Raised arterial blood pressure in neurokinin-1 receptor deficient mice (NK1R-/-): evidence for a neural rather than a vascular mechanism.

Amie J. Moyes[1], S. Clare Stanford[2], Patrick S. Hosford[2], Adrian J. Hobbs[1] and Andrew G. Ramage[2]

[1]The William Harvey Research Institute, Barts & The London School of Medicine & Dentistry, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ and [2]Department of Neuroscience, Physiology and Pharmacology, University College London, Gower Street, London WC1E 6BT U.K.

Running title: NK1 receptors and arterial blood pressure

Key words: NK1 receptors, blood pressure, substance P

Total number of words (excluding ref & fig legends) = 3041
Total number of references = 37
Subject area: Cardiovascular Control

Corresponding Author: Dr A. J. Moyes; The William Harvey Research Institute, Barts & The London School of Medicine & Dentistry, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ
E-mail. a.j.moyes@qmul.ac.uk

New Findings

- **What is the central question of this study?** Does genetic ablation of NK1 receptors alter arterial blood pressure?
- **What is the main finding and its importance?** NK1R-/- mice have raised mean arterial blood pressure, but without a concomitant change in vascular reactivity. This finding suggests NK1R play a role in the neural regulation of blood pressure

Abstract

Mice with functional ablation of the neurokinin-1 receptor gene (NK1R-/-) express behavioural abnormalities equivalent to those seen in Attention Deficit Hyperactivity Disorder (ADHD). An established model of ADHD is the spontaneously hypertensive rat (SHR), which exhibits high blood pressure due to increased central sympathetic drive. In light of the evidence that NK1R also influence cardiovascular haemodynamics, we have investigated whether NK1R-/- similarly exhibit raised blood pressure. Cardiovascular parameters were recorded for 24 h in conscious mice using radiotelemetry. Vascular function was assessed in mesenteric resistance arteries by wire myography. NK1R-/- mice exhibited a higher blood pressure than wildtype animals throughout the 24 h period. Heart rate and locomotor activity in NK1R-/- mice were higher than wildtypes during the night period (active phase), consistent with an ADHD-like phenotype, but not during the day. Mesenteric and renal arteries from NK1R-/- mice exhibited normal vascular function: the responses to vasoconstrictors (U46619 & phenylephrine) and the endothelium-dependent vasodilator, acetylcholine, were not altered in these animals, suggesting that NK1R do not regulate vascular tone. Analysis of heart rate variability revealed a higher low frequency: high frequency (LF:HF) ratio in NK1R-/- indicative of increased cardiac sympathetic activity. We propose that the raised blood pressure in NK1R-/- mice could be due a neural mechanism, rather than a change in vascular reactivity. Further studies are required to understand this mechanism and to establish whether a subgroup of ADHD patients, with polymorphism of the equivalent (*TACR1*) gene, are similarly affected.

Introduction

The major endogenous mammalian tachykinin is substance P, which is the cognate ligand for neurokinin-1 receptors ('NK1R' see: Maggi, 1995; Severini et al., 2002). Two organs have a relatively high concentration of substance P. One is the brain, although the concentration differs from one region to another (Kanazwaa and Jessell, 1976; Douglas et al., 1982). The other is the enteric nervous system of the gut, from which substance P was originally isolated (see: Holzer and Holzer-Petsche, 1997). Substance P is also found in several regions of the vasculature. In endothelial cells, it induces endothelium-dependent relaxation, a response that causes peripheral dilatation and a reduction in blood pressure (Euler & Gaddum, 1931; Constantine et al., 1991; Bachelard et al., 1992; Severini et al., 2002). Substance P is also located in sensory nerve endings in blood vessels (Scotland et al., 2004; Severini et al., 2002) and in the terminals of cardiovascular afferents terminating in the nucleus tractus solitarius (NTS), a brain-stem nucleus with a pivotal role in the regulation of blood pressure and implicated in baroreceptor regulation (see: Helke and Seagard, 2004). Consistent with this, are reports that plasma concentrations of substance P are reduced in stroke-prone Spontaneously Hypertensive rats (Mori et al., 2003) and in patients with essential hypertension (Faulhaber et al., 1983). Collectively, these findings suggest that a reduction in substance P signalling could contribute to the pathogenesis of these diseases.

Substance P, via activation of NK1 receptors, also influences behaviour; for instance, when injected intracerebroventricularly a "defence reaction" is observed which involves sympathoexcitation and a rise in blood pressure (Unger et al 1995; Culmam et al., 1997). This has led to the suggestion that central NK1 receptors are involved in the mediation of stress responses (Culmam et al., 1997). More recently, studies of NK1R-/- mice have revealed behavioural and cognitive deficits equivalent to those seen in patients with Attention Deficit Hyperactivity Disorder (ADHD; see: Yan et al., 2010; 2011). In this respect, there is evidence, from single nucleotide polymorphism (SNP) markers, for an association between polymorphism(s) of the *TACR1* gene (the human equivalent of the *nk1r* gene in rodents) and susceptibility to ADHD in humans (Yan et al., 2009; Sharp et al., 2014).

Because the most widely studied rodent model of ADHD is the Spontaneously Hypertensive Rat (Myers et al., 1982; see Sagvolden et al., 2005), the present investigation determined whether NK1R-/- mice also have raised blood pressure and, if so, whether the reactivity of

resistance arteries, isolated from these mice, exhibit any abnormalities in endothelial and vascular smooth muscle function.

