Journal of Molecular and Cellular Cardiology 52 (2012) 667–676

Contents lists available at SciVerse ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

The cardiac sympathetic co-transmitter galanin reduces acetylcholine release and vagal bradycardia: Implications for neural control of cardiac excitability

Neil Herring [a,*], James Cranley [a], Michael N. Lokale [a], Dan Li [a], Julia Shanks [a], Eric N. Alston [b], Beatrice M. Girard [c], Emma Carter [a], Rodney L. Parsons [c], Beth A. Habecker [b], David J. Paterson [a]

[a] Burdon Sanderson Cardiac Science Centre, Department of Physiology, Anatomy and Genetics, BHF Centre of Research Excellence, University of Oxford, Parks Road, OX1 3PT, UK
[b] Department of Physiology and Pharmacology, Oregon Health & Science University, Portland, OR, USA
[c] Dept. of Anatomy and Neurobiology, University of Vermont College of Medicine, Burlington, VT, USA

ARTICLE INFO

Article history:
Received 8 July 2011
Received in revised form 6 November 2011
Accepted 28 November 2011
Available online 7 December 2011

Keywords:
Autonomic nervous system
Sympathetic
Vagus
Co-transmitters
Acetylcholine
Heart rate

ABSTRACT

The autonomic phenotype of congestive cardiac failure is characterised by high sympathetic drive and impaired vagal tone, which are independent predictors of mortality. We hypothesize that impaired bradycardia to peripheral vagal stimulation following high-level sympathetic drive is due to sympatho-vagal crosstalk by the adrenergic co-transmitters galanin and neuropeptide-Y (NPY). Moreover we hypothesize that galanin acts similarly to NPY by reducing vagal acetylcholine release via a receptor mediated, protein kinase-dependent pathway. Prolonged right stellate ganglion stimulation (10 Hz, 2 min, in the presence of 10 μM metoprolol) in an isolated guinea pig atrial preparation with dual autonomic innervation leads to a significant ($p<0.05$) reduction in the magnitude of vagal bradycardia (5 Hz) maintained over the subsequent 20 min ($n=6$). Immunohistochemistry demonstrated the presence of galanin in a small number of tyrosine hydroxylase positive neurons from freshly dissected stellate ganglion tissue sections. Following 3 days of tissue culture however, most stellate neurons expressed galanin. Stellate stimulation caused the release of low levels of galanin and significantly higher levels of NPY into the surrounding perfusate ($n=6$, using ELISA). The reduction in vagal bradycardia post sympathetic stimulation was partially reversed by the galanin receptor antagonist M40 after 10 min (1 μM, $n=5$), and completely reversed with the NPY Y_2 receptor antagonist BIIE 0246 at all time points (1 μM, $n=6$). Exogenous galanin ($n=6$, 50–500 nM) also reduced the heart rate response to vagal stimulation but had no effect on the response to carbamylcholine that produced similar degrees of bradycardia ($n=6$). Galanin (500 nM) also significantly attenuated the release of ^{3}H-acetylcholine from isolated atria during field stimulation (5 Hz, $n=5$). The effect of galanin on vagal bradycardia could be abolished by the galanin receptor antagonist M40 ($n=5$). Importantly the $GalR_1$ receptor was immunofluorescently co-localised with choline acetyl-transferase containing neurons at the sinoatrial node. The protein kinase C inhibitor calphostin (100 nM, $n=6$) abolished the effect of galanin on vagal bradycardia whilst the protein kinase A inhibitor H89 (500 nM, $n=6$) had no effect. These results demonstrate that prolonged sympathetic activation releases the slowly diffusing adrenergic co-transmitter galanin in addition to NPY, and that this contributes to the attenuation in vagal bradycardia via a reduction in acetylcholine release. This effect is mediated by $GalR_1$ receptors on vagal neurons coupled to protein kinase C dependent signalling pathways. The role of galanin may become more important following an acute injury response where galanin expression is increased.

1. Introduction

Sympathetic neurons throughout the autonomic nervous system contain co-transmitters such as ATP, neuropeptide-Y and galanin, in addition to the main neurotransmitter norepineprhine [1–3]. The release of co-transmitters is highly dependent on the level of neuronal stimulation, and they tend to be slowly diffusing molecules that often function as neuromodulators rather than classical neurotransmitters [3]. High-level cardiac sympathetic stimulation in the presence of beta-adrenergic blockade is associated with reduced chronotropic responses to peripheral stimulation of the right cardiac vagus nerve [4,5]. One possibility is that sympathetic co-transmitters are responsible for this phenomenon by acting locally within the heart at the site of cholinergic neurons to reduce acetylcholine release, and thus contribute to a potentially pro-arrhythmic shift in autonomic balance [6]. High cardiac sympathetic drive with reduced vagal tone is the characteristic autonomic phenotype associated with myocardial infarction and congestive heart failure and is a poor prognostic indicator (e.g. [7–10]). Interestingly, elevated

* Corresponding author. Tel.: +44 1865 272518.
E-mail address: neilherring@doctors.org.uk (N. Herring).

0022-2828
doi:10.1016/j.yjmcc.2011.11.016

plasma neuropeptide-Y has been demonstrated during both of these conditions where levels also correlate with mortality [11–13].

Whilst others have shown that adrenergic or purinergic receptor stimulation is unable to alter cardiac acetylcholine release in humans [14] or guinea pigs [15,16], we have recently reported direct evidence showing how neuropeptide-Y inhibits cardiac acetylcholine release and vagal bradycardia via Y_2 receptors on cholinergic neurons which couple to protein kinase C dependent signalling pathways [17]. Moreover, the Y_2 receptor antagonist BIIE 0246 also partially reverses impaired heart rate responsiveness by the cardiac vagus after prolonged sympathetic stimulation in-vivo [in the mouse [18], and dog [19]] during which neuropeptide-Y release has been directly measured [in the dog [20]]. Genetic knockout of the Y_2 receptor has similar effects to BIIE 0246 [21].

The neuropeptide co-transmitter galanin is known to be involved in the central regulation of the cardiovascular system at the level of the brainstem and, along with related peptides, may also act as a vasoactive substance in the vasculature (see [22] for a review). Interestingly, knockout of the galanin $GalR_1$ receptor also partially prevents the impaired vagal bradycardia post peripheral sympathetic stimulation in the mouse [4,18]. The cardiac release of galanin in these conditions has not been demonstrated however, and the mechanism behind the effect of $GalR_1$ gene knockout is unclear. We therefore hypothesized that galanin is expressed in tyrosine hydroxylase positive neurons of the stellate ganglion and is released upon high frequency sympathetic stimulation in an isolated guinea pig sinoatrial node preparation with intact right stellate and vagal innervation. Moreover, we hypothesize that galanin receptors are located on cholinergic neurons that innervate the sinoatrial node, and that endogenously released or exogenously applied galanin reduces vagal bradycardia via a $GalR_1$ receptor mediated pathway. We also investigated if galanin acts similarly to neuropeptide-Y by inhibiting acetylcholine release via a protein kinase dependent pathway.

2. Methods

Experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and the Animals (Scientific Procedures) Act 1986 (UK). Adult (300–400 g, n = 90) female guinea pigs (*Cavia porcellus*, Dunkin Hartley) were killed by cervical dislocation and exsanguinated according to Schedule 1. Experiments were performed under British Home Office Project License PPL 30/2130 and PPL 30/2630.

2.1. Immunohistochemistry

The midbrain, right atria and stellate ganglia were dissected free (n = 6 animals) and placed in separate vials containing fresh fixative (2% paraformaldehyde containing 0.2% picric acid) over night and then placed in 0.1 M phosphate-buffered saline (PBS) containing 20% sucrose for cryoprotection until sectioned. Tissue sections (20 μm stellate, 40 μm right atria and guinea pig midbrain) were processed by direct mounting techniques as described previously [23,24]. In a separate set of experiments stellate neurons were dissociated and maintained in culture media for 3 days as described in detail previously [25–27] prior to a 1 hour exposure to fixative (2% paraformaldehyde containing 0.2% picric acid). The fixed tissue sections or dissociated cells were rinsed in PBS, permeabilized with 0.5% Triton X-100, and incubated at 4 °C overnight with a primary antiserum directed against tyrosine hydroxylase (TH) (mouse monoclonal 1:400 from RBI, Natick MA) and a primary antiserum against galanin (rabbit polyclonal 1:1000 from Phoenix Pharmaceuticals, Burlingame CA). Following removal of the primary antisera, the ganglion sections/dissociated cells were incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)-conjugated or indocarbocyanine (Cy3)-conjugated secondary antiserum (Jackson Immunoresearch Laboratories, west Grove, PA, 1:100 and 1:500 respectively).

The whole-mount stellate preparations and dissociated neurons were viewed with an Olympus AX70 fluorescence microscope equipped with HBO 100-W UV light source and filters for FITC and Cy3. Digital images were obtained with a CCD camera (MagnaFire SP; Optronics; Optical Analysis Corp., Nashua, NH) and imported into Adobe Photoshop CS3 (Adobe Corporation, Mountain View, CA) to assemble figures, which were minimally adjusted for contrast and brightness. Both antisera used are well characterised and have been employed in prior studies (e.g. [28]). In addition, neuronal staining was not observed when whole mounts were treated only with secondary antiserum.

Fixed right atrial slices (or guinea pig midbrain as a positive control) were incubated with rabbit anti-$GalR_1$ (Sigma, 1:200), anti-$GalR_2$ (Affinity Bioreagents, 1:150) or anti-$GalR_3$ (Sigma, 1:150) primary antibodies followed by biotinylated goat anti-rabbit (Vector, 1:200). Conjugation was then performed using a fluorescein stain followed by blocking of remaining unbound biotin with a Streptavidin Biotin Blocking Kit. To assess galanin receptor–choline acetyltransferase (ChAT) co-localization, neurons were further labelled with goat anti-ChAT (Vector, 1:200), followed by secondary, biotinylated horse anti-goat (Vector, 1:200). Conjugation was then performed using a Texas Red stain. Images were obtained using a Nikon Eclipse TE2000-U inversion fluorescent light microscope and appropriate filters (560/40 nm Texas Red and 460–500 nm fluorescein filters). Neuronal staining was not observed when whole mounts were treated only with secondary antiserum.

2.2. Reverse transcription-polymerase chain reaction

Stellate ganglia, cardiac ganglia and brain were dissected (n = 3 animals) under RNase-free conditions and total RNA was extracted from individual preparations using Tri reagent (Sigma) as described previously [29]. The total RNA quantity for each whole-mount preparation was determined with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). One microgram of RNA per sample was used to synthesize complementary DNA with the Omniscript reverse transcription (Qiagen, Valencia, CA) and a mix of oligo-dT and random hexamer primers. Amplified MPG DNA product from specific primers was ligated into pCR2.1 TOPO using TOPO TA cloning kit (Invitrogen, Carlsbad, CA) to generate plasmid standards. The nucleotide sequences of the inserts were verified by automated fluorescent dideoxy dye terminator sequencing (Vermont Cancer Center DNA Analysis Facility), and ten-fold serial dilutions of stock plasmid were prepared to generate assay standard curves. Amplification of the cDNA templates and plasmid standards was performed using HotStart IT® SYBR® Green qPCR Master Mix (USB). Real-time quantitative PCR was performed on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Norwalk, CT).

The primers (designed using the Ensembl sequence) and conditions used were: Galanin: (+)TCCTGCTCCTCTCGCTCC, (−)CTTCTCCTTTGCCGGGAA; 53 °C for 30 s; $GALR_1$: (+)ACTTTATCACGCTGGTGGTGTTTG, (−)ACCGTGAAGACATAGTGGGTGAA; 60 °C for 30 s; $GALR_2$: (+)ACTTCCTCATCTTCCTCACCA, (−)TGCCAGCGCGTTTCGAG; 54 °C for 30 s; $GALR_3$: (+)AGGGAGTGTGGGGGCTGTG, (−)AAGAGCