Elevated blood pressure, heart rate and body temperature in mice lacking the XLαs protein of the Gnas locus is due to increased sympathetic tone

Nicolas Nunn†, Claire H. Feetham2, Jennifer Martin1, Richard Barrett-Jolley2* and Antonius Plagge1*

1 Cellular and Molecular Physiology, Institute of Translational Medicine, University of Liverpool, Liverpool, UK
2 Institute of Ageing and Chronic Disease, University of Liverpool, Liverpool, UK

New Findings

- What is the central question of this study?
  Previously, we showed that Gnasxl knock-out mice are lean and hypermetabolic, with increased sympathetic stimulation of adipose tissue. Do these mice also display elevated sympathetic cardiovascular tone? Is the brain glucagon-like peptide-1 system involved?
- What is the main finding and its importance?
  Gnasxl knock-outs have increased blood pressure, heart rate and body temperature. Heart rate variability analysis suggests an elevated sympathetic tone. The sympatholytic reserpine had stronger effects on blood pressure, heart rate and heart rate variability in knock-out compared with wild-type mice. Stimulation of the glucagon-like peptide-1 system inhibited parasympathetic tone to a similar extent in both genotypes, with a stronger associated increase in heart rate in knock-outs. Deficiency of Gnasxl increases sympathetic cardiovascular tone.

Imbalances of energy homeostasis are often associated with cardiovascular complications. Previous work has shown that Gnasxl-deficient mice have a lean and hypermetabolic phenotype, with increased sympathetic stimulation of adipose tissue. The Gnasxl transcript from the imprinted Gnas locus encodes the trimeric G-protein subunit XLαs, which is expressed in brain regions that regulate energy homeostasis and sympathetic nervous system (SNS) activity. To determine whether Gnasxl knock-out (KO) mice display additional SNS-related phenotypes, we have now investigated the cardiovascular system. The Gnasxl KO mice were ~20 mmHg hypertensive in comparison to wild-type (WT) littermates (P ≤ 0.05) and hypersensitive to the sympatholytic drug reserpine. Using telemetry, we detected an increased waking heart rate in conscious KOs (630 ± 10 versus 584 ± 12 beats min⁻¹, KO versus WT, P ≤ 0.05). Body temperature was also elevated (38.1 ± 0.3 versus 36.9 ± 0.4°C, KO versus WT, P ≤ 0.05). To investigate autonomic nervous system influences, we used heart rate variability analyses. We empirically defined frequency power bands using atropine and reserpine and verified high-frequency (HF) power and low-frequency (LF) LF/HF power ratio to be indicators of parasympathetic and sympathetic activity, respectively. The LF/HF power ratio was greater in KOs and more sensitive to reserpine than in WTs, consistent with elevated SNS activity. In contrast, atropine and exendin-4, a centrally acting agonist of the glucagon-like peptide-1 receptor, which influences cardiovascular physiology and metabolism, reduced HF power equally in both genotypes. This was associated with a greater increase in heart rate in KOs. Mild stress had a blunted effect on the LF/HF ratio in KOs consistent with elevated basal sympathetic tone.
Sympathetic cardiovascular stimulation in Gnaxl knock-out mice

It has been increasingly recognized over recent years that disorders of energy balance and metabolism are often associated with cardiovascular disease. Typically, these symptoms occur jointly when central effects are involved. Specifically, the sympathetic nervous system (SNS) has been recognized as a major regulator of metabolic rate and cardiovascular physiology (Matsumura et al. 2003; Hall et al. 2010; Malpas 2010). Obesity-related cardiovascular symptoms involve central actions of the adipokine leptin, which downstream effector, the melanocortin system, and subsequently increased sympathetic nerve activity (Hall et al. 2010). Much less is known about cardiovascular complications in disorders of hypermetabolism and leanness. Genetic manipulations of the renin–angiotensin system have been shown to increase metabolic rate and affect blood pressure (BP) through central as well as peripheral mechanisms (Dumont & Brouwers, 2010; Grobe et al. 2010). Another model with elevated sympathetic and cardiovascular tone is the Schlager inbred mouse line, which also displays reduced body weight (Davern et al. 2009). However, the underlying genetic cause for their phenotype is unknown. The anatomical organization of brain regions involved in the control of sympathetic activity is well known, although any differential topographical regulation of SNS outflow towards distinct peripheral tissues is less clear (Sved et al. 2001; Malpas, 2010; Nunn et al. 2011). Retrograde tracing studies from various peripheral tissues have indicated a common set of hierarchically organized brainstem and hypothalamic areas to be involved in the control of SNS outflow to diverse organs (Sved et al. 2001).

