunnett's multiple comparison test; $p < 0.05$, Figure 5C).

To determine if vagal efferent nerves were involved in responses to tragus stimulation, physiological parameters were recorded in vagotomised preparations. Confirming vagal section, HR was significantly higher post transection (320 $\pm$ 4.8 bpm) than control (309 $\pm$ 5.6 bpm; $p < 0.05$; paired t-test). Tragus stimulation in the vagotomised groups elicited no significant effects on the HR and PP. However, there was a significant reduction in SND at 1 (3.4 $\pm$ 0.2 AUC) and 10 (3.2 $\pm$ 0.3 AUC) minutes post stimulation compared to control (4.0 ± 0.2 AUC; $p < 0.05$, Figure 5D).

Since neuronal tracing from the tragus revealed an abundance of labelled afferent terminals in the cervical spinal cord dorsal horn, the contribution of the C1-C3 dorsal roots to tVNS mediated SND reductions was tested by sectioning these roots in the WHBP. Tragal stimulation in C1-C3 transected WHBP had no effect on heart rate or perfusion pressure. However, an arterial depressor response was evident at 10 minute post-stimulation (from 65.4 ± 5.1 mmHg baseline to 61.9 ± 5.9 mmHg;

Friedman’s ANOVA and Dunn's multiple comparisons test, data not shown). Unlike stimulation in intact preparations, the integrated SND recorded from the lumbar sympathetic trunk remained unaltered across all time points (Figure 5E).

4. Discussion:

This study reveals that in the rat the most dense central termination site of sensory afferent fibres from the tragus is the cervical dorsal horn of the spinal cord and that this pathway is involved in mediating reductions in sympathetic nerve activity elicited by electrical stimulation of the tragus. In addition, circadian rhythm is present in sympathetic nerve activity and respiratory rate in the WHBP, indicating that such rhythms are maintained in the absence of the central circadian clock, the suprachiasmatic nucleus.

4.1 The cervical spinal cord dorsal horn is a major termination site for sensory afferents from the tragus.

A significant finding was that the major sensory projections from the tragus were to the dorsal horn of the C1-C3 cervical spinal cord. The labelled afferents in the upper cervical cord covered laminae I, III & IV. Similar labelling in the dorsal horn has been mentioned previously when CTB was injected into the inner concha of rats, but no images or other analyses were shown (19). This is therefore the first study to reveal the sizeable extent of afferent signalling from the tragus to the cervical spinal cord. The physiological significance from this innervation was then tested in the WHBP.

4.2 Possible neuronal pathways via the cervical cord dorsal horn mediating the effects of tragus stimulation.

Dorsal horn neurons are typically associated with conveying sensory information from the skin and internal organs into the spinothalamic tract for pain perception processing (28). Although perhaps less widely appreciated, neurons in the superficial laminae of the cervical dorsal horn also provide synaptic input into cardiovascular centres in the medullary reticular formation. Injections of anterograde tracer into the superficial (I-II) of the cervical dorsal horn in rats revealed projections predominantly concentrated in the medial part of the commissural subnuclei of the NTS at caudal levels, whilst the deeper laminae showed predominant labelled fibres and terminals only in the ventrolateral and dorsolateral portions of the caudal NTS (29). In a similar experiment, microinjection of an anterograde neuronal tracer covering laminae I-V of the cervical spinal dorsal horn of rats, revealed labelled axons in the medial, dorsomedial and commissural subdivisions of the caudal NTS, with most of the staining found ipsilaterally (30). In addition, projections to other brainstem regions involved in autonomic control were also observed – including the caudal and rostral ventrolateral medulla, which play significant roles in the control of sympathetic function. Hence, this suggests afferent projections from the tragus can indirectly influence brainstem regions involved in sympathetic function via the cervical spinal cord. Future studies could investigate this proposition by injecting the tragus with viruses that travel transynaptically only in the anterograde direction, such as select rabies viruses (31).

