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Hypothalamic paraventricular nucleus neuronal nitric oxide synthase activity is a major determinant of renal sympathetic discharge in conscious Wistar rats.

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Abstract

What is the central question of this study?

Does chronic reduction of neuronally generated nitric oxide in the hypothalamic paraventricular nucleus affect the set-point regulation of blood pressure and sympathetic activity destined to the kidneys?

What is the main finding and its importance?

Within the hypothalamic paraventricular nucleus, nitric oxide generated by neuronal nitric oxide synthase plays a major constitutive role in suppressing long term the levels of both ongoing renal sympathetic activity and arterial pressure in conscious Wistar rats. This finding unequivocally demonstrates a mechanism by which the diencephalon exerts a tonic influence on sympathetic discharge to the kidney and may provide the basis for both blood volume and osmolality homeostasis.

The paraventricular nucleus of the hypothalamus (PVN) plays a crucial role in cardiovascular and neuroendocrine regulation. Application of nitric oxide donors to the PVN stimulates GABAergic transmission, and may suppress sympathetic nerve activity (SNA) to lower arterial pressure. However, the role of endogenous nitric oxide within PVN in regulating renal SNA *chronically* remains to be established in conscious animals. To address this, we used our previously established lentiviral vectors to selectively knock down neuronal nitric oxide synthase (nNOS) in the PVN of conscious Wistar rats. Blood pressure (BP) and renal SNA were monitored simultaneously and continuously for 21 days (n=14) using radio-telemetry. Renal SNA was normalised to maximal evoked discharge and expressed as a percent change from baseline. The PVN were microinjected bilaterally with a neurone-specific tetracycline-controllable lentiviral vector, expressing a short hairpin miRNA-30 (shRNA) interference system

targeting nNOS (n=7) or expressing a mis-sense as control (n=7). Recordings continued for a further 18 days. The vectors also expressed GFP, and successful expression in the PVN, and nNOSknockdown, was confirmed histologically *post-hoc*. Knock-down of nNOS expression in the PVN resulted in a sustained increase in BP (95±2 to 104±3 mmHg, P<0.05), with robust concurrent sustained activation of renal sympathetic nervous activity (>70%, P<0.05). The study reveals a major role for nNOS-derived nitric oxide within the PVN in chronic set-set point regulation of cardiovascular autonomic activity in the conscious, normotensive rat.

Introduction

The paraventricular nucleus (PVN) of the hypothalamus is known to play a key role in the central regulation of sympathetic tone in health and disease.(Saper *et al.*, 1976; Ciriello & Calaresu, 1980; Swanson & Sawchenko, 1983; Caverson *et al.*, 1984; Coote *et al.*, 1998; Ranson *et al.*, 1998; Patel, 2000; Pyner & Coote, 2000; Kenney *et al.*, 2003) Recently, there has been a particular focus on the regulatory role of nitric oxide within the PVN (Li *et al.*, 2002a; Li *et al.*, 2002b; Wang *et al.*, 2005; Pyner, 2009; Watkins *et al.*, 2009; Sharma *et al.*, 2011). Neuronal nitric oxide synthase (nNOS) is the enzyme largely responsible for the production of nitric oxide in neurons, and is expressed in a number of brain regions important in autonomic control, including the PVN (Garthwaite & Boulton, 1995). nNOS expression in the PVN has been shown to be reduced in sympathoexcitatory diseases including heart failure (Zhang *et al.*, 1998; Schultz, 2009; Pyner, 2014) and chronic intermittent hypoxia-induced hypertension (Huang *et al.*, 2007). In contrast, nNOS expression is *increased* in the PVN of spontaneously hypertensive (Plochocka-Zulinska & Krukoff, 1997) and renovascular hypertensive rats (Krukoff *et al.*, 1995), where it is hypothesised to compensate for the sympathetic hyperactivity characteristic of these models.

Functionally, experimental approaches which either increase neuronal excitability or decrease nitric oxide transmission in the PVN, tend to *increase* sympathetic drive and arterial blood pressure, at least as observed in the acute setting (Kannan *et al.*, 1989; Martin & Haywood, 1992; Li *et al.*, 2001; Hirooka *et al.*, 2011). In conscious rats, the short-term blockade of nNOS transmission, by infusing nNOS-specific antisense oligonucleotides into the PVN, has been shown to produces increases in blood pressure and heart rate for several hours (Wang *et al.*, 2005), with evidence that a reduction in nitric oxide transmission in the PVN may contribute to sympathoexcitation in heart failure (Zhang *et al.*, 1998). Conversely, acute treatments which either increase synaptic inhibition or increase nitric oxide transmission in the PVN, produce *decreases* in sympathetic drive and blood pressure (Zhang & Patel, 1998; Li *et al.*, 2002b; Akine *et al.*, 2003). These functional data, together with neuroanatomical findings (Watkins *et al.*, 2009), suggest that nNOS-derived nitric oxide in the PVN plays a key role in the acute regulation of sympathetic outflow and arterial pressure.

Taken together, these data suggest an important role for nNOS in the PVN in regulation of the sympathetic outflow in both health and disease (Coote, 2005). However, direct evidence that endogenous nNOS activity in the PVN plays a role in the long-term physiological regulation of cardiovascular sympathetic tone is lacking.

Previous studies examining a role for nitric oxide in the PVN for sympathetic control have been largely limited to acute effects, observed over a scale of minutes or hours in anesthetized animals (Zhang *et al.*, 1997; Zhang *et al.*, 2001). Longer time scale studies have involved nNOS overexpression in the PVN; however, interpretation here is confounded as cells that do not normally produce nNOS now express it, which may limit relevance to the physiological setting.(Li *et al.*, 2002b) Thus, we set out to identify the role of nNOS in the PVN in determining long-term sympathetic outflow and cardiovascular control, in conscious, unrestrained healthy rats. In order to chronically decrease endogenous levels of nNOS in the PVN, we have used our previously published lentiviral vector knock-down system reported to cause a ~55% reduction in nNOS protein (Liu *et al.*, 2010; Liu *et al.*, 2011).

Methods

Ethical Approval

All experiments performed were carried out according to the guidelines laid down by our institution's animal welfare committee, and conform to the principles and regulations described by Grundy (2015). Experiments were in accordance with the European Commission Directive 86/609/EEC (European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes) and the UK Home Office (Scientific Procedures) Act (1986), with project approval from the University of Bristol Animal Care and Use Committees.

Experiments were carried out on adult male Wistar rats bred within the University of Bristol's animal facility (n=25, 280-320g), individually housed with standard rat chow and water available *ad libitum* and kept on a 12 hour light-12 hour dark cycle. Surgeries were performed aseptically under ketamine (60 mg kg^{-1}; i.m.) and medetomidine (250 µg kg^{-1}, i.m.) anaesthesia, with sufficient depth of anaesthesia ensured by testing

intermittently (approximately every 20 min) for the absence of limb withdrawal reflexes. Analgesia was administered post-surgically.