In this study, we analyse mice deficient for XLαs, a G-protein α-subunit encoded by the Gnaxl transcript of the complex Gnas locus (Plagge et al. 2008). Gnaxl constitutes an alternative splice variant of Gnas and differs only in the NH2-terminal exon (Fig. S1). Like Gα, XLα links a number of G-protein-coupled receptors in vitro, which results in activation of adenylate cyclase and production of cAMP (Liu et al. 2011). However, the specific receptor(s) that signal through XLαs in vivo remain unknown. Gene expression at the Gnas locus is regulated by the epigenetic mechanism of ‘genomic imprinting’, which leads to the silencing of one allele depending on its parental origin (Plagge et al. 2008). Thus, Gnaxl expression occurs exclusively from the paternal allele. By contrast, Gnα remains expressed biallelically in most cells, with the exception of some tissues where its expression is restricted to the maternal allele, e.g. in the paraventricular nucleus (PVN) of the hypothalamus (Fig. S1; Chen et al. 2009). Loss of XLαs in mice results in a lean and hypermetabolic phenotype, caused by increased sympathetic stimulation of brown and white adipose tissues, and it has been proposed that there may be a systemic increase in SNS outflow (Xie et al. 2006). Expression of XLαs in adult mice is limited almost exclusively to the brain and correlates with SNS control centres, including the intermediolateral layer (IML) of the spinal cord, the ventrolateral medulla, medullary raphe nuclei, the nucleus tractus solitarii (NTS), hypothalamic nuclei (PVN, dorsomedial nucleus, lateral hypothalamic area, arcuate and suprachiasmatic nuclei) and the preoptic area (Pasolli & Huttner, 2001; Krechowec et al. 2012). Its expression in regions such as the PVN and dorsomedial nucleus suggests that it may influence wider sympathetic outflow, including targets within the cardiovascular system (Coote, 2007; Womack et al. 2007).

Here, we explore the cardiovascular phenotype of Gnaxl knock-out (KO) mice and its regulation by the autonomic nervous system. The effects of inhibition of the sympathetic and parasympathetic nervous system (PNS), respectively, were examined using reserpine, which blocks the vesicular monoamine transporter at synapses, and atropine, which inhibits muscarinic acetylcholine receptors (Janssen et al. 2000; Young & Davisson, 2011). To begin an investigation into potentially deregulated neuropeptide systems that might be involved in the Gnasl KO phenotype, we explored responses to activation of the brain glucagon-like peptide-1 (GLP-1) system. Glucagon-like peptide-1, apart from its peripheral role, also acts as a neuromodulator produced in the NTS of the medulla (Llewellyn-Smith et al. 2011). As the GLP-1 receptor signals via an α-stimulatory G-protein and cAMP, this pathway could potentially be affected in XLαs KO mice. Activation of the central GLP-1 receptor has dual autonomic effects. It inhibits parasympathetic control of the cardiovascular system, resulting in increased heart rate (HR) and BP (Barragan et al. 1999; Yamamoto et al. 2002; Hayes et al. 2008; Griffioen et al. 2011), while it also stimulates sympathetic outflow towards metabolically relevant tissues (Nogueiras et al. 2009).
Furthermore, the c-fos response to the comparatively stable GLP-1 receptor agonist exendin-4 (Ex-4), which elicits identical central effects independent of its route of injection (intraperitoneal or intracerebroventricular), coincides with brain regions that control sympathetic outflow in the hypothalamus, medulla and spinal cord (Yamamoto et al. 2002; Baraboi et al. 2011).

In this study, we present BP and HR data obtained from anaesthetized and conscious mice, respectively. Autonomic influences on HR were examined by heart rate variability (HRV) analyses. Heart rate and HRV responses to reserpine, atropine, Ex-4 and handling stress were measured. Additionally, neuronal activation in response to Ex-4 was quantified histologically via c-fos expression analysis in wild-type (WT) and KO mice.

**Methods**

**Ethical approval**

All animal work was approved by the Ethical Review Committee of the University of Liverpool and carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 (UK Home Office Project Licences PPL40/3009 and PPL40/3351). All surgery was performed under general anaesthesia as described in detail below.

**Animals**

Gnasxl mutant mice (Plagge et al. 2004) were maintained on a CD1 background. Gnasxl KO offspring (Gnasxl^m+/p−, i.e. lacking XLαs expression from the paternally inherited allele) were produced by mating CD1 females with Gnasxl mutation-carrying males. All experiments were performed on young adult (~4-month-old) male Gnasxl KO and WT siblings. Mice were housed in the animal facility of the University of Liverpool on a 12 h–12 h light–dark cycle and had unlimited access to water and standard chow diet.