There is also a possibility that some of the effects of activating cervical sensory afferents are mediated through connections within the spinal cord. There are neurons in the cervical spinal cord that are pre- sympathetic as they are labelled following transneuronal virus application to the stellate ganglion (32), but they are located laterally in the white matter and so appear unlikely to be innervated by the tragus

afferents, although their dendritic architecture is unknown and it is possible that these dendrites lie within the afferent termination fields. The circuitry underlying the cervical cord mediation of tragus evoked responses therefore requires further examination.

4.3 Pa5 is likely to be involved in the tragus evoked responses

Following injection of CTB into the tragus, a prominent projection was to the paratrigminal nucleus (Pa5) in the brainstem. The Pa5 is a small collection of neurons within the dorsal lateral medullary spinal trigeminal tract that receives input from the jugular ganglion since injection of CTB^{488} into the Pa5 of guinea pigs labelled jugular ganglion neurons, whilst injection of CTB^{555} into the NTS labelled predominantly the nodose ganglion (33). The input from the jugular ganglion to the Pa5 is consistent with observations in the cat that the ABVN sensory somata are within the jugular ganglion (35) and the Pa5 tragus projections observed in this study.

Previous studies have revealed roles for the Pa5 consistent with a role in mediating the tragus influences. The Pa5 also receives sensory input from airways - anterograde trans- neuronal viral tracing from the trachea of rats identified tracheal sensory inputs towards the Pa5 that were relayed in the brainstem to the spinal trigeminal complex (33). This input is involved in respiratory reflexes, revealed by a study performed on guinea pigs with a selective recurrent laryngeal nerve section to remove nodose ganglion inputs (eliminating the cough reflex), but leaving afferents via the jugular ganglion intact. Electrical laryngeal stimulation evoked a frequency dependent respiratory slowing and a mild decrease in blood pressure. Both of these responses were abolished when the Pa5 was inhibited by muscimol injections (35). Since approximately 4% of the human population coughs when cleaning their ears, the so-

called Arnold's reflex, this may reflect a role for the tragus afferents to the Pa5 in some cases.

Neurons in Pa5 have also been suggested to have a role in baroreceptor reflex modulation. Electrophysiological recording from Pa5 neurons in anaesthetic free rats showed that a large percentage (~35%) increased firing rate in response to intravenous phenylepinephrine injection (36). The functional role of the Pa5 in baroreflex control was later tested in anaesthetic free rats that underwent Pa5 ablation. Not only was a reduction in the baroreceptor reflex sensitivity ($\Delta HR/\Delta AP$) observed, resting AP and HR decreased, whilst respiratory rate increased; comparable to basal changes on lesioned NTS rats (36). It therefore seems possible that the tragus projections to the Pa5 could be involved in the stimulation evoked sympathetic nerve reductions, but direct experimental clarification is required.

4.4 The afferent projection from the tragus to the NTS in the rat is limited.

Although the NTS is the main target for vagal and glossopharyngeal sensory afferents (39), very few labelled afferents were found in the NTS following tragus injections of CTB in this study. This is similar to labelling from the concha, which was minimal and restricted to the medial NTS (19). Although according to human cadaveric dissections both of these auricular regions are vagally innervated, they are also innervated by other nerves (21,37). Therefore, the projection to the NTS may reflect that each of these areas only receives a small innervation from the ABVN. There have been no studies where the ABVN in rats has been specifically labelled, probably due to relative inaccessibility for recovery surgery. However, in cats application of HRP to the ABVN also resulted in labelling that was not predominantly in the NTS, but rather the spinal

trigeminal nucleus and cervical cord dorsal horn (34). It is therefore possible that the NTS is not in fact a major target of the ABVN sensory afferents.

The lack of projection from the external ear direct to the NTS appears at first glance contradictory to a functional study - when the auricular dermatomes in anaesthetised rats were stimulated by electrical stimulation (100 Hz, 1mA) or manual acupuncture and the pattern of cardiovascular and gastric responses were documented. A mild depressor response (6%-12%) was noted in the blood pressure, HR, and intragastric pressure from manual and electrical stimulation of the ear regardless of the stimulation area (38). Further, manual stimulation on the auricular area with the ABVN innervation (e.g. concha) revealed significant activation of the neurons in the NTS with cardiac related activity (presumed baroreceptive cells) which was associated with inhibition of the blood pressure and HR (39). However, it is possible that the pathway underlying activation of these NTS 'baroreceptive' neurons is not through the NTS, but through the spinal cord cervical dorsal horn as discussed above.

4.5 Comparing tVNS in WHBP to human studies

As with most experimental preparations, the WHBP offers both advantages and limitations. Advantages include ease of access to peripheral nerves for recording (sympathetic in this case) or transecting (vagus and C1-C3) as well as the absence of anaesthetic which may modulate cardiorespiratory function. Limitations include the fact that arterial pressure and heart rate are significantly influenced by the perfusion rate, possibly limiting ability to detect changes in these parameters. In addition, whilst cardiorespiratory reflexes in the WHBP have previously been shown to be similar to those of mature animals (26, 27), the relative immaturity of the nervous system in the age of rats used may influence responses. Despite such caveats, there are similarities

between the outcomes of tragal tVNS in the WHBP to those in humans. For example, tragal stimulation reduced SND in the WHBP, similar to the decrease in single unit SND upon tVNS in healthy humans (12). Interestingly, reductions in SND was observed in the WHBP only when higher (in night time rats), similar to observations in healthy humans that tVNS reduced single unit SND only when it was relatively high, in subjects also exhibiting a low heart rate variability (12). It may therefore be of interest to determine the effects of tVNS in animal models of other human conditions where SND is elevated, such as hypertension and heart failure, albeit maintaining consideration of differences between animal models and human subjects.

4.6 Persistence of circadian rhythms in SND and respiratory rate in WHBP.

An interesting finding in this study was that sympathetic nerve activity was higher in WHBP preparations of night time compared to day time animals. Such circadian patterns of SND as well as BP are found in humans (40, 41). Microneurography recordings from healthy subjects in dark phase while sleeping (overnight) showed decreases in sympathetic outflow specifically during non-rapid eye movement (non-REM) sleep and progressively into deeper sleeping stages (42). The rapid eye movement (REM) sleeping stage which is most manifest toward the end of sleep, before arousal, showed profound sympathetic activation with the HR and BP returning to levels similar to those during wakefulness (43).

Unlike humans, direct measurement of the sympathetic nerve activity in an effort to study the circadian variation in rodents is scarce. The nearest study was performed in rats where the sympathetic nerve activity measured indirectly through plasma noradrenaline and adrenaline level in free moving rats, collected hourly during 12 hour light and dark period. It was found that the circulating adrenaline and noradrenaline

levels were significantly higher during the night time, along with the more active behaviour pattern (e.g. grooming, feeding, drinking, resting) (44). Similarly, a 24 hour telemetric recording of heart rate variability showed higher LF/HF ratio in the dark-phase, suggesting predominant sympathetic nervous activity at night (45). Hence, the experiments here are consistent with different circadian autonomic profiles between humans and rats, but both exhibiting a circadian rhythm. This is evident as the current WHBP preparation showed the lumbar sympathetic activity was significantly higher at night.

An important point to note is that the circadian rhythms in SND and PND persisted in the WHBP, in which the hypothalamus has been removed. The circadian rhythms are set by the neuronal network from the SCN; nevertheless, how do these rhythms persist in its absence? Notably, circadian rhythms are driven by gene expression changes in the master clock in the SCN, but these serve to entrain similar rhythms in gene expression in cells of other organs and tissues. For example, oscillations of clock genes (per2, bmal1 and clock) have been reported in CNS regions controlling the baroreflex functions, such as the NTS and RVLM (46). How fluctuations in expression of these genes influence the behaviour of neurons in these areas, for e.g. by controlling expression of other genes such as ion channels, is unknown. Indeed, it is not known if specific cells are under circadian influence, or indeed how other regions controlling autonomic outflow, such as the IML, may also display circadian rhythms. Crucially, since the WHBP is a short term preparation, it is clear that circadian changes in the brainstem circuits controlling PND and SND are sufficient to influence these activities. Future studies may address the changes in specific cell types and how they contribute to the circadian differences observed in this study.

5. Conclusion:

Sensory afferents from the rat tragus projected heavily to the dorsal horn of the upper cervical spinal cord, and in the brainstem significantly to the Pa5 but to a lesser extent in the NTS. Stimulating the tragus resulted in sympathoinhibition recorded from the lumbar sympathetic chain. Since the neuronal tracing showed the upper cervical cord was significantly innervated by afferents originating from the tragus, the importance of this sensory afferent innervation to tragus evoked responses was then tested in C1-C3 ipsilateral nerve transected WHBP. The absence of sympathoinhibition in this preparation suggests reductions in sympathetic nerve activity were mediated at least in part through these projections.

6. Author's contribution:

The project was conceived by JD and SAD. MKM performed WHBP following tuition from VKL, who participated in some experiments. Injections for anatomical tracing were performed by MKM with JD and MKM processed tissue. Analysis was performed by MKM, with input from JD and SAD. MKM wrote the manuscript with JD and SAD.

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8. Conflicts of interest:

None declared.

9. References:

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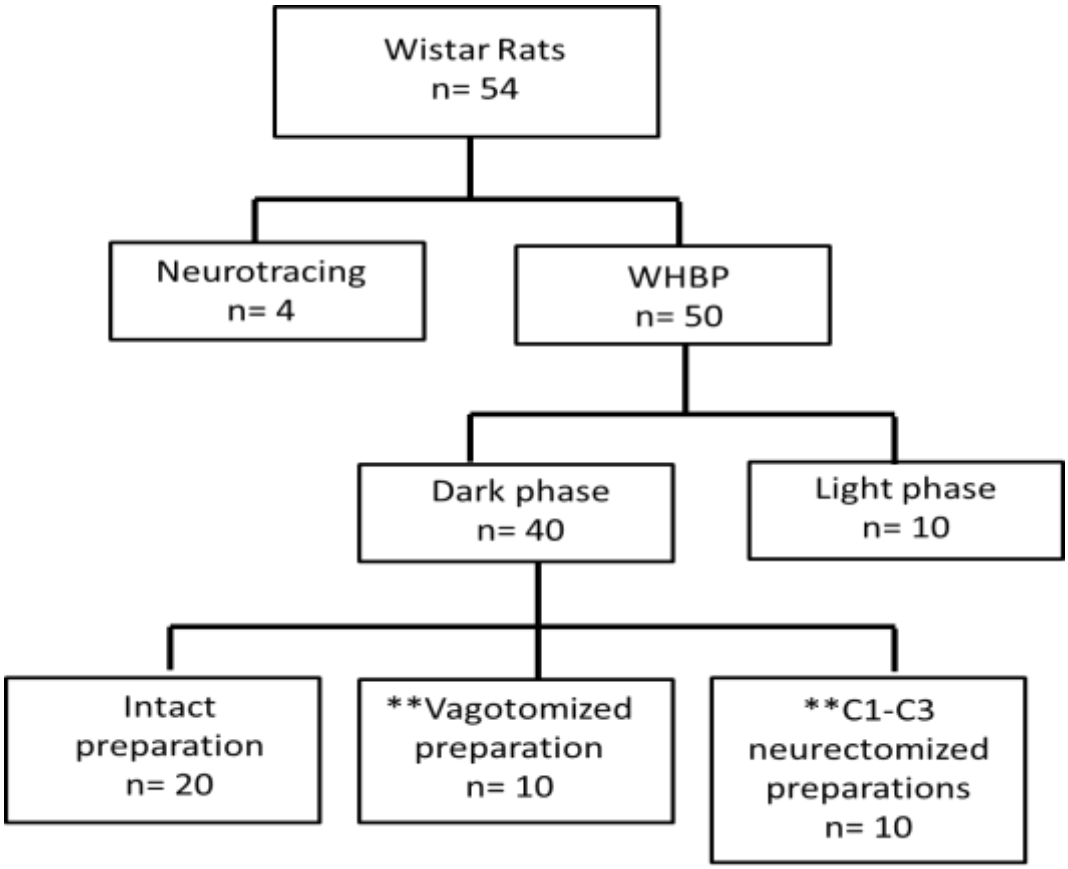
Figure 1: Groupings of animals used throughout experiments.