Telemetric blood pressure and renal nerve recordings

Arterial pressure and renal sympathetic nerve activity (renal SNA) were monitored continuously in rats using an implanted telemetric device (model TRBSNA; Telemetry Research Ltd, Auckland, New Zealand) as per (McBryde *et al.*, 2013). Briefly, rats were anaesthetised and the abdominal aorta exposed and cannulated just above the iliac bifurcation. The catheter was inserted until the tip rested just below the left renal artery branch point, and held in place using a tissue adhesive (VetBond, 3M, USA) and cellulose matrix. The transmitter body was placed in the abdominal cavity, and the electrode wire leads tunnelled through to an incision on the left flank. The left renal artery was exposed via a retroperitoneal approach, the renal nerves gently freed from surrounding connective tissue and placed over the bipolar recording electrodes. The electrodes themselves were secured in place with sutures to the wall of the renal artery and abdominal aorta, and a biocompatible silicone elastomer (Kiwk-Sil, WPI, Eu) used to isolate both electrode and nerve. The incisions were closed, and rats recovered for at least 7 days post-operatively, with analgesic treatment once daily for 3 days (Buprenorphine, 0.01 mg/kg per dose).

Arterial pressure and renal SNA signals were sampled at 500 Hz using an analog-digital data acquisition card (PCI 6024E National Instruments, Austin, Texas) and displayed by a data acquisition program (Universal Acquisition 11, University of Auckland, Auckland, New Zealand). Heart rate (HR) was derived from the inter-pulse interval of the arterial pressure waveform. The renal SNA signal was amplified, filtered between 50-5000 Hz, full-wave rectified and integrated using a low pass filter with a 20 ms time constant. At least 7 days were allowed for recovery before a 3 day baseline period was recorded. Arterial pressure, renal SNA and heart rate were recorded for 24 hours a day as 2 second averages, with high frequency (500 Hz) periods of 5 minutes saved every hour to verify signal quality. Two days before and 7 after lentiviral injection, rats were briefly exposed to a bolus of cigarette smoke (5 ml in air), which activates the nasopharyngeal nerve endings and provokes a powerful reflex increase in renal SNA. The magnitude of this response was used to check that the renal SNA signal remained viable and responsive throughout the experiment. The success rate

for renal SNA recordings for the full experimental protocol (24 days) was approximately 65%.

Lentiviral (LVV) knock-down of nNOS in neurones

For nNOS knock-down, we used our binary LVV system which has been validated previously in hippocampus and dorsal vagal complex (Liu *et al.*, 2008; Liu *et al.*, 2010; Liu *et al.*, 2011). This system requires co-operative action of two LVVs. The first vector – LV-Tretight-eGFP-miR30-shRNA/nNOS harbours an expression cassette for an miRNA30 (miR30)-based short hairpin (shRNA) interference system (Stegmeier *et al.*, 2005) under control of a Tet-sensitive promoter. The system expresses a miRNA30-like hairpin targeting nNOS fused to the 3' end of the sequence encoding enhanced green fluorescent protein (eGFP); this design facilitates targeting of the RNA duplex into the RNA-induced silencing pathway. The second vector – LV-mCMV/SYN-tTA - expresses tetracycline-sensitive transactivator Tet-off under control of an enhanced synapsin-1 promoter. This neurone-specific nNOS knock-down system works by expression of the Tet-off transactivator (tTA) from LV-mCMV/SYN-tTA which contains the bidirectional amplified SYN promoter to ensure high level of tTA. tTA binds to Tretight promoter in LV-Tretight-GFP-miR30-shRNA/nNOS and activates the expression of shRNA/nNOS. For simplicity, this system is hereafter referred to as LVV-shRNA/nNOS. As a control, we used a similar viral system directed against firefly luciferase (Luc); LV-Tretight-GFP-miR30-shRNA/Luc plus LV-mCMV/SYN-tTA. This construct does not affect nNOS protein *in vivo:* (Liu *et al.*, 2010; Liu *et al.*, 2011) and will be further referred to as LVV-control for simplicity.

LVV protocol and microinjections

On day 0, rats were anaesthetised as above, placed in a stereotaxic frame using non-traumatic ear bars, and underwent bilateral microinjections of viral vectors (LVV-shRNA/nNOS and LV-control) into the PVN using glass micropipettes with tip diameters of ~25 µm. Microinjections were made 1.8 mm caudal to bregma, ±0.3 mm lateral to the midline and 7.5 mm below the dorsal surface of the cerebrum. Titres of both LVV used were $2–6 \times 10^9$ TU ml^{-1}, with an injected volume of 0.5 µL per site. MAP, HR and RSNA were recorded for a further 21 days. In two rats injected with LVV-nNOS the recording window was extended to 55 days to investigate the persistence of the observed pressor responses.

Because of the known brain region differences in the efficacy of the viral system used (Liu *et al.*, 2010) and to validate the efficacy of nNOS knock-down in the PVN specifically, LVV-nNOS was microinjected unilaterally in a sub-group of rats (n=3), and nNOS expression both compared and quantified immunocytochemically (see below) in the injected vs non-injected sides. For this, Fiji binary representation particle analysis of eGFP and nNOS was used to determine the % of each protein in both the left and right PVN regions.

Immunocytochemistry

Rats were terminally and deeply anaesthetised (induction with halothane, 5% in oxygen followed by sodium pentobarbital 40 mg/kg i.p.). The depth was confirmed by failure of the animal to respond to a noxious pinch of a limb or the tail and evidence of suppression of respiratory depth and frequency. Subsequently, rats were perfused transcardially with fixative (5% formaldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C) and their brains removed and post fixed for 2 h in the same solution. They were cryoprotected overnight using 30% sucrose in phosphate buffer at 4°C. The brains were trimmed above the dorsal 3rd ventricle and lateral to the reticular thalamic nucleus. Coronal sections 40 μm were cut on a freezing microtome with every section collected into a series of 3 wells. The freely floating sections were stained using standard immunofluorescence double labelling techniques (blocking using 10% goat serum and permeabilisation using 0.1% Triton X) and employed primary antibodies against eGFP (A11122, Life Technologies Ltd, UK) in a concentration of 1:2000, and NeuN Chemicon International, USA) in a concentration of 1/200. Sections from animals with unilateral LVV-nNOS microinjections were incubated with primary antibodies against eGFP (as above) and NOS1 (sc-5302, Santa Cruz Biotechnology Inc, GE) in concentrations of (1:25, 1:50 and 1:100). The eGFP antibody was visualised with secondary antibody Goat-anti-Rabbit-AF488 (A11008, Life Technologies Ltd, UK) used 1:500 and the NeuN or NOS1 antibody with 1:500 biotinylated anti-mouse IgG (BA9200, Vector Laboratories Ltd, UK) tagged with 1:500 Streptavidin-AF594 (S11227, Molecular Probes, UK). All incubations were performed for 1h at room temperature except the primary antibody incubation which lasted 24h at 4°C. Upon staining the sections were mounted on Super frost slides, airdried at room temperature and cover slipped using vectashield (H-1000, Vector Laboratories

Ltd, UK). Slides were inspected on a Leica fluorescent microscope under 5-20x magnification, and images captured with AxioVision software. Omission of the primary antibody resulted in no detectable staining.