Methods

Ethical Approval

Experiments were authorised under the Animals (Scientific Procedures) Act, 1986 [2010/63/EU], and had received local Animal Welfare and Ethical Approval Body at University College London (UCL).

Animals

Male wildtype (NK1R+/+) and NK1R-/- mice, 10-12 weeks of age and from the same background strain (129/Sv*x*C57BL/6J, crossed with an outbred MF1 strain many generations ago (see: de Felipe et al., 1998) were used for both *in vivo* and *in vitro* experiments. The animals were bred and housed at UCL in a facility held at 21 ± 2^{o}C, 45 ± 5% humidity, with a 12: 12 h light: dark cycle and *ad libitum* access to water and food.

Measurement of cardiovascular haemodynamics and locomotor activity

Cardiovascular parameters, along with locomotor activity, were recorded by radiotelemetry (TA11PA-C10, Data Sciences International). Wildtype and NK1R-/- mice (n=6 per genotype) were anaesthetized with 2% isoflurane delivered with oxygen (0.4 L/min) and radiotelemetric probes were inserted into the aortic arch via the left common carotid artery. The transmitter body was positioned in a subcutaneous abdominal pouch. Animals were allowed to recover and acclimatise to their environment for ten days prior to telemetric monitoring. Blood pressure (systolic and diastolic), heart rate and locomotor activity were recorded for 24 h during weekends, so as to minimise variability due to extraneous noise and other sources of disturbance. Studies were conducted in each genotype simultaneously and mice were singly housed. Data were acquired for a 2 min period every 15 min and the average values for mean arterial blood pressure (MAP), heart rate (HR) and activity were calculated for these time points (Dataquest Art Acquisition System, Data Sciences International).

Heart rate variability (HRV) data were analyzed in both frequency and time domains using standard HRV parameters (Baudrie et al., 2007; Zuberi et al., 2008). A threshold sensing

algorithm was applied to detect R-R intervals from the telemetric blood pressure traces. Artefacts and ectopic beats (defined as those that were 2-fold above or below the average R-R interval) were excluded from the analysis. Four waveforms (2 min periods), during a phase of inactivity with no erratic fluctuations, were selected from data recorded during the light period. Frequency domain analysis was performed after fast Fourier transform (FFT) using 1024 spectral points and a half overlap within a Welch window. Cut-off frequencies, previously determined to be accurate for mice, were used to divide signal into low frequency (LF 0.4–1.5 Hz) and high frequency (HF 1.5–4.0 Hz) components.

Telemetric blood pressure data was imported into Spike2 software (Cambridge Electronic Design, Cambridge, UK) for offline analysis. Two separate Spike2 scripts (available from CED) were applied to the data. 1) HRBP8.s2s was used to determine respiratory frequency based on breathing-induced modulation of pulse pressure. 2) sBRG.s2s was used to calculate baroreflex sensitivity based on the method of Oosting et al., 1997. Spontaneous fluctuations in blood pressure were smoothed over 10 heart beats and episodes of continual pressure changes were inspected. These ramp changes in blood pressure were plotted against corresponding pulse interval with a delay of 4 beats and a linear best fit was calculated. Ramps with a R^2 value of less than 0.7 were rejected. Baroreflex gain was expressed as the slope of the best fit in ms $mmHg^{-1}$.

Functional reactivity of isolated arteries

Male WT and NK1R-/- mice (n=6 per genotype), 10-12 weeks of age, were killed by cervical dislocation. The mesentery or kidney was removed and placed in Krebs' solution (composition: 119 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 5.5 mM Glucose) and gassed with 95% O_2/5% CO_2. Mesenteric arteries (100-150µM diameter) and renal arteries (~300 µM) were mounted in a tension myograph (Danish Myotechnology, Aarhus, Denmark) and allowed to equilibrate for 45 min before the start of the experiment.

Automated normalization was performed using the inbuilt software of the myograph interface. This procedure involves stretching the vessels in a stepwise manner to determine the relationship between passive tension and internal circumference (according to Laplace's law): from this relationship, the internal diameter and effective pressure can be determined (Mulvany & Halpern 1977). The stepwise distension is stopped when the effective pressure

exceeds 100 mmHg (13.3 kPa). An exponential curve is then fitted to the internal circumference pressure data and, using Laplace's equation, the point on the curve corresponding to 100 mmHg is determined. Vessels are then stretched to 90% of the diameter achieved under a transmural pressure of 100 mmHg as this is the internal circumference at which maximal active force is produced. Following normalization, the arteries were contracted repeatedly, by application of the thromboxane-A_2 mimetic U46619 (1µM) or KCl (125 mM), until successive responses were of consistent amplitude (±10% of previous contraction). Following this, the vessels were washed with Krebs' solution to restore basal tone before construction of cumulative concentration-response curves to U46619 (1nM – 1µM) or the alpha1-adrenoceptor agonist phenylephrine (1nM – 100µM). In order to study the effect of vasodilators, the vessels were pre-contracted to 50% of the maximum U46619 response and, once a stable contraction was achieved, cumulative concentration-response curves to the endothelium-dependent vasodilator, acetylcholine (ACh; 1nM – 10µM), and substance P (1pM - 1µM) were constructed.

In order to investigate relaxation induced by endothelial-derived hyperpolarising factor (EDHF) alone, a subset of vessels were pre-treated with the nitric oxide synthase inhibitor, L-NAME (300 µM) and the cyclooxygenase inhibitor, indomethacin (5 µM), for 30 min (Scotland et al., 2005). These inhibitors were used to abolish responses to nitric oxide (NO) and prostacyclin (PGI_2) before commencing the concentration-response curve to ACh, In certain experiments, tissues were treated with the NK1R antagonist, RP 67580 (1 µM), to investigate the effect of NK1R blockade on endothelium-dependent relaxation to ACh and substance P.