A total number of 54 rats were used in the experiments which were divided into the neuroanatomical study (n=4) and physiological examination (n=50). The physiological examinations upon auricular stimulations were performed with the Working Heart Brainstem Preparation (n=50). The circadian profiles of the preparations were examined using intact preparations only. ** the neurectomized preparations were excluded in circadian profile analysis.



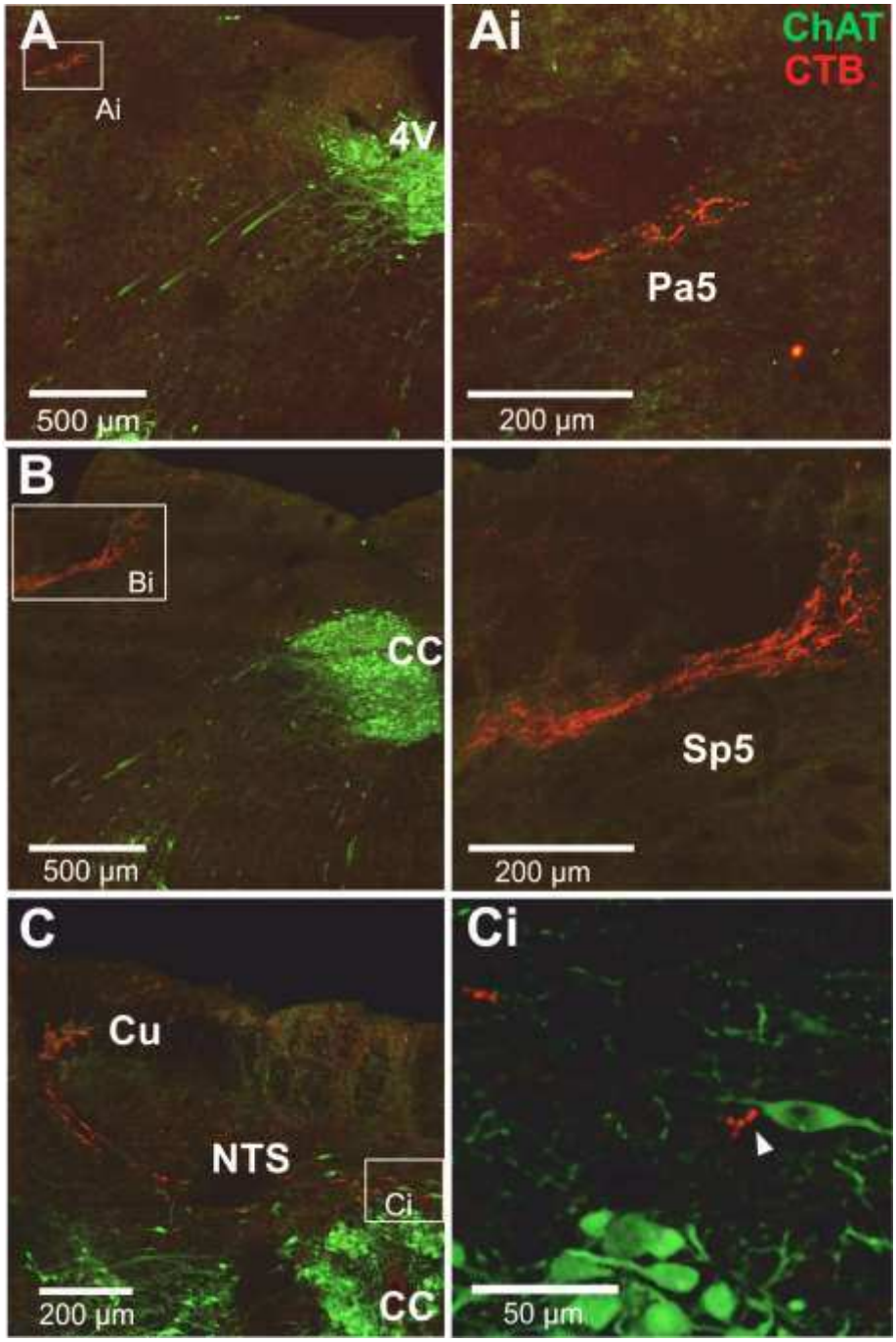


Figure 2: Labelled afferents in the brainstem following injection of CTB into the tragus, with co-staining for ChAT immunofluorescence

A) Labelled afferent in the caudal brainstem, coursing from Cu to dorso-medio lateral NTS. Example of a rare apposition between a labelled afferent and a ChAT immunoreactive cell (Ai) B) Afferents located more rostrally in Sp5 (magnified:Bi). C) Rostral brainstem with afferents labelled in the