Data analysis

From the arterial pressure waveform, values for systolic, diastolic, mean and pulse pressure, and heart rate were calculated. Spontaneous ramps in arterial pressure and heart rate were used to derive spontaneous cardiac baroreceptor reflex gain, and low and high frequency spectral components of the blood pressure and heart rate signals assessed to give indirect insight in to changes in global autonomic tone as described previously (Waki *et al.*, 2006). Renal SNA is expressed as a percentage, where 100% was set as the average integrated value across the baseline period as previously described by us (McBryde et al. 2013). Values are presented as daily average mean ± standard deviation (SD). Data were compared between baseline and nNOS time points, by repeated measures 2-way ANOVA with time and experimental group as factors (SigmaPlot v.12.0, Systat Software Inc, 2011). Where appropriate, post-hoc comparisons were made using the Holm-Sidek multiple comparison test. Differences within or between groups with p values <0.05 were considered significant.

Results

Cardiovascular response to nNOS knock-down.

Baseline (Days -3 to 0) mean arterial pressure (MAP) was similar between LVV-shRNA/nNOS and LVV-control rats (95±5 mmHg vs. 97±6 mmHg, N.S.). As can be seen in Fig 1, following microinjection of LVV-shRNA/nNOS on Day 0 MAP increased slowly from ~Day 7 reaching a stable plateau around Day 10 (104±7 mmHg; p=0.041). LVV-control animals showed no change in MAP across the entire experimental period. Figure 2 (panel A) shows the individual data from a rat where the BP recording was continued out to 55 days; in this rat the blood pressure remained above baseline levels as was found in a second rat that was followed out to this extended time point. Heart rate was not significantly different between LVV-shRNA/nNOS and LVV-control groups at pre- (311±27 vs. 330±45 bpm, respectively) and did not change significantly in either group post- viral microinjection. The increased BP persisted in the two LVV-

shRNA/nNOS rats that were maintained for 55 days, which is consistent with the known long-acting expression of transgenes by Lentiviruses (Naldini *et al.*, 1996).

Renal sympathetic response to nNOS knock-down.

Figure 1 shows the temporal association between the rise in MAP and the concurrent rise in renal SNA in LVV-shRNA/nNOS rats. Renal SNA in LVV-shRNA/nNOS rats increased to over 70% above baseline (+75±44%, p=0.034), while there was no significant change in LVV-control rats (-7±37%, NS; Figs 2 & 3). Figures 2B and 2C show traces of raw renal SNA, cardiac association of the renal SNA signal, and the renal SNA responses to activation with nasopharyngeal stimuli before (Day -1) and after (Day 15) nNOS knock-down; comparable responses were evoked confirming the validity of the recording with time.

Baroreceptor reflex and heart rate variability.

No change in the spontaneous cardiac baroreceptor reflex gain (sBRG) was seen with nNOS knock-down at baseline (LVV-control: -6.7±0.3 versus LVV-shRNA/nNOS -6.6±0.5 mmHg.bpm^{-1}, NS) or at week 3 (LVV-control: -6.3±0.3 versus LVV-shRNA/nNOS: -6.3±0.5 mmHg.bpm^{-1}, NS, Fig 3). Heart rate variability, as measured by the ratio of low frequency and high frequency power (LF:HF) of pulse interval, showed a significant increase from baseline to week 3 in LVV-shRNA/nNOS rats (0.13±0.08 to 0.22±0.07, p=0.032) but not in the LVV-control group (0.11±0.05 to 0.10±0.03, NS, Fig 3). This increase in LF:HF ratio gives indirect evidence for a shift towards sympathetic dominance in cardiac sympatho-vagal balance. The LF component of SBP increased significantly in LVV-shRNA/nNOS rats from baseline to week 3 (3.20±0.33 to 3.66±0.34 mmHg^2, p=0.016) but not in LVV-control rats (3.12±0.32 to 3.21±0.34 mmHg^2, NS), indicating a general increase in vasomotor sympathetic drive, and consistent with the increase in renal SNA. Taken together, these observations suggest that the increased sympathetic outflow in LVV-shRNA/nNOS rats is global.

Immunohistochemical confirmation of vector expression.

Figure 4 shows immunohistochemical validation of nNOS knock-down, with LVV-shRNA/nNOS expression associated with a ~50% reduction in nNOS expression

compared to the contralateral PVN that was devoid of viruses mediating nNOS knockdown; this was consistent in all rats studied.

Discussion

The data reported herein demonstrate for the first time that LVV-mediated long term knock down of nNOS activity targeted to the PVN produces sustained increases in both renal sympathetic drive and arterial pressure in healthy conscious rats. Our observation of a 70% increase in renal SNA and a shift towards sympathetic predominance in the autonomic balance in LVV-shRNA/nNOS transfected rats suggests that the hypertension in these animals is driven at least partially by sympathetic actions on the kidney and heart. These results have important implications for interpreting findings by others that nitric oxide levels in the PVN are depressed in cardiovascular disease, and supports the hypothesis that chronically reduced (endogenous) nitric oxide signalling in the PVN can cause sustained sympathetic hyperactivity (Huang *et al.*, 2007; Schultz, 2009).

Consideration of the methodology employed

The present study used lentiviral shRNA to knock-down endogenous nNOS in the PVN that revealed a long term role for this protein in regulating the circulation. We have previously shown, using western blot analysis, that this binary system reduces nNOS protein expression in cell lines *in vitro* by up to 82% and *in vivo* in dorsomedial medullary neurons by ~55%, while the control construct caused no measurable effects (Montero *et al.*, 2010; Liu *et al.*, 2011). The latter is consistent with the nNOS knockdown we found in the PVN herein.

In the *in vivo* animal, it is unknown how long expression takes to reach a functionally effective reduction in nNOS protein. The use of a viral vector approach, as opposed to a knockout mouse, gives the benefit of allowing a highly restricted circumscribed brain region to be targeted. In comparison to over-expression of nNOS, the use of knock down is advantageous because only neurones that endogenously express nNOS will be affected; in contrast to the situation when a newly expressed exogenous protein may appear in uncontrolled concentrations as well as in cells which normally do not produce it (Li *et al.*, 2001; Zheng *et al.*, 2011). Moreover, the use of LVV permits relatively long term (up to several months) of stable expression (Coleman *et al.*, 2003).

In addition, LVV are essentially non-immunogenic in the CNS. This may be important because pro-inflamatory properties of previously used adenoviral vectors (Liu & Muruve, 2003) could lead to up-regulation of nNOS, complicating interpretation of data. While a viral vector strategy to knock down nNOS limits compensation, we cannot rule out compensatory changes, perhaps in the form of up regulation of eNOS and/or iNOS, which can also occur after surgical trauma (Petrov & Rafols, 2001). However, any such changes would be expected to oppose a reduction in nitric oxide, and thus reduce the observed pressor and sympathoexcitatory responses. If this has occurred, then the magnitude of the rise in arterial pressure and renal SNA will have been underestimated.

It might be expected that multiple intrinsic physiological systems would oppose any increase in arterial pressure such as that we have observed. The fact that nNOS knock-down in the PVN of a normal adult Wistar rat is able to produce such a dramatic *and* sustained increase in renal SNA, with a moderate increase in arterial pressure, supports the notion of the PVN as a 'central command' centre for the sympathetic outflow (Dampney *et al.*, 2005). Although we did not detect any changes in the spontaneous cardiac baroreceptor reflex gain, we speculate that the baroreflex control of sympathetic outflow to the arterioles, including those in the kidney, is re-set to permit adjustment of the set-point of vasomotor tone, and hence arterial pressure. Our data is compatible with the notion that the PVN is a powerful and important regulator of the long-term level of sympathetic outflow in health.

nNOS activity in the PVN: a major regulator of physiological levels of autonomic activity

Mechanistically, it has been shown that nitric oxide inhibits the firing of PVN neurons projecting to autonomic centres in the medulla oblongata, such as the rostral ventrolateral medulla, and spinal cord (Li *et al.*, 2002a; Li *et al.*, 2003). Sutton *et al* have published findings showing that as well as directly projecting to the NTS, nNOS-positive PVN neurons also directly innervate pre-ganglionic sympathetic output neurons in the thoracic spinal cord (Sutton *et al.*, 2014). This suggests that nNOS-dependent alterations in PVN outflow may influence sympathetic tone via this direct pathway. Alternatively, substantial neuroanatomical and functional evidence supports

nNOS-released nitric oxide driving an inhibitory (GABA$_A$-ergic) system regulating PVN output activity affecting sympathetic tone and arterial pressure (Li *et al.*, 2003; Dampney *et al.*, 2005; Pyner, 2009; Patel & Zheng, 2012). In rats with heart failure, the inhibitory effects of nitric oxide in the PVN on sympathetic nerve discharge appear to be impaired (Sharma *et al.*, 2011) and this is associated with both a down-regulation of nNOS via an AT1 receptor mediated mechanism (Sharma *et al.*, 2013) and a loss of GABAergic inhibition (Zhang *et al.*, 2002). In contrast, ectopic nNOS expression in the PVN of heart failure rats has been shown to reduce the glutamatergic drive in this region (Zhang *et al.*, 2001; Zheng *et al.*, 2011).

It has been suggested that nitric oxide acts via excitation of GABAergic interneurones impinging onto PVN sympathetic pre-motor neurons (Biancardi *et al.*, 2011) thereby acting as a 'brake' on sympathetic outflow (Zhang *et al.*, 1997; Hirooka *et al.*, 2011). This is supported by immunohistochemical investigations showing that although spinally-projecting PVN neurons rarely contain either GABA or nNOS, they are surrounded by dense populations of interneurons which show positive staining for GABA or nNOS, with co-localization being rare (Watkins *et al.*, 2009). A somewhat similar situation exists in the NTS where nitric oxide was shown to potentiate GABA release via cADP-rybose pathway and sensitisation of ryanodine-sensitive stores (Wang *et al.*, 2006b). We have previously shown that nitric oxide potentiates GABA release via cGMP-mediated sensitisation of intracellular Ca^{2+} stores (Wang *et al.*, 2006a; Wang *et al.*, 2006b) while confirming that nitric oxide within the PVN is important for determining the long-term level of sympathetic outflow, our results do not determine whether this occurs via direct projections, or changes in local GABAergic inhibition.

In contrast to the present study, Ramchandra et al. (2013) injected muscimol or glycine into the PVN of conscious sheep and reported no change in blood pressure

or renal sympathetic nerve activity, suggesting that there was no tonic sympathoexcitatory drive from the PVN regulating arterial pressure. Species differences aside, this pan-neuronal approach could be explained by simultaneous inactivation of opposing excitatory and inhibitory drives regulating sympathetic activity with a nulling out of a response on blood pressure. In contrast, our lentiviral approach used an amplified synapsin promoter that will only affect neurones (not astrocytes) and, importantly, only affects those neurones in which nNOS is active _constituitively_. Our findings support the presence of constituitively active nNOS affecting preferentially an inhibitory system. The presence of GABA- and nNOS-containing interneurons in the PVN (Watkins _et al._, 2009) and their close apposition provides the neuro-anatomical substrate for discrete cross-talk allowing selective activation of an inhibitory synapse on to excitatory PVN sympathoexcitatory neurones.

Our results did not show a change in cardiac baroreflex gain or heart rate variability, as has been previously observed with global, chronic changes to PVN neuronal activity (Geraldes _et al._, 2014). This suggests that nitric oxide regulation of PVN activity may not be critical for cardiac parasympathetic activity and baroreceptor reflex control of heart rate. However, our data do support that nNOS activity in the PVN is an important factor regulating sympathetic activity to the heart, as indicated by alterations in the HF:LF ratio of the heart rate spectra, as well as vasomotor sympathetic control. We acknowledge that this will need further confirmation, but our data lead us to propose that nitric oxide in the PVN predominantly modulates sympathetic rather than parasympathetic outflows.

The Regulation of nNOS Activity in Health and Disease:

The factors which drive the normal expression and activity of nNOS have not been fully elucidated. Afferent connections from the nucleus tractus solitarii have been shown to impinge on GABAergic and nitric oxide-producing interneurons in the PVN (Affleck *et al.*, 2012), which supports a role for homeostatic reflex activity (e.g. baroreceptor, peripheral chemoreceptor, Bainbridge reflexes) in the control of sympathetic outflow via PVN-nNOS pathways. At a cellular level, nNOS expression has been shown to be regulated by the inducible transcription factor c-jun,(Cheng *et al.*, 2012; Cheng *et al.*, 2014) implying that nNOS expression may be responsive to stimuli such as stress, infection and inflammation.

In disease states, nitric oxide levels are typically depleted by oxidative stress or NOS uncoupling (Moens *et al.*, 2008; Antoniades *et al.*, 2009; Crabtree & Channon, 2011) both of which are known to occur in the brain under conditions of hypertension (Zimmerman *et al.*, 2004; Oliveira-Sales *et al.*, 2010; Hirooka *et al.*, 2011; Francis & Davisson, 2014) and heart failure (Zucker, 2006; Patel & Schultz, 2013). Together with the present data, these studies implicate insufficient nNOS signalling in the PVN as a potential causal mediator of sympathetic excess, which could underpin a plethora of disease states.

In conclusion, our data fully support the notion that nNOS-generated nitric oxide in the PVN of Wistar rats is acting as a physiological 'brake' on sympathetic outflow. Moreover, the tonicity of the inhibitory system in the PVN provides neural plasticity permitting the set-point control of arterial pressure to be adjusted. A future challenge will be to see under what physiological conditions this plasticity is harnessed to maintain homeostasis.

Author contributions

Figure 1
Chronic reductions in Nitric Oxide in the PVN produce sustained sympathoexcitation and hypertension. Group daily averages of mean arterial pressure (MAP) heart rate (HR) and renal SNA responses to nNOS knock-down in the PVN (Microinjection of LVV-shRNA/nNOS on Day 0 indicated by dashed line). Mean±sem, * p<0.05 from Baseline (repeated measures ANOVA, Factors: Time and Group). Note the similar temporal profile of the increase in renal SNA and MAP.

Figure 2
A: Typical individual response to nNOS knock-down (Microinjection of LVV-shRNA/nNOS indicated by a dashed line). 2 hour averages of mean arterial pressure (MAP), heart rate (HR) and renal SNA shown, and the MAP and HR responses on days 50-55. **B: Representative high-frequency recordings before and after nNOS knock-down**. Arterial pressure (AP), renal SNA integrated (RSNAi) and original (RSNAo) during taken while the rat was quiescent at baseline (Day -2) and peak response (Day 20). **C: Confirmation of renal SNA recording viability over time**. This figure shows the cardiac association (integrated renal SNA signal overlaid and averaged over successive systolic beats), and the response to nasopharyngeal activation with a brief exposure to cigarette smoke (insets, smoke exposure indicated by grey arrow) of the renal SNA recordings 1 days before and 15 days after LVV-nNOS microinjection.

Figure 3
Group data showing cardiovascular and sympathetic responses to nNOS knock-down in the PVN. Mean arterial pressure (MAP), renal SNA, low frequency to high frequency ratio of R-R interval (LF:HF) and spontaneous baroreflex gain (sBRG) during baseline (Days -3 to -1) and nNOS knock-down (Day 15-18). # indicates p<0.05, nNOS vs. Control. **Note**: the LF:HF ratio is increased in nNOS vs. control rats indicating that cardiac sympatho-vagal tone may be decreased.

Figure 4
Demonstrating efficacy of nNOS knockdown in the PVN using lentiviral mediated shRNA. *Top and middle:* Immuno-histologically stained nNOS (red) in a brain section at Bregma -2.0mm showing how an ipsilateral injection (left side) of nNOS knockdown virus (green; LV-Tretight-eGFP-miR30-shRNA/nNOS) into the PVN reduced nNOS expression. Note that the occurrence of eGFP corresponds with a diminution of nNOS immunofluorescence (compare regions c with d), whereas the more lateral regions of the PVN (e.g. a & f) are relatively unaffected. *Bottom:* Quantitative assessment of nNOS knockdown. This was computed using Fiji binary representations particle analysis of eGFP and nNOS to determine the % of each region (a-f) of eGFP versus nNOS. A schematic representation of the 3rd ventricle is overlaid in blue.
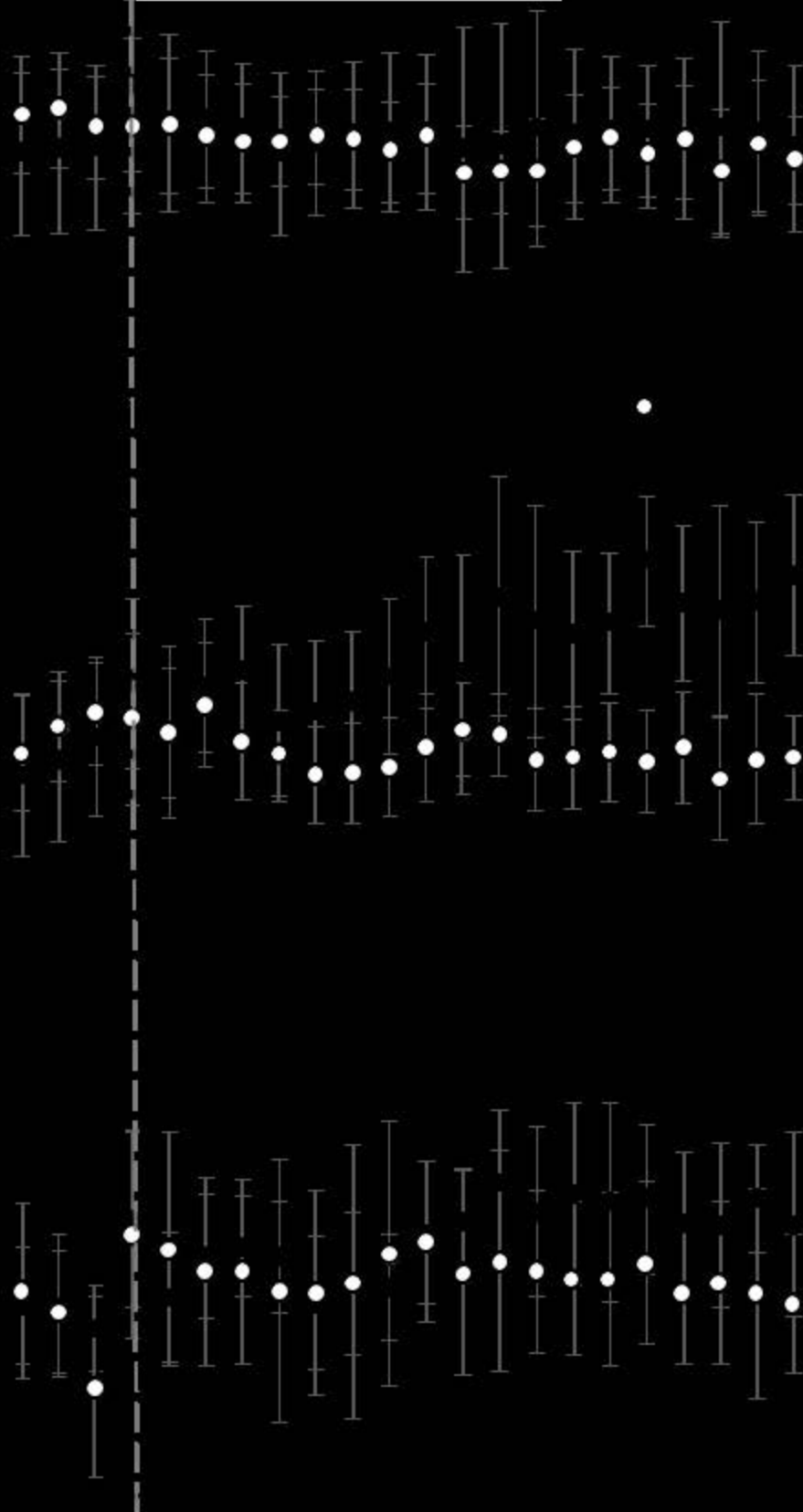
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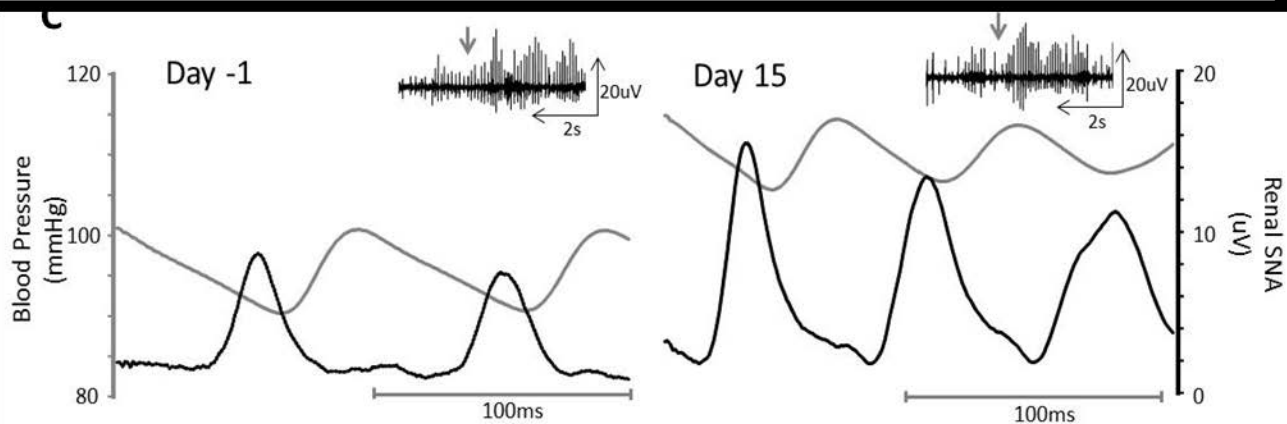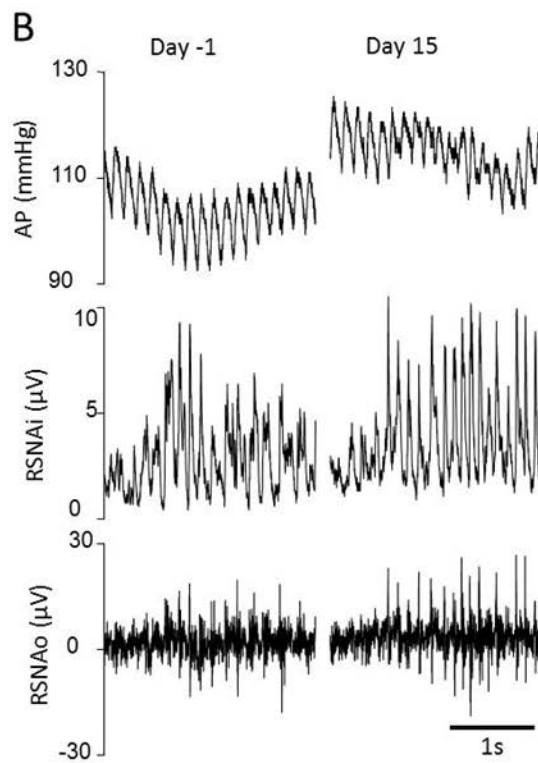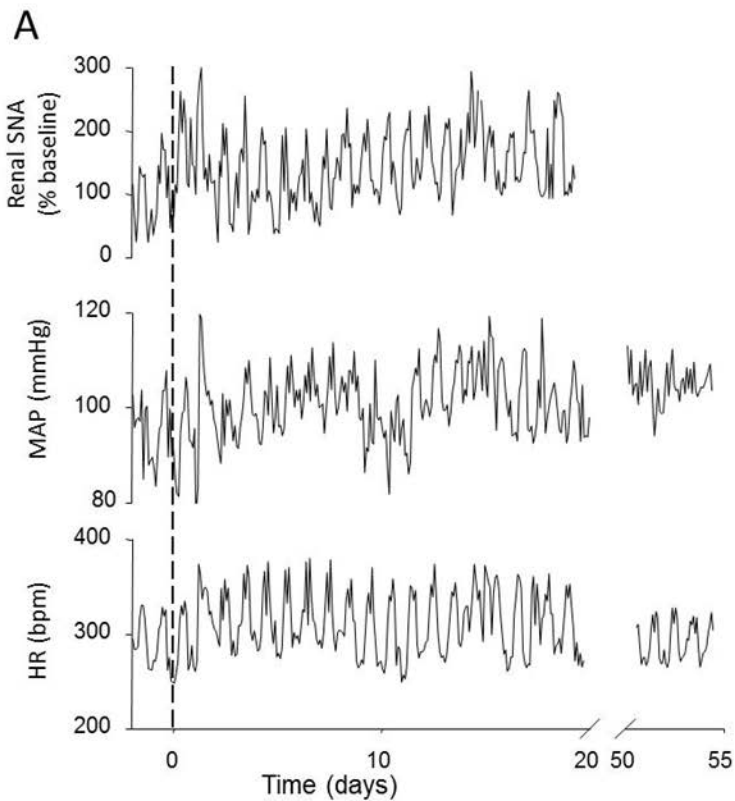
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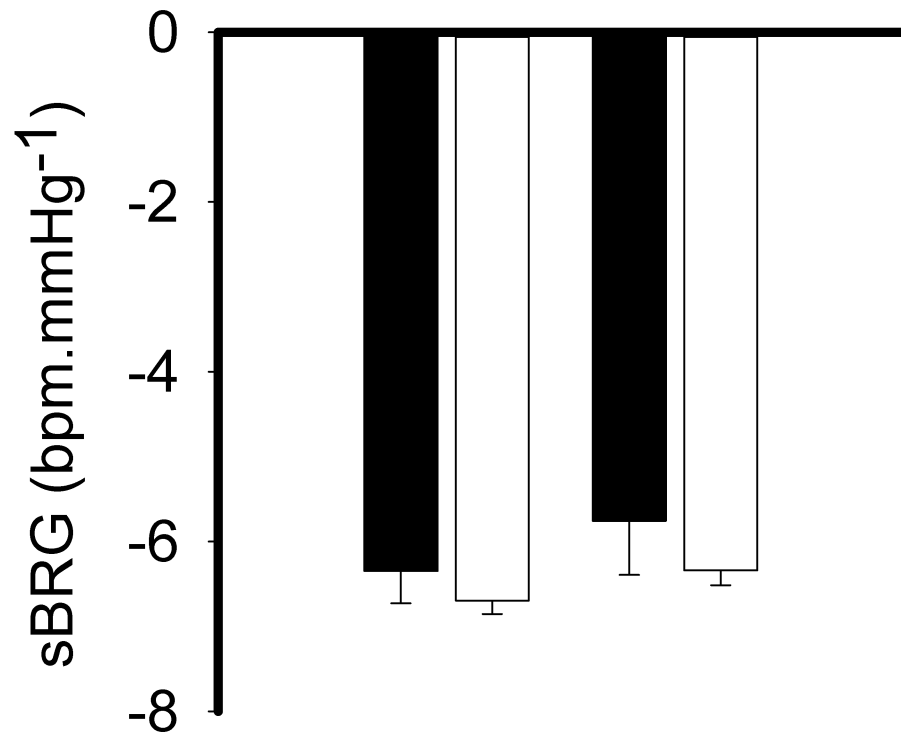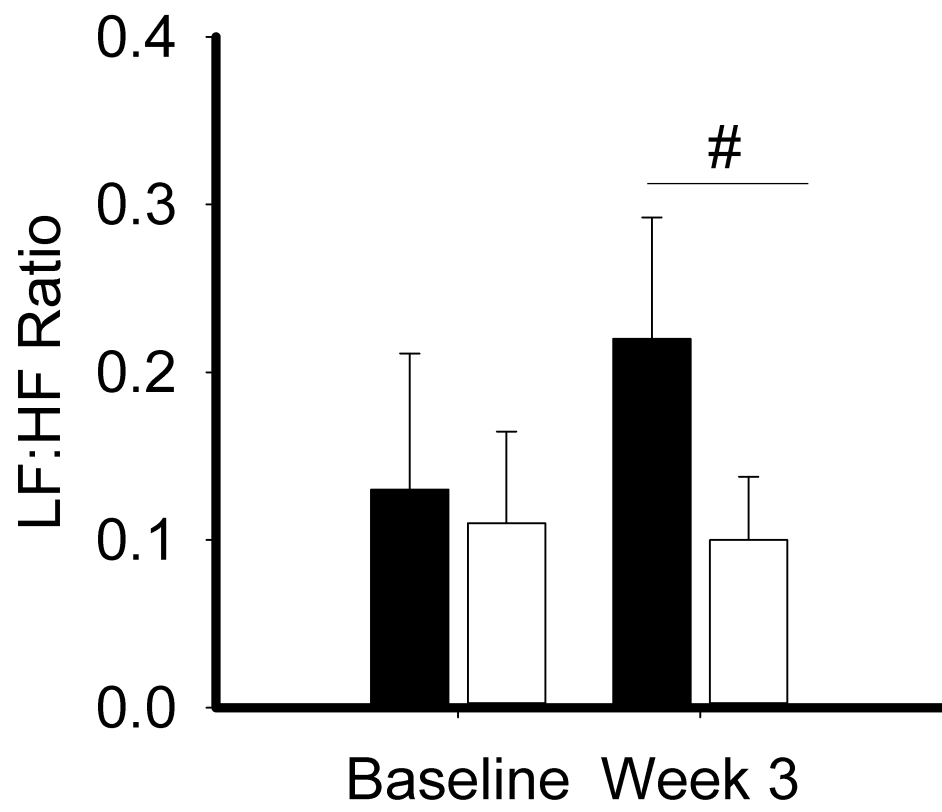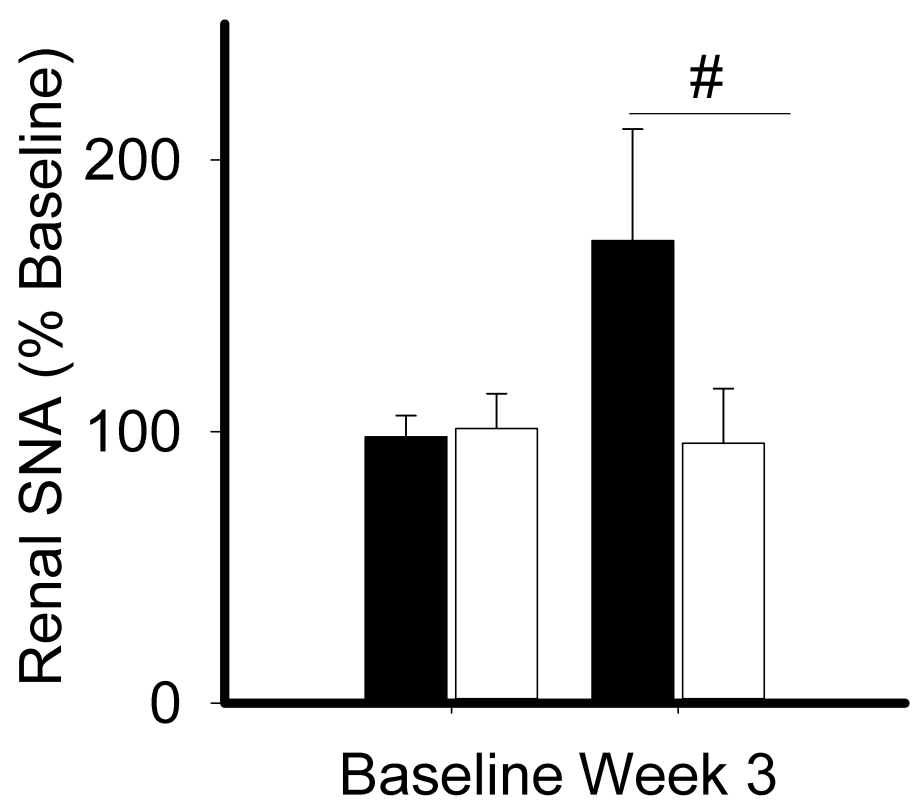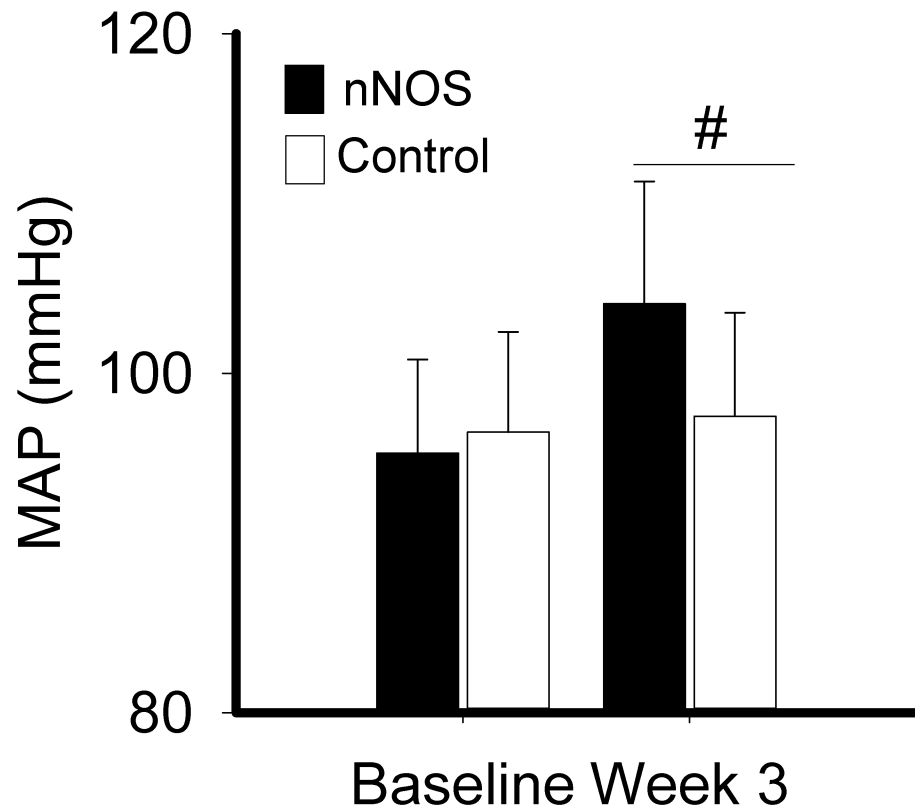
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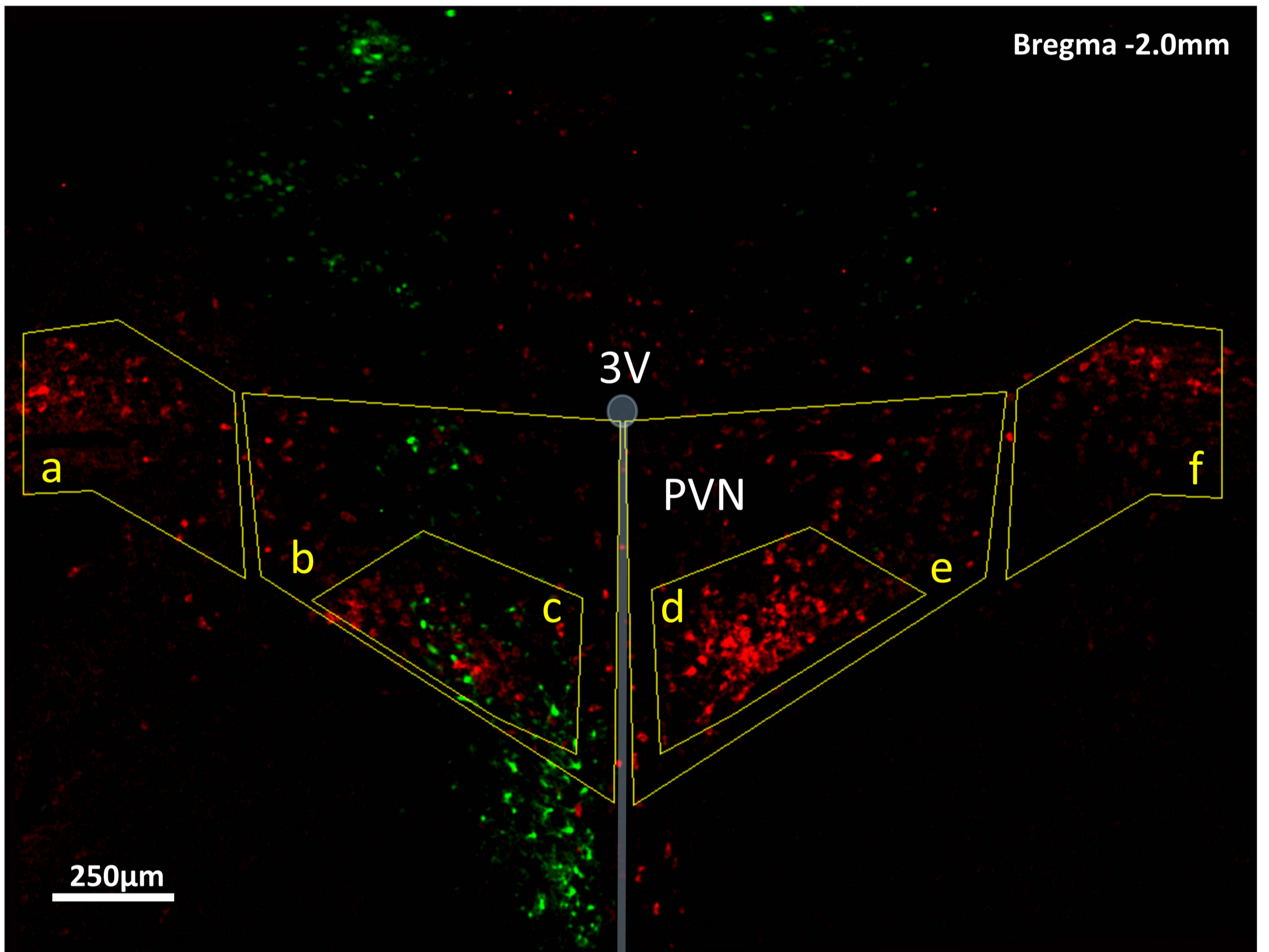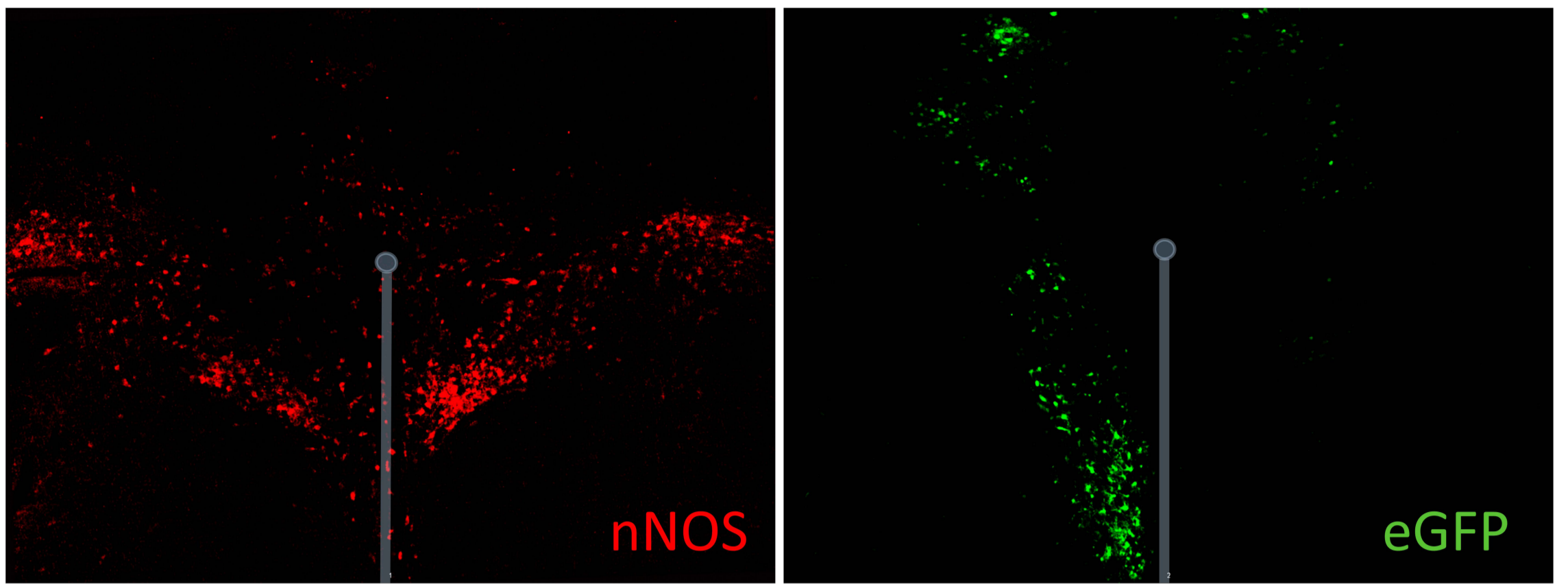
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Anti nNOS LVV Injection

nNOS

eGFP

Bregma -2.0mm

3V

PVN

a

b

c

d

e

f

250µm

Percent of selected area

virus positive cells

nNOS positive cells

%

PVN regions