Drugs and chemicals

RP 67580 (3a*R*,7a*R*)-Octahydro-2-[1-imino-2-(2-methoxyphenyl)ethyl]-7,7-diphenyl-4*H*-isoindol) was purchased from Tocris Cookson Ltd., Avonmouth, Bristol. U46619 (9,11-Dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$) was purchased from Enzo Life Sciences, UK. Acetylcholine (Cl); phenylephrine HCl; L-NAME; indomethacin; substance P acetate salt hydrate and Krebs solution (10x concentrate) were purchased from Sigma-Aldrich, Poole, Dorset.

Statistical Analysis

Differences in mean cardiovascular parameters were compared using an unpaired Student's t-test. Concentration-response curves were analysed by two-way ANOVA and Bonferroni post-hoc analysis. GraphPad Prism Software (San Diego California USA, version 5.0) was used for statistical analysis. A *P* value less than 0.05 was considered the threshold to declare significance.

Results

Cardiovascular variables and locomotor activity in conscious WT and NK1R-/- mice

Over the 24 h period, MAP, HR and locomotor activity were higher in NK1R -/- mice in comparison to WT. Specifically, MAP was increased by 6 ± 1 mmHg: this was due to a rise in both systolic and diastolic pressure ($p<0.0001$, Table 1). HR was increased by 11 ± 4 bpm ($p=0.0074$) and locomotor activity increased by 3 ± 0.5 auc. ($p<0.0001$, Figure 1).

However, these differences changed through the 24 h cycle. Compared with WT animals, the MAP in NK1R-/- mice in the dark period (active phase), was raised by 8 ± 1 mmHg ($p<0.0001$), resulting from elevations in both systolic and diastolic pressures (Figure 1; Table 1). In the first few hours of the dark phase, in particular, between 20.00 – 02.00 h, the HR (KO: 555± 6 versus WT, 530 ± 6 bpm; $p=0.0001$) and locomotor activity, (KO: 18.2 ± 0.9 versus WT, 11.9 ± 0.8 auc.; $p<0.0001$) in NK1R-/- mice were significantly higher than WT animals.

During the light period, the average MAP in NK1R-/- mice was also higher than wildtypes (4 ± 1 mmHg; Figure 1; Table 1; $p=0.0011$). However, unlike the dark period, there was no difference in HR or locomotor activity between the two genotypes during the daytime. In addition, the difference in systolic pressure (7 ± 1 mmHg) was greater than that of diastolic pressure (2 ± 1 mmHg). Despite this small increase, diastolic pressure differed in NK1R-/- and WT mice ($p=0.0261$).

Spectral analysis of HRV revealed a higher LF:HF ratio in the NK1R -/- (Table 2; $p=0.0164$), that was predominantly due to an increase in the LF component. However, total HRV (standard deviation of heart rate interval [SDNN]) was not altered in NK1R -/- mice (Table 2). There were no significant differences in respiratory rate between WT and NK1R-/- at rest or during activity (Table 3). There was a trend toward a reduction in spontaneous baroreflex

gain both during rest and activity in the NK1R -/- mice (Table 3) but this did not reach significance.

An *in vitro* comparison of vascular reactivity in WT and NK1R-/- mice

Vasoconstrictor responses

Contractions of mesenteric arteries from NK1R-/- and wildtype mice, induced by the thromboxane receptor agonist, U46619, were almost identical (pEC_{50} = 8.23 ± 0.3 versus WT, pEC_{50} = 7.90 ± 0.1, P>0.05, Figure 2a). Likewise, the arterial concentration-response curve to phenylephrine did not differ in the two genotypes (pEC_{50} = 5.74 ± 0.1 versus WT, pEC_{50} = 5.96 ± 0.2, P>0.05, Figure 2b). Similar results were obtained in renal arteries: there was no difference in response to U46619 in tissues from WT and NK1 -/- mice (pEC_{50} = 8.11 ± 0.07 versus WT, pEC_{50} = 8.13 ± 0.05, P>0.05, Figure 4a). Data shown are the mean ± S.E.M, however, individual data points for each replicate are presented in supplementary Figure 1a & b and supplementary Figure 3a.

Endothelial vasodilator responses

Endothelium-dependent relaxations induced by ACh in mesenteric and renal arteries were similar in NK1R-/- and WT mice (mesenteric arteries: pEC_{50} = 6.94 ± 0.2 versus WT, pEC_{50} = 6.91 ± 0.3, P>0.05, Figure 2c; renal arteries: pEC_{50} =7.35 ± 0.1 versus WT, pEC_{50} = 7.5 ± 0.1, P>0.05, Figure 4b). The concentration-response curve to ACh was shifted to the right (Figure 2d) in arteries incubated with L-NAME and indomethacin, which abolish the production of NO and PGI_2, respectively, and so permit study of EDHF-dependent responses in isolation. This rightward shift of the ACh concentration-response curve in vessels treated with L-NAME and indomethacin has been well documented and it is the residual relaxation (due to EDHF release) that was of interest in these particular experiments. However, there was no difference in EDHF-dependent relaxations in arteries from the two genotypes (pEC_{50} = 5.52 ± 0.4 versus WT, pEC_{50} = 5.35 ± 0.3, Figure 2d, n=4, p= 0.82). Furthermore, ACh-induced relaxations were not inhibited by the NK1R antagonist RP 67580 (data not shown). Data shown are the mean ± S.E.M, however, individual data points for each replicate are presented in Supplementary Figure 1c & d and supplementary Figure 3b.

Involvement of NK_1 receptors in vasoreactivity

Substance P did not alter vascular tone at physiological concentrations (10^{-12}M – 10^{-8}M) in mesenteric or renal arteries. A contractile effect of this agonist was evident at high concentrations (mesenteric arteries: 1µM induced 48 ± 6% contraction; versus WT 42 ± 18%, Figure 3a; renal arteries: 1µM induced 14 ± 14% contraction; versus WT 16 ± 9%, Figure 4c) and this response was not altered by incubation with the NK1R antagonist RP 67580 (Figure 3b, n=3). The contractile response to substance P was also observed in vessels without pre-contraction and reached a level similar to that induced by KCl (Figure 3C). Experiments using single doses of substance P (10^{-10}M, 10^{-8}M & 10^{-6}M) gave similar responses to those observed in the cumulative concentration-response curves, suggesting that the lack of substance P response at physiological concentrations is not due to tachyphylaxis (data not shown). Data shown are the mean ± S.E.M, however, individual data points for each replicate are presented in supplementary Figure 2 and supplementary Figure 3c.

Discussion

This study demonstrates that NK1R-/- mice have raised MAP and HR, along with increased locomotor activity, compared with WT. It is unlikely that the haemodynamic changes are a secondary consequence of the animals' hyperactivity because MAP was raised in the NK1R-/- mice throughout the 24 h cycle, even during the day when there was no genotype difference in the locomotor activity or HR. The rise in MAP also cannot be attributed to changes in vascular reactivity of NK1R-/- mice because contractile and vasorelaxant responses of the mesenteric and renal arteries were almost identical to those of the WT.

The findings suggest that NK1R are not involved in the local regulation of vascular tone via the release of vasodilatory substances such as NO and EDHF (see Furchgott, 1983; Miike et al., 2009). This is inferred from the lack of any difference in the response to ACh in mesenteric and renal arteries from the two genotypes. In fact, only high concentrations of substance P trigger a significant response in this tissue and this contractile effect cannot be blocked by the selective NK1R antagonist RP 67580 (Garret et al., 1991). This suggests that vascular endothelial/smooth muscle NK1R do not play a role in the regulation of contractility of mesenteric or renal arteries.

This inference is at variance with evidence that activation of NK1R causes constriction of the perfused mesenteric vascular bed (D'Orléans-Juste et al., 1991) and regulates the myogenic

response to changes in intraluminal pressure (Bubb et al., 2013 & Scotland et al., 2008). These differences could be due to fact that arteries need to be pressurised in order to observe NK1R-mediated responses. However, if vascular NK1R are involved in mediating myogenic constriction, as others have proposed, then NK1R-/- would be expected to be hypotensive rather than hypertensive. Thus, there must be an alternative explanation for the alterations in blood pressure observed in these mice.

There are conflicting data regarding the effect of substance P *in vivo*. For example, administration of substance P in conscious rats causes vasoconstriction of the mesenteric bed (Bachelard et al., 1992). By contrast, substance P induces vasodilation in the kidney and the hindquarter vascular beds, a response that is mediated by NK1R receptors on the endothelium (Constantine et al., 1991). It appears that substance P can induce different effects in different vascular beds. However, here we studied both the mesenteric and renal vasculature of NK1R-/- mice and found a similar response in both vessels. The discrepancy between these different studies could be due to the fact we used isolated vessels, rather than studying vascular responses *in vivo*. Species differences in the NK1R and the ensuing response to substance P are also possible. Nevertheless, our data suggest that NK1R located in the endothelium/smooth muscle of mice are not involved in regulating the functional reactivity of vessels, indicating that the rise in MAP in NK1R-/- mice may be due to a different mechanism.

Substance P and NK1R are also known to regulate respiratory activity and so we analysed the telemetry data to determine whether changes in ventilation could account for the elevated blood pressure observed in the NK1R -/- mice. We found no difference in respiration rate at rest or during activity in NK1R -/- mice when compared to WT. This is in accordance with studies by Ptak et al. (2000 & 2002) who have shown resting breathing parameters are unaltered in NK1R-/- mice.

Another mechanism by which substance P/ NK1R signalling could contribute to the regulation of the cardiovascular system is via modulation of the baroreceptor reflex. Afferent fibres within and surrounding baroreceptor areas contain substance P (Gillis et al., 1980 & Helke et al., 1980). Moreover, NK1R are found within the nucleus of the solitary tract (NTS), the region in the brain innervated by baroreceptor afferent nerves (Danks et al., 1986 & Maley et al., 1988). However, the role of this pathway in regulating baroreceptor activity is

not entirely clear; some studies have shown that microinjection of substance P into the NTS causes a decrease in blood pressure (Hall et al., 1989) while other investigators have shown no change (Talman et al., 1981). Moreover, one report suggests that administration of a NK1R antagonist into the NTS attenuates baroreflex function and increases blood pressure (Paton et al., 1999), whereas other studies have either found no change or the opposite response (Abdala et al., 2003). Despite this, there is a possibility that a lack of functional NK1R could affect baroreflex sensitivity and, as a consequence, resting blood pressure.