Pa5 (magnified: Ci).Pa5 paratrigeminal nucleus, Sp5- spinal trigeminal tract, Cu-cuneate nucleus, NTS- nucleus tractus solitarius, ChAT – Choline acetyl transferase, CTB – Cholera Toxin B.

Figure 3: CTB-positive afferents labelled in the upper cervical cord following injection of CTB into the tragus.

A) Cervical section at C1 with CTB-positive afferents terminating in laminae III and IV. B) Cervical section at C2 with CTB-positive afferents terminating in laminae I, III and IV. C) Cervical section at C2 with CTB-positive afferents terminating in laminae I, III and IV. Ai-Ci) Potential cell contact of the labelled afferents with ChAT immunopositive cells mostly detected in laminae IV. ChAT – Choline acetyl transferase, CTB – Cholera Toxin B.

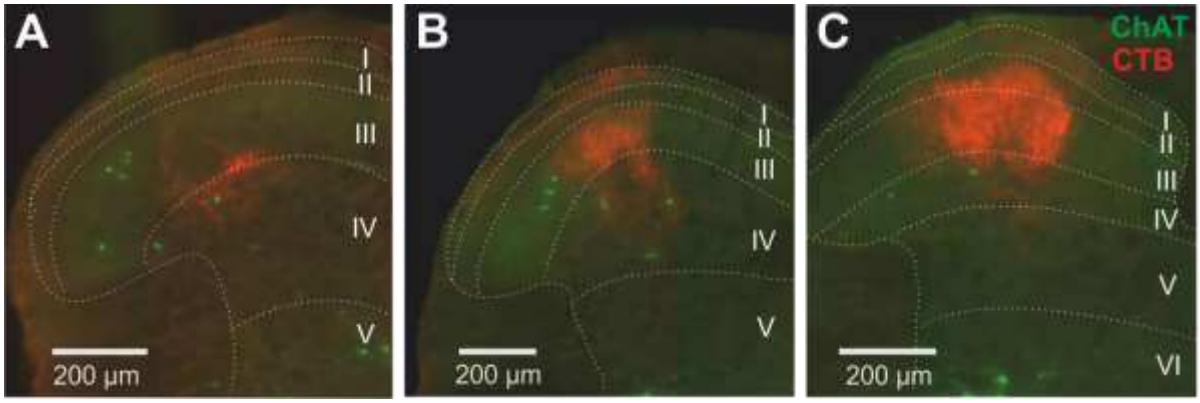


Figure 4: Sympathetic nerve activity and phrenic nerve discharge rates are higher in night time compared to day time WHBPs.