**Blood pressure recordings and body temperature measurements**

For BP recordings via tail volume pressure recording (VPR) plethysmography, which measures systolic and diastolic pressure (CODA VPR system; Kent Scientific, Torrington, CT, USA), mice were lightly anaesthetized (urethane, 1.5 mg kg⁻¹ I.P.; Sigma-Aldrich, UK) and kept in thermoneutral conditions (ambient temperature 30°C) throughout recording, which commonly results in lower BP values compared with conscious mice at room temperature (Swoap et al. 2004). A minimum of 10 readings were taken from each mouse; any values with low (<15 ml) displacement values were disregarded. For BP recordings via cannulation, mice were anaesthetized with urethane (1.4–2.2 mg kg⁻¹) and α-chloralose (7–11 μg kg⁻¹; Sigma-Aldrich, UK), administered I.P. in saline; doses were matched within sibling groups. Body temperature was recorded by rectal probe 3 min after injection of anaesthetic agents and prior to application of external heat. After this, temperature was continuously monitored and maintained at 37 ± 0.5°C by heat pad and infra-red lamp. Following loss of reflexes, the trachea was intubated to facilitate breathing, and the carotid artery was cannulated with stretched PP25 tubing filled with heparinized saline, connected to a pressure transducer. The raw blood pressure signal was digitized to a PC with a CED Micro1401 (CED, Cambridge, UK) using Spike2 at 5 kHz. The raw signal was also split, AC coupled and amplified between 10 and 100 times, depending on signal strength, and recorded on Spike2 at 5 kHz. Heartbeats were annotated to the amplified AC-coupled blood pressure signal using Wabp from the Physionet suite of programs (Goldberger et al. 2000). The numbers of animals used are indicated in the Results section.

**Telemetry surgery, recording and mild stress handling**

Electrocardiogram transmitters (ETA-F20; Data Sciences International, St Paul, MN, USA) were implanted subcutaneously into adult mice under isoflurane–N₂O gas anaesthesia. The mice were given preoperative S.C. injections of the analgesic buprenorphine (Temgesic, 1.5 mg kg⁻¹; Reckitt Benckiser, Slough, UK), the anti-inflammatory meloxicam (Metacam, 100 μg kg⁻¹; Boehringer Ingelheim, Ingelheim, Germany) and the anti-inflammatory enrofloxacin (Baytril, 0.2 ml kg⁻¹; Bayer AG, Leverkusen, Germany) and the anti-inflammatory meloxicam (Metacam, 100 μg kg⁻¹; Boehringer Ingelheim, Ingelheim, Germany). The mice were monitored postoperatively, and were allowed at least 5 days of recovery before any further procedures were begun. This recovery period was found to be sufficient for the re-establishment of normal HR patterns (Thireau et al. 2008); we observed no differences compared with data from later time points. Mice were housed individually over receiver pads (Data Sciences International); ECG and locomotor activity were recorded continuously. The ECG signal was digitized to a PC with a CED Micro1401 using Spike2 at 5 kHz; locomotor activity was recorded at 100 Hz. Heart rate and locomotor activity were annotated using a custom program. Mild stress was induced by handling of the mice (restrained by holding the scruff; Meijer et al. 2006). Heart rate responses were evaluated between 120 and 180 min after reserpine injection, for 15 min after stress, and between 60 and 120 min after injection of atropine and Ex-4. The numbers of animals used are indicated in the Results section.

**Heart rate variability**

Heart rate variability analysis was performed using the Kubios HRV program (Niskanen et al. 2004). For power
Peripheral drug injections

Reserpine (Sigma-Aldrich, UK) was dissolved in 1% acetic acid solution; it was injected at 2 mg kg\(^{-1}\) in anaesthetized mice (cannulation experiments) and at 1 mg kg\(^{-1}\) in conscious mice (telemetry experiments). Atropine (Sigma-Aldrich, UK) and Ex-4 (Tocris, UK) were dissolved in physiological saline (0.9% NaCl) and injected at doses of 2 mg kg\(^{-1}\) and 50 μg kg\(^{-1}\), respectively. All injections were performed i.p. in 50 μl volume.

c-fos immunostaining

Exendin-4 was injected i.p. at 50 μg kg\(^{-1}\); 2 h later mice were terminally anaesthetized with Pentobarbitone (Pentoject, Animalcare, York, UK; 160 mg kg\(^{-1}\) i.p.) and perfused transcardially with 4% paraformaldehyde in PBS. Tissues were removed, dehydrated with 30% sucrose in PBS and coronal cryostat sections prepared. c-fos immunohistochemistry was performed using rabbit anti-c-fos primary antibody (Ab5, 1:50,000 dilution; Calbiochem-Merck, Feltham, UK), followed by signal amplification using the VECTASTAIN Elite ABC kit (Vector Laboratories, Peterborough, UK) with diaminobenzidine with 0.05% (w/v) NiCl\(_{2}\) substrate (Sigma-Aldrich, UK). Immunofluorescence was used to co-localize c-fos with XLαs on WT brain sections after Ex-4 stimulation. The same c-fos primary antibody (1:2000 dilution) was combined with donkey anti-rabbit Alexa 488 secondary antibody (1:1000 dilution; Life Technologies, Paisley, UK); XLαs was detected using primary goat anti-XLαs (sc-18993, 1:200 dilution; Santa Cruz, Heidelberg, Germany) and donkey anti-goat Alexa 594 secondary antibody (1:1000 dilution; Life Technologies). Tissues from five WT and five KO mice were analysed.

Statistical analyses

For the measurements in anaesthetized dose-matched siblings, Student’s paired t tests were used (Minitab Ltd, Coventry, UK). Except where stated otherwise, the telemetry data were analysed by Student’s unpaired t tests or repeated-measures ANOVA (for circadian time points). All graphs show data as means ± SEM.

Results

Elevated BP in Gnasxl KO mice

Blood pressure was recorded from lightly anaesthetized male Gnasxl KO mice and their WT littermates via tail VPR plethysmography in thermoneutral conditions. We found mean BP to be significantly higher in KO mice than in WT siblings (Fig. 1A; 84 ± 6 versus 64 ± 4 mmHg, KO versus WT, \(P < 0.05\), \(n = 9\) and 8). Responses to the sympatholytic drug reserpine were recorded via arterial cannulation, to allow continuous measurement of BP. Knock-out mice had a significantly greater response to reserpine than WT mice (Fig. 1B; −7 ± 1 versus −1 ± 2 mmHg, KO versus WT, \(P < 0.05\), \(n = 5\)). Responses to vehicle were similar in both genotypes (data not shown). These findings suggest that elevated sympathetic activity could be the underlying cause for the increased BP in mutant mice. Additionally, and in line with the previously described elevated energy expenditure (Xie et al. 2006), KO mice had elevated body temperature (Fig. 1C; 38.1 ± 0.3 versus 36.9 ± 0.4°C, KO versus WT, \(P < 0.05\), \(n = 7\)).
Increased basal HR in conscious Gnasxl KO mice

Heart rate data were obtained in anaesthesia-free conditions following subcutaneous implantation of telemetric transmitters. The ECG was recorded continuously in freely moving mice, and HR was derived using a custom program (see Fig. S2 for representative telemetry data). Circadian variation in HR and locomotor activity were plotted as averages per hour over a 24 h period (Fig. 2A and C). Heart rate was found to be

Figure 2. Circadian heart rate (HR), activity and HR responses in conscious Gnasxl KO mice

Heart rate and activity were recorded continuously in freely moving KO mice and WT siblings via ECG telemetry. A, 1 h averaged circadian HR over 24 h. Significantly different time points are indicated (*P ≤ 0.05, n = 12, repeated-measures ANOVA). Filled bar, night period; open bar, day period. B, mean HR is increased at night in KO compared with WT mice (*P ≤ 0.05), but did not reach significance during the daytime. C, 1 h averaged circadian activity over 24 h. D, mean activity is unchanged in KO compared with WT mice. E–G, treatments and HR recordings were undertaken during the light period. E, following i.p. injection of 1 mg kg⁻¹ of the sympatholytic reserpine the HR was significantly decreased in KO mice and trended to be lower in WT mice. F, HR was significantly increased in KO mice following i.p. injection of 2 mg kg⁻¹ of the parasympatholytic atropine. G, HR was significantly increased, to a similar extent, in both genotypes following mild handling stress. *P ≤ 0.05 by Student’s paired t test. Error bars indicate SEM.
Sympathetic cardiovascular stimulation in Gnasxl knock-out mice

Autonomic control of HR in conscious Gnasxl KO mice

The cause of the elevated HR in Gnasxl KO mice was investigated by injection of reserpine and atropine to inhibit sympathetic and parasympathetic stimulation, respectively, of the cardiovascular system (Janssen et al. 2000; Young & Davisson, 2011). All treatments were carried out during the daytime (light) period. Reserpine at a dose of 1 mg kg−1 i.p. caused a significant decrease in HR in KO mice (Fig. 2E; KO, from 531 ± 23 to 455 ± 12 beats min−1, P ≤ 0.05, n = 8; and WT, from 516 ± 21 to 471 ± 41 beats min−1, n.s., n = 8; Student’s paired t test). Atropine at a dose of 2 mg kg−1 i.p. caused a significant increase in HR in KO mice (Fig. 2F; from 551 ± 16 to 591 ± 19 beats min−1, P ≤ 0.05, Student’s paired t test, n = 6), but not in WT siblings (from 533 ± 28 to 552 ± 17 beats min−1, n = 6). These data, combined with the HRV analysis described below, which shows the effects of reserpine and atropine more clearly, suggest that increased sympathetic tone causes elevated HR in Gnasxl-deficient mice and that inhibition of the parasympathetic system emphasizes this effect. Additionally, we investigated the cardiovascular reactivity of KO mice to mild handling stress (Meijer et al. 2006). Heart rate increased to a similar degree in response to stress in both WT mice (Fig. 2G; from 568 ± 7 to 675 ± 39 beats min−1, P ≤ 0.05, n = 9) and KO mice (from 615 ± 20 to 716 ± 11 beats min−1, P ≤ 0.05, n = 9).

Analysis of HRV

In the context of abnormal HR phenotypes, HRV analysis, especially the ratio of LF to HF HRV bandings, can provide additional information on sympathetic versus parasympathetic balance (Malpas, 2002; Baudrie et al. 2007; Laude et al. 2008; Thireau et al. 2008). We used this approach via power spectrum analysis and tested autonomic influences on the cardiovascular system in conscious mice. The LF and HF HRV bandings were validated empirically following inhibition of sympathetic and parasympathetic cardiovascular tone of WT mice with reserpine and atropine, respectively (Fig. S3). High-frequency power was then used to indicate the degree of parasympathetic stimulation of the cardiovascular system, and the LF/HF ratio was used to indicate the degree of sympathetic stimulation, specifically as a measure of sympathovagal balance (Young & Davisson, 2011).

Autonomic control of HRV in conscious Gnasxl KO mice was examined by measuring LF/HF responses to reserpine, as well as HF power responses to atropine during the day (light) period. The LF/HF ratio was significantly reduced by reserpine in both WT and KO mice (Fig. 3A; WT, from 1.38 ± 0.31 to 0.47 ± 0.09, P ≤ 0.05; and KO, from 1.39 ± 0.22 to 0.26 ± 0.06, P ≤ 0.05; both by Student’s paired t test, n = 8). However, the decrease in LF/HF ratio was significantly greater in KO compared with WT mice (Fig. 3B; −80 ± 5 versus −48 ± 13%, KO versus WT, P ≤ 0.05, n = 8). High-frequency power was significantly reduced in response to atropine in both WT and KO mice (Fig. 3C; WT, from 3.0 ± 0.6 to 1.2 ± 0.5 ms² Hz−1, P ≤ 0.05; and KO, from 2.7 ± 0.7 to 0.6 ± 0.2 ms² Hz−1, P ≤ 0.05; both by Student’s paired t test, n = 6). There was no difference in HF power decrease between WT and KO (Fig. 3D; −61 ± 16 versus −77 ± 6% in WT versus KO, n = 6). Finally, with regard to the stress response, mean LF/HF ratio was increased in WT (Fig. 3E; from 1.12 ± 0.15 to 1.85 ± 0.29, P ≤ 0.05, n = 8) but not in KO mice (from 1.83 ± 0.46 to 1.62 ± 0.45), whose basal LF/HF ratio was already as high as that of the stressed WT siblings. The relative change in LF/HF ratio was short of a significant difference between both genotypes (Fig. 3F).

Having validated the effects of reserpine and atropine on HRV of both genotypes, we investigated basal HRV during the night phase, when HR and activity are highest. Fast Fourier transform spectra were produced from a minimum of 10 3 min windows for each mouse as discussed by Thireau et al. (2008; Fig. 4A). The Gnasxl KO mice had an increased LF/HF ratio at night (Fig. 4B; 1.79 ± 0.18 versus 1.23 ± 0.14, KO versus WT, P ≤ 0.05, n = 9 and 12), which is consistent with an elevated sympathetic tone as the main cause of their cardiovascular phenotype.

Cardiovascular responses to Ex-4

Given that the GLP-1 neuropeptide system impacts on BP, HR and metabolic rate and that the GLP-1 receptor couples via an α-stimulatory G-protein, we investigated this system using the agonist Ex-4. This agonist elicits identical cardiovascular and brain c-fos responses independent of its route of injection (Barragan et al. 1999; Yamamoto et al. 2002; Hayes et al. 2008; Nogueiras et al. 2009; Baraboi et al. 2011; Griffioen et al. 2011). First, the HR response to 50 μg kg−1 Ex-4 was examined (injected i.p. during the daytime); it resulted in a significant increase in HR in KO mice (Fig. 5A and B; from 542 ± 27 to 622 ± 14 beats min−1 in KO...
versus from 533 ± 21 to 561 ± 27 beats min⁻¹ in WT; $P \leq 0.05$ by Student’s paired t test, $n = 7$). Heart rate variability analysis indicated little effect on LF/HF ratio (sympathetic stimulation) in WT or KO mice (Fig. 5C and D). However, HF power (parasympathetic activity) was decreased significantly by Ex-4 to a similar degree in both genotypes (Fig. 5E and F; WT, −76 ± 4%; and KO, −77 ± 9%, $P \leq 0.001$; both by Student’s paired t test, $n = 7$), suggesting that the regulation of parasympathetic tone by the GLP-1 system (Griffioen et al. 2011) is functioning normally in the absence of XLαs. The effects of Ex-4 were very similar to those of the parasympatholytic atropine (Figs 2F and 3C and D). Both drugs caused a similar HRV (HF) response in WT and KO mice, as well as an increase in HR in KOs.

### Neuronal c-fos responses to Ex-4

c-fos immunohistochemistry provides a useful histological indicator of neuronal activation patterns in response to various stimuli. In contrast, neuronal inhibition does not result in c-fos induction (Dampney & Horiuchi, 2003). We investigated potential changes in the c-fos response to Ex-4 in Gnasxl KO mice using the same dose as in the HR experiment. Exendin-4 resulted in similar numbers of c-fos-positive neurons in the expected brain regions (PVN, NTS, area postrema and amygdala) in both genotypes (Fig. 6 and Figs S4 and S5). There was also no difference in c-fos counts when a more detailed analysis of PVN subregions along its rostral–caudal axis was undertaken (data not shown). The unchanged c-fos response in sympathetic control regions of XLαs-deficient mice further supports the view that the functions of the GLP-1 system are not impaired in KO mice. We also examined whether the Ex-4-induced c-fos response occurs in XLαs-expressing neurons in WT mice. There was no overlap of expression of the two proteins in the PVN or in the anterior part of the NTS, indicating that XLαs-positive neurons are not activated by Ex-4 (Fig. 7 and Figs S6 and S7).

### Discussion

In this study, we demonstrate, for the first time, that Gnasxl-deficient mice have increased BP, HR and body temperature, which is not associated with increased locomotor activity. To examine the degree of sympathetic stimulation of the cardiovascular system, mice were injected with the sympatholytic reserpine, which inhibits catecholamine uptake into secretory vesicles and thereby release at sympathetic nerve terminals (Iversen et al. 1965). Thus, it selectively diminishes sympathetic stimulation of peripheral tissues. Reserpine caused a greater reduction in BP, HR and LF/HF ratio of HRV in KO compared with WT
mice, which concurs with the hypothesis of an increased sympathetic stimulation of the cardiovascular system. In contrast, inhibition of parasympathetic activity with the muscarinic antagonist atropine (Janssen et al. 2000; Laude et al. 2008; Young & Davisson, 2011) or the GLP-1 receptor agonist Ex-4, which was only recently discovered to affect parasympathetic cardiovascular control (Griffioen et al. 2011), caused similar reductions in HF power of HRV in both WT and KO mice. This indicates normal levels of parasympathetic tone in XLαs-deficient mice. The increased HR in KO mice after atropine or Ex-4 injection can be attributed to an underlying elevated basic level of sympathetic activity in mutants compared with WT mice.

For BP experiments, we chose an anaesthetic agent that affects the cardiovascular system less than other substances (Carruba et al. 1987). Furthermore, the anaesthetic was carefully dose matched among WT and KO littermate groups, to equalize any effect. Thermoneutral conditions, as used in our tail-cuff VPR experiment, also tend to lower mean BP (Swoap et al. 2004). Nevertheless, we consistently found BP and the reserpine-mediated reduction in BP to be greater in Gnasxl KO mice. The cardiovascular phenotype was further investigated in conscious, freely moving mice using telemetry for ECG monitoring. Knockout mice showed an increased HR mainly during the active night period. The reserpine-mediated reduction in HR of KO mice suggests increased sympathetic stimulation of the cardiovascular system. Furthermore, a significant increase in the HR of Gnasxl-deficient mice was observed after application of the muscarinic blocker atropine. These findings led us further to investigate HRV data, which inform on autonomic control of the cardiovascular system and would exclude potential peripheral causes (Young & Davisson, 2011).

Heart rate variability analysis is a method for quantifying autonomic influences on the cardiovascular system based on the inherent rhythms involved in HR variation over time. These occur at characteristic frequencies associated with SNS and PNS influences. Heart rate variability is widely used as a helpful and accurate indicator of autonomic balance (Baudrie et al. 2007; Laude et al. 2008; Thireau et al. 2008) and autonomic responses (Farah et al. 2006; Griffioen et al. 2011). As there are no universal HRV indicators that precisely map to sympathetic activity (Malpas, 2002; Thireau et al. 2008), we defined these empirically by using the classical sympatholytic and parasympatholytic drugs reserpine and atropine, respectively (Iversen et al. 1965; Janssen et al. 2000; Laude et al. 2008; Young & Davisson, 2011). Our empirical validation led us to set our HF band between 1 and 5 Hz. This is in agreement with other analyses (Thireau et al. 2008). We determined that reserpine resulted in loss of virtually all LF power density down to 0.15 Hz. We therefore set our LF range between 0.15 and 1 Hz. In our verifications of drug effects on LF,
HF and the LF/HF ratio, atropine decreased LF and HF but had minimal effect on the LF/HF ratio, whilst reserpine decreased LF and the LF/HF ratio, without significant change in HF power. Therefore, the LF/HF ratio appropriately indicates sympathetic tone.

Using these HRV bandings, we found that KO mice had significantly increased LF/HF ratio during the active night period. In KO mice, the LF/HF change after reserpine was significantly more pronounced than in WT mice, suggesting increased sympathetic tone. In contrast, atropine induced a similar HF power response in both genotypes, suggesting no difference in basal PNS activity. The combined HRV and HR data after atropine suppression of the PNS are consistent with elevated basal SNS activity as the prominent cause of the cardiovascular phenotype of Gnasxl KO mice.

**Figure 5. Heart rate and HRV responses to exendin-4 (Ex-4)**

Treatments and HR recordings were carried out during the light period as in Figs 2 and 3. **A**, HR response over time in response to i.p. injection of 50 μg kg⁻¹ Ex-4 (arrow). **B**, HR is significantly increased in KO mice following Ex-4 and trended higher in WT mice. **C and D**, there was no significant difference in LF/HF ratio or relative change in LF/HF ratio following Ex-4 in either genotype. **E and F**, both genotypes had a decrease in HF power following Ex-4, although this reached significance only in WT. The relative decrease in HF power was significant to a similar extent. *P ≤ 0.05 and ***P ≤ 0.001 by Student’s paired t test. Error bars indicate SEM.
Several neuropeptides have been shown to influence the central control of the autonomic nervous system and cardiovascular phenotype (Matsumura et al. 2003; Dupont & Brouwers, 2010; Hall et al. 2010). To begin an analysis of potentially deregulated neuropeptide pathways, which use α-stimulatory G-protein-coupled receptors for signal transduction, we analysed the GLP-1 system. In the CNS, GLP-1 is produced by neurons in the medulla oblongata (Llewellyn-Smith et al. 2011). It increases BP, HR and sympathetic outflow towards metabolically

![Image](image_url)

Figure 6. Neuronal c-fos response to Ex-4 in the hypothalamic paraventricular nucleus (PVN) c-fos immunohistochemistry of coronal brain sections after injection of 50 μg kg⁻¹ i.p. Ex-4. A, representative images showing high levels of c-fos in the PVN of both genotypes. B, no significant c-fos response was observed following saline injection. C, there was no significant difference in numbers of c-fos-positive neurones (per section and left/right brain side) between genotypes. This was also confirmed in a more detailed anterior–posterior PVN subregion-specific assessment (data not shown). Error bars indicate SEM.

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relevant tissues (Barragan et al. 1999; Yamamoto et al. 2002; Hayes et al. 2008; Nogueiras et al. 2009). We detected an exaggerated HR response to Ex-4 in Gnasxl KO mice. Our findings are consistent with a recent report on Ex-4-induced changes in HR and in the HF component of HRV (Griffioen et al. 2011). Together, these studies strongly suggest that Ex-4 increases HR by suppressing cardiac parasympathetic tone. Although Ex-4 reduced the HF component of HRV to the same degree in WT and KO mice, the increase in HR was more pronounced in XLαs-deficient mice, which we attribute to a higher underlying basal sympathetic tone.

In parallel to the suppression of parasympathetic cardiovascular tone, the GLP-1 system also affects the SNS and the hypothalamic–pituitary–adrenal axis (Gil-Lozano et al. 2010). Exendin-4 induces c-fos expression in neurons that project to the sympathetic preganglionic neurons of the spinal cord, as well as in corticotropin-releasing factor-positive neurons of the PVN (Yamamoto et al. 2002; Sarkar et al. 2003). It also stimulates sympathetic nerve activity to white adipose tissue (Nogueiras et al. 2009). A normal c-fos response and an unchanged HRV LF/HF ratio in Gnasxl KO mice indicate that Ex-4 did not directly increase sympathetic stimulation of the heart. It is also interesting to note that XLαs-positive neurons did not show a c-fos response to Ex-4, which concurs with previous findings that XLαs is expressed in a neuron population different from, but in proximity to, sympathetic control neurons at several levels of the CNS (Krechowec et al. 2012).

Glucagon-like peptide-1 is not the only example of a neuropeptide that regulates the PNS and SNS reciprocally. A recent study showed that the melanocortin peptide (α-MSH) system inhibits brainstem parasympathetic preganglionic neurons, but activates sympathetic preganglionic neurons in the spinal cord via its MC4R receptor (Sohn et al. 2013). Stimulation of the melanocortin system results in increased sympathetic tone and hypertension (Hall et al. 2010; Sohn et al. 2013). As the MC4R receptor signals through Gαs, the main protein product of the Gnas locus, there is an interesting antithesis between the two splice variants XLαs and Gαs (Xie et al. 2006; Plagge et al. 2008; Chen et al. 2009; Sohn et al. 2013). Knock-out mice for XLαs and Gαs, respectively, show opposite physiological phenotypes. While XLαs deficiency is associated with leanness and hypermetabolism (Xie et al. 2006), lack of Gαs expression (from the maternal allele, i.e. Gnas<sup>m−/p+</sup>) in the brain results in obesity and hypometabolism (Chen et al. 2009). This Gαs-mutant phenotype resembles MC4R deficiency (Balthasar et al. 2005). Brain-specific Gnas<sup>m−/p+</sup> mice are also hypotensive, with reduced HR and a global decrease in SNS activity (Chen et al. 2009, 2012). In contrast, we show here that Gnasxl KO mice are hypertensive and tachycardic.