Spectral analysis of low frequency (LF) and high frequency (HF) components of HRV has been used frequently to assess autonomic regulation of the cardiovascular system (Baudrie et al., 2007; Thireau et al., 2007). The HF component of HRV is believed to reflect parasympathetic vagal activity, whereas the LF component is believed to reflect sympathetic activity. Thus, the LF/HF ratio has been used as a tool to evaluate sympathovagal balance. On this basis, our data indicate that NK1R-/- mice exhibit an increase in sympathetic drive due to a higher LF:HF ratio.

However, recent evidence makes this proposal uncertain because the interplay between the two branches of the autonomic nervous system is complex and the frequency components of heart rate do not simply reflect input from one system alone (Parati et al., 2006). Mean HRV parameters are likely to provide information on the net effects of all components influencing cardiac function and may not accurately assess sympathovagal regulation. Also, variables, such as mechanical events (atrial stretch), respiration and prevailing heart rate can profoundly alter the results (Billman, 2013). Therefore, we have to be cautious with the interpretation of our results. Analysis of baroreflex sensitivity was performed to determine if the changes in HRV truly reflect an alteration in autonomic balance, these results showed a trend toward a reduction in spontaneous baroreflex gain in NK1R -/-. Although this data does not give us a definitive answer, it does suggest a more detailed investigation would be worthwhile to gain a better understanding of the role of NK1R in regulating baroreflex responses.

In addition to the central effects of substance P on the autonomic nervous system, there is evidence suggesting this neuropeptide is released by peripheral sensory nerves in the heart, where it induces negative inotropic and chronotropic effects in Langendorff preparations via the release of ACh from cholinergic nerves (Chiao et al., 1995). It is not known if this occurs *in vivo* but, if so, this raises the possibility heart function could be altered in mice lacking

NK1R. This proposal would fit with our data showing a greater increase in systolic arterial pressure: this possibility of an increase in cardiac output in NK1R -/- mice merits investigation in future studies.

The raised blood pressure in NK1R-/- mice is not as severe as that observed in SHR rats, in which hypertension is considered to be due to a general increase in sympathetic nerve activity (Judy and Farrell, 1979; Lundin et al., 1984). Unlike NK1R -/- mice, SHR have elevated heart rates, measured by radiotelemetry and under anaesthesia (Komolova et al., 2012), suggesting that an alteration in locomoter activity is not the cause of their tachycardia (Shanks et al 2013). Increased norepinephrine release, or a reduction in its clearance, as well as changes in Ca^{2+} signalling, are thought to contribute to this phenotype (Shanks et al., 2013). It should be noted that one of the limitations of using transgenic animals is that the abnormal physiology observed could be a secondary consequence of NK1R knockout, rather than a direct one. Nevertheless, it is interesting that substance P and NK1R expression is altered in SHR dorsal root ganglia and renal sensory neurons, which is thought to contribute to the hypertension in these rats (Aline Boer et al 2005).

Heart rate is also higher in children with ADHD, compared with normal subjects, when monitored over 24 h (Imeraj et al., 2011) but whether or not this relates to their hyperactivity, remains to be determined. Although the behavioural abnormalities of both NK1R-/- mice and SHR echo those of ADHD patients, there are no reports that blood pressure is higher in ADHD patients. However, it is striking that some children with renal hypertension express behaviour abnormalities that resemble those seen in ADHD (Krause et al. 2009).

In conclusion, the NK1R-/- mouse model of ADHD exhibits higher MAP, HR and nocturnal locomotor activity. These genetically-altered animals are also mildly hypertensive, even during periods of rest, suggesting that the raised arterial pressure may, in part, be due to changes in the autonomic control of blood pressure. These results lead to the prediction that that there could be an, as yet undetected, subgroup of ADHD patients with polymorphism of the *TACR1* gene, who exhibit increased susceptibility to hypertension and cardiovascular disease.

References

Abdala AP, Haibara AS, Colombari E (2003). Cardiovascular responses to substance P in the nucleus tractus solitarii: microinjection study in conscious rats. *Am J Physiol Heart Circ Physiol*. **285**, H891-8.

Aline Boer P, Ueno M, Sant'ana JS, Saad MJ, Gontijo JA **(2005).** Expression and localization of NK(1)R, substance P and CGRP are altered in dorsal root ganglia neurons of spontaneously hypertensive rats (SHR). *Brain Res Mol Brain Res.* **29**, 35-44.

Azar T, Sharp J & Lawson D (2011). Heart Rates of Male and Female Sprague–Dawley and Spontaneously Hypertensive Rats Housed Singly or in Groups. *J Am Assoc Lab Anim Sc* **50**, 175-184.

Bachelard H, Gardiner SM, Kemp PA & Bennett T (1992). Involvement of capsaicin-sensitive neurones in the haemodynamic effects of exogenous vasoactive peptides: studies in conscious, adult Long Evans rats treated neonatally with capsaicin. *Br J Pharmacol* **105**, 202-210.

Baudrie V, Laude D & Elghozi J (2007). Optimal frequency ranges for extracting information on cardiovascular autonomic control from the blood pressure and pulse interval spectrograms in mice. *Am. J. Physiol. Regul. Integr. Comp. Physiol* **292**:R904-R912.

Billman GE (2013). The LF/HF ratio does not accurately measure cardiac sympatho-vagal balance. *Front Physiol.* **20**,4-26.

Bubb KJ, Wen H, Panayiotou CM, Finsterbusch M, Khan FJ, Chan MV, Priestley JV, Baker MD & Ahluwalia A (2013). *Hypertension* **62**:426-433.

Busse R, Edwards G, Feletou M, Fleming I, Vanhoutte PM & Weston AH (2002). EDHF: bringing the concepts together. *TiPs* **23**, 374–380.

Chan JYH, Barnes CD & Chan SHH (1990). Tonic enhancement of the sensitivity of baroreceptor reflex response by endogenous substance P in the rat. *Regul Pept* **29**,199-213.

Chiao H, Caldwell RW (1995). Local cardiac effects of substance P: roles of acetylcholine and noradrenaline. *Br J Pharmacol.***114**, 283-8.

Constantine JW, Lebel WS &Woody HA (1991). Inhibition of tachykinin-induced hypotension in dogs by CP-96,345, a selective blocker of NK-1 receptors. *Naunyn-Schmied Arch Pharmacol* **344**, 471-477.

Culman J, Klee S, Ohlendorf C & Unger T (1997). Effect of tachykinin receptor inhibition in the brain on cardiovascular and behavioral responses to stress. *J Pharmacol Exp Ther* **280**, 238-246.

Danks JA, Rothman RB, Cascieri MA, Chicchi GG, Liang T, Herkenham M (1986). A comparative autoradiographic study of the distributions of substance P and eledoisin binding sites in rat brain. *Brain Res.* **385**, 273-81.

De Felipe C, Herrero JF, O'Brien JA, Palmer JA, Doyle CA, Smith AJH, Laird JMA, Belmonte C, Cervero F 7 Hunt SP (1998). Altered nociception, analgesia and aggression in mice lacking the substance P receptor. *Nature* **392**, 394–397.

D'Orléans-Juste P, Claing A, Télémaque S, Warner TD & Regoli D (1991). Neurokinins produce selective venoconstriction via NK-3 receptors in the rat mesenteric vascular bed. *Eur J Pharmacol* **204**, 329-334.

Euler von US & Gaddum JH (1931). An unidentified depressor substance in certain tissue extracts. *J Physiol* **72**, 74-87.

Faulhaber HD, Oehme P, Baumann R, Enderlein J, Rathsack R, Rostock G & Naumann E (1986). Substance P in Human Essential Hypertension. *J Cardiovas Pharmacol* **10** (suppl.12). S172-S176.

Gillis RA, Helke CJ, Hamilton BL, Norman WP, Jacobowitz DM (1980). Evidence that substance P is a neurotransmitter of baro- and chemoreceptor afferents in nucleus tractus solitarius. *Brain Res.* **181**, 476-81.

Helke CJ, O'Donohue TL, Jacobowitz DM.Substance P as a baro- and chemoreceptor afferent neurotransmitter: immunocytochemical and neurochemical evidence in the rat (1980). *Peptides.* **1**,1-9.

Helke CJ & Seagard JL (2004). Substance P in the baroreceptor reflex: 25 years. *Peptides* 25, 413‑423.

Frank L. Douglas FL, Palkovits M & Brownstein, MJ (1982). Regional distribution of substance P-like immunoreactivity in the lower brainstem of the rat. *Brain Res* **243**, 376‑378.

Furchgott, RF (1983). Role of endothelium in responses of vascular smooth muscle. *Circ Res* **53**, 557-573.

Garret C, Carruette A, Fardin V, Moussaoui, S, Peyronel JF, Blanchard JC & Laduron, PM (1991). Pharmacological Properties of a Potent and Selective Nonpeptide Substance P Antagonist. *Proc Natl Acad Sci* **88**, 10208-10212.

Hall ME, Miley FB, Stewart JM (1989). Cardiovascular effects of substance P peptides in the nucleus of the solitary tract. *Brain Res.* **497**, 280-90.

Holzer P & Holzer-Petsche U (1997). Tachykinins in the gut. Part I. Expression, release and motor function. *Pharmacol Ther* **73**, 173‑217.

Imeraj L, Antrop I, Roeyers H, Deschepper E, Bal S & Deboutte D (2011). Diurnal variations in arousal: a naturalistic heart rate study in children with ADHD. *Eur Child Adolesc Psychiatry* **20**, 381-392.

Judy WV & Farrell SK (1979). Arterial baroreceptor reflex control of sympathetic nerve activity in the spontaneously hypertensive rat. *Hypertension* **1**, 605–614.

Kanazawa I & Jessel T (1976). Post morten changes and regional distribution of substance P in the rat and mouse nervous system. Brain Res **117**,362- 367.

Komolova M, Friberg P, Adams MA (2012). Altered vascular resistance properties and acute pressure-natriuresis mechanism in neonatal and weaning spontaneously hypertensive rats. *Hypertension.* **59**, 979-84.

Krause I, Cleper R, Kovalski Y, Sinai L & Davidovits M (2009). Changes in behavior as an early symptom of renovascular hypertension in children. *Pediatr Nephrol* **24**, 2271-2274.

Lundin S, Ricksten SE & Thoren P (1984). Renal sympathetic activity in spontaneously hypertensive rats and normotensive controls, as studied by three different methods. *Acta Physiol Scand* **120**, 265–272.

Maggi A (1995). The mammalian tachykinin receptors. Gen Pharmacol, **26**, 911–944.

Maley BE, Sasek CA, Seybold VS (1988). Substance P binding sites in the nucleus tractus solitarius of the cat. *Peptides* **9**, 1301-6.

Miike T, Shirahase H, Kanda M, Kunishiro K & Kurahashi K (2009). NK_1 receptor-mediated endothelium-dependent relaxation and contraction with different sensitivity to post-receptor signaling in pulmonary arteries. Vasc *Pharmacol* **51**, 147-153.