Examples of original WHBP traces showing the baseline heart rate, phrenic and sympathetic nerve recordings, and perfusion pressure recorded from A) day time and B) night time animals. The SND C) and respiration rate D) were significantly higher ($p < 0.05$) in night time compared to day-time preparations.

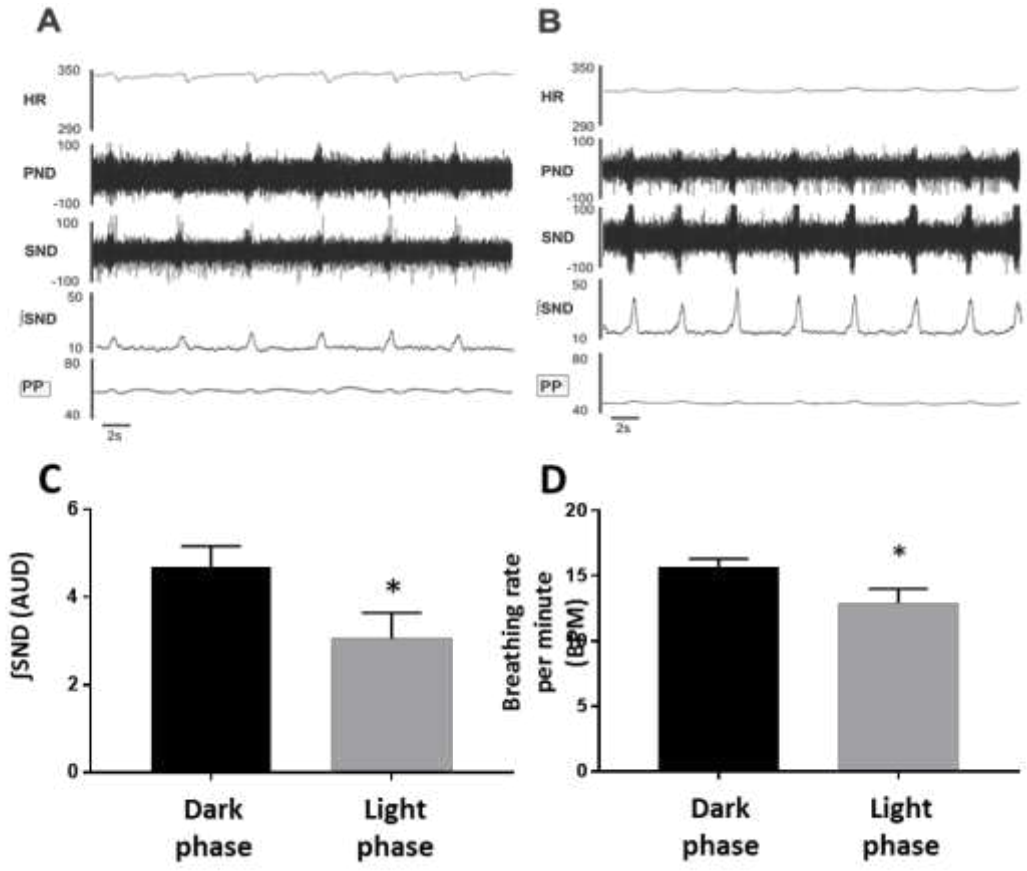


Figure 5: Tragus stimulation elicited sympathoinhibition in intact and vagotomised WHBP, but not in C1-C3 transected preparations.

A) Indicating application of electrical stimuli on the tragus using a modified alligator clip

B) Experimental parameters recorded after the preparation reached eupnic breathing pattern for at least 30 minutes. The top black arrows indicate specific timings when the parameters were recorded. The effects of tragus stimulation were analysed and compared during baseline, 1 minute post-stimulation and 10 minute post-stimulation. The effects of tragus stimulation were also compared in groups that were vagotomised (D) or with C1-C3 nerve transection (E). Significant sympathoinhibition was observed in the intact (dark phase) and vagotomised preparations only. SND - sympathetic nerve discharge.