![Figure 7. c-fos response to Ex-4 in correlation to XLαs expression in the PVN](image)
due to increased sympathetic tone, which also causes elevated energy expenditure (Xie et al. 2006). Whether deficiency of XLαs might lead to an increased activity of the melanocortin–MC4R–Gα pathway remains to be tested in future studies. It is also noteworthy that XLαs and MC4R show a similar pattern of expression in some subdivisions of the hypothalamic PVN (Kishi et al. 2003; Liu et al. 2003; Krechowec et al. 2012). However, whether coexpression occurs or whether separate neighbouring neuron populations are involved remains to be clarified.

The Gnasxl KO phenotype is also consistent with a permanently activated stress reaction (Grippo et al. 2003; Farah et al. 2006). We found that WT and KO mice had comparable HR increases in response to stress, but the increase in the LF/HF ratio of HRV seen in WT mice was absent in KO mice. This would be consistent with a model whereby the classic reduction in parasympathetic cardiac inhibition by stress (Farah et al. 2006) still occurs, but the sympathetic excitation does not increase further in KO mice due to an already elevated basal cardiovascular sympathetic tone.

In summary, our data indicate that Gnasxl KO mice display a systemic increase in SNS activity, being responsible for the cardiovascular as well as the metabolic phenotype described previously (Xie et al. 2006). Our findings imply that the in vivo role of XLαs involves suppression of SNS outflow. This hypothesis is further supported by the defined expression pattern of XLαs in specific SNS control regions of the hypothalamus and brainstem, including the PVN, dorsomedial nucleus, lateral hypothalamic area, arcuate nucleus, medullary raphe nuclei, NTS and spinal cord (Krechowec et al. 2012). XLαs might, for example, mediate signal transduction from a G-protein-coupled receptor(s) (Liu et al. 2011) in a population of neurons that inhibits neighbouring sympathetic control neurons. The nature of such a receptor currently remains unclear, however. Future experiments will be aimed at characterizing XLαs-expressing neurons and the mechanisms by which this protein inhibits sympathetic activity, including analyses of additional neuroactive substances known to function in XLαs-expressing brain regions, such as α-MSH, angiotensin or substance P (Womack & Barrett-Jolley, 2007; Womack et al. 2007; Nunn et al. 2011).

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Sympathetic cardiovascular stimulation in Gnasxl knock-out mice and WT siblings were injected with 50 μg/kg I.P. Ex-4 and tissues collected two hours later. Brain sections were stained for c-fos by immunohistochemistry. A, Representative images showing c-fos response in the amygdala of both genotypes. B, Representative images showing no significant c-fos response in the same region following saline injection. C, There was no significant difference in numbers of c-fos positive neurones (per section and left/right brain side) between genotypes. Error bars indicate SEM.

Figure S5. Neuronal c-fos responses to Ex-4 in the area postrema (AP) and medial region of the NTS
Gnasxl KO mice and WT siblings were injected with 50 μg/kg I.P. Ex-4 and tissues collected two hours later. Brain sections were stained for c-fos by immunohistochemistry. A, Representative images showing c-fos response in the AP and NTS of both genotypes. B, Representative images showing no significant c-fos response in the same brain regions following saline injection. C, There was no significant difference in numbers of c-fos positive neurones between genotypes. Error bars indicate SEM.

Figure S6. Control immunofluorescence for XLas and c-fos in response to Ex-4 in the hypothalamic PVN of Gnasxl KO
Gnasxl KO mice were injected with 50 μg/kg I.P. Ex-4 and tissues collected two hours later. Brain sections were co-stained for c-fos (green) and XLas (red). A representative image is shown, demonstrating the specificity of the XLas antibody and a similar c-fos response as in WTs.

Figure S7. c-fos response to Ex-4 in XLas-expressing neurones of the amygdala
WT and KO mice were injected with 50 μg/kg I.P. Ex-4 and tissues collected two hours later. Brain sections were co-stained for c-fos (green) and XLas (red). A representative image is shown, demonstrating that XLas-expressing neurones and c-fos responsive neurones constitute noticeably separate populations. Of 5 WT mice investigated, there were no co-localised neurones found.