Mori K, Asakura S, Ogawa H, Sasagawa S & Takeyama M. (1993). Decreases in substance P and vasoactive intestinal peptide concentrations in plasma of stroke-prone spontaneously hypertensive rats. *Jpn Heart J* **34**,785-794.

Mulvany MJ, Halpern W. (1977). Contractile properties of small arterial resistance vessels in spontaneously hypertensive and normotensive rats. *Circ Res* **41**, 19–26.

Myers MM, Musty RE & Hendley ED (1982). Attenuation of hyperactivity in the spontaneously hypertensive rat by amphetamine. *Behav Neural Biol* **34**, 42‑54.

Oosting J, Struijker-Boudier HA, Janssen BJ (1997). Validation of a continuous baroreceptor reflex sensitivity index calculated from spontaneous fluctuations of blood pressure and pulse interval in rats. *Journal of hypertension* **15**, 391-399.

Paton JF, Kasparov S. J Physiol (1999). Differential effects of angiotensin II on cardiorespiratory reflexes mediated by nucleus tractus solitarii - a microinjection study in the rat. **521,** 213-25.

Parati G, Mancia G, Rienzo M, Castiglioni P (2006). Point: Counterpoint: Cardiovascular variability is/is not an index of autonomic control of circulation. *J Appl Physiol* **688**, 690-1.

Ptak K, Hunt SP, Monteau R (2000). Substance P and central respiratory activity: a comparative in vitro study in NK1 receptor knockout and wild-type mice. *Pflugers Arch.* **440**, 446-51.

Ptak K, Burnet H, Blanchi B, Sieweke M, De Felipe C, Hunt SP, Monteau R, Hilaire G (2002). The murine neurokinin NK1 receptor gene contributes to the adult hypoxic facilitation of ventilation. *Eur J Neurosci.***16**, 2245-52.

Sagvolden T, Russell VA, Aase H, Johansen EB & Farshbaf M. (2005). Rodent models of attention-deficit/hyperactivity disorder. *Biol Psychiatry* **57**, 1239-1247.

Scotland RS, Chauhan S, Davis C, De Felipe C, Hunt S, Kabir J, Ahluwalia, A. (2004) Vanilloid receptor TRPV1, sensory C-fibers, and vascular autoregulation - A novel mechanism involved in myogenic constriction. *Circ Research* **95** (10) 1027 - 1034.

Scotland RS, Madhani, M, Chauhan S, Moncada S, Andresen J, Nilsson H, Hobbs AJ & Ahluwalia A (2005). Investigation of vascular responses in endothelial nitric oxide synthase/cyclooxygenase-1 double-knockout mice: key role for endothelium-derived hyperpolarizing factor in the regulation of blood pressure in vivo. *Circ* **111**,796-803.

Severini C, Improta G. Falconieri-Erspamer G. Salvadori S & Erspamer V (2002). The tachykinin peptide family. *Pharmacol Rev* **54**,285–322.

Shanks J, Manou-Stathopoulou S, Lu CJ, Li D, Paterson DJ, Herring N (2013). Cardiac sympathetic dysfunction in the prehypertensive spontaneously hypertensive rat. *Am J Physiol Heart Circ Physiol*. **305**, H980-6.

Sharp SI, McQuillin A, Marks M, Hunt SP, Stanford SC, Lydall GJ, Morgan MY, Asherson P, Curtis D & Gurling HM (2014). Genetic association of the tachykinin receptor 1 TACR1 gene in bipolar disorder, attention deficit hyperactivity disorder, and the alcohol dependence syndrome. *Am J Med Genet B Neuropsychiatr Gene* **165B**, 373-380.

Talman WT, Reis DJ (1981). Baroreflex actions of substance P microinjected into the nucleus tractus solitarii in rat: a consequence of local distortion. *Brain Res.* **220**,402-7.

Thireau J, Zhang B, Poisson D & Babuty D. (2007). Heart rate variability in mice: a theoretical and practical guide. *Exp Physiol* **93.1**; 83-94.

Unger T, Becker H, Petty M, Demmert G, Schneider B, Ganten D & Lang RE (1985). Differential effects of angiotensin II and substance P on sympathetic nerve activity in conscious rats. Implications for cardiovascular adaptation to behavioral responses. *Cir Res* **56**, 563-575.

Yan, TC, Hunt SP & Stanford SC (2009). Behavioural and neurochemical abnormalities in mice lacking functional tachykinin-1 (NK1) receptors: A model of attention deficit hyperactivity disorder. *Neuropharmacol* **57**, 627‑635.

Yan TC, McQuillin A, Thapar A, Asherson P, Hunt SP, Stanford SC & Gurling H (2010). NK_1 ($TACR_1$) receptor gene 'knockout' mouse phenotype predicts genetic association with ADHD. *J Psychopharmacol* **24**, 27-38.

Yan TC, Dudley JA, Weir RK, Grabowska EM, Peña-Oliver Y, Ripley TL, Hunt SP, Stephens DN, Stanford SC (2011). Performance deficits of NK1 receptor knockout mice in the 5-choice serial reaction-time task: effects of d-amphetamine, stress and time of day. *PLoS One.*;**6**(3):e17586. doi: 10.1371/journal.pone.0017586.

Zuberi Z, Birnbaumer L & Tinker A (2008). The role of inhibitory heterotrimeric G proteins in the control of in vivo heart rate dynamics. *Am J Physiol Regul. Integr. Comp Physiol* **295**:R1822-R1830.

Acknowledgements

None

Author Contributions

AM contributed to the conception, study design, data acquisition, data interpretation and drafting of the work. PH contributed to data acquisition, analysis and drafting of the work. SS, AH & AR contributed to the conception, design, data interpretation, drafting and critical revision of the work for this paper.

Competing Interests

None of the authors have any conflicts of interest

Funding

This work was funded by the Medical Research Council, grant reference G0801038.

Table 1: Mean (MAP), systolic (SBP) and diastolic (DBP) arterial blood pressure and heart rate (HR) in WT and NK1R-/- mice over 24h (n=6), **p<0.01,*p<0.001 vs WT.**

	MAP (mmHg)	SBP(mmHg)	DBP (mmHg)	HR(bpm)
24h				
WT	116 ± 1	134 ± 1	100 ± 1	493± 4
NK1R-/-	122 ± 1***	143 ± 1***	103 ± 1***	504± 4**
Light (12h)				
WT	110 ± 1	127 ± 1	94 ± 1	455± 5
NK1R-/-	114 ± 1**	134 ± 1***	96 ± 1*	453± 5
Dark (12h)				
WT	123 ± 1.0	142 ± 1	106 ± 1	530± 6
NK1R-/-	131 ± 1.0***	153 ± 1***	110 ± 1***	555± 6***

Table 2: HRV Parameters in WT and NK1R-/- mice (n=6), * p<0.05 vs WT

	SDNN (ms)	LF (ms^2)	HF (ms^2)	LF/HF Ratio
WT	11.12 ± 0.63	15.82 ± 3.3	41.18 ± 7.2	0.38 ± 0.05
NK1R-/-	11.68 ± 0.64	22.76 ± 0.8	43.17 ± 3.2	0.57 ± 0.03*

Table 3: Respiratory frequency & sBRG in WT & NK1R-/- mice (n=6)

	Respiration Rate (rest) (breaths/min)	Respiration Rate (active) (breaths/min)	sBRG (rest) (ms/mmHg)	sBRG (active) (ms/mmHg)
WT	124 ± 9.13	138 ± 5.48	5.07 ± 1.25	1.739 ± 0.63
NK1R-/-	166 ± 11.59	167 ± 11.81	3.48 ± 0.40	1.011 ± 0.19

Figure 1. Circadian rhythm of cardiovascular parameters measured by radiotelemetry. Diurnal variations in mean blood pressure (MAP; a), heart rate (HR; c) and locomotor activity (e) in WT and NK1R -/- mice. Histograms (b, d & f) show the mean values obtained for MAP (b), HR (d) and locomotor activity (f) over 24 h, during the light period (7am – 7pm) and during the dark period (7pm-7am). Data shown is the mean ± SEM. Data were analysed using a Student's *t*-test. ** $P<0.01$; *** $P<0.01$ vs WT.

Figure 2. Vascular reactivity of isolated mesenteric arteries from WT and NK1R-/- mice. Concentration-response curves to the vasoconstrictor agents (a) U46619 (n=4) and (b) phenylephrine (n=3) are expressed as the percentage of the maximum contraction elicited by KCl (125mM) to normalize for differences in vessel size. Concentration-response curves to the vasodilator ACh (n=3) were performed in the absence (c) and presence (d) of L-NAME (300 µM) and indomethacin (5 µM). Data is represented as mean ± SEM. Data were analysed by two-way ANOVA and showed no significant differences.

Figure 3. Responses to substance P in isolated mesenteric arteries from WT and NK1R-/- mice. Vessels were pre-contracted with U46619 (a) (n=3). The effect of the NK1R antagonist RP67580 (1 µM) against substance P responses in WT vessels (b) (n=3). Concentration-response curves to substance P were also performed in non pre-contracted vessels from WT and NK1R-/- mice (c) (n=3). Data is represented as mean ± SEM. Data were analysed by two-way ANOVA and showed no significant differences.

Figure 4. Vascular reactivity of isolated renal arteries from WT and NK1R-/- mice. Concentration-response curves to the vasoconstrictor agent (a) U46619 (n=4), the endothelium-dependent vasodilator, ACh (b) (n=4) and substance P (c) (n=4) in renal arteries. Data were analysed by two-way ANOVA and showed no significant differences.

Supplementary Figure 1. Vascular reactivity of isolated mesenteric arteries from WT and NK1R-/- mice. Concentration-response curves to the vasoconstrictor agents (a) U46619 (n=4) and (b) phenylephrine (n=3) are expressed as the percentage of the maximum contraction elicited by KCl (125mM) to normalize for differences in vessel size. Concentration-response curves to the vasodilator ACh (n=3) were performed in the absence (c) and presence (d) of L-NAME (300 µM) and indomethacin (5 µM). Individual data points from each replicate are shown.

Supplementary Figure 2. Vascular reactivity of isolated renal arteries from WT and NK1R-/- mice. Concentration-response curves to the vasoconstrictor agent (a) U46619 (n=4), the endothelium-dependent vasodilator, ACh (b) (n=4) and substance P (c) (n=4) in renal arteries. Individual data points from each replicate are shown.

Supplementary Figure 3. Responses to substance P in isolated mesenteric arteries from WT and NK1R-/- mice. Vessels were pre-contracted with U46619 (a) (n=3). The effect of the NK1R antagonist RP67580 (1 μM) against substance P responses in WT vessels (b) (n=3). Concentration-response curves to substance P were also performed in non pre-contracted vessels from WT and NK1R-/- mice (c) (n=3). Individual data points from each replicate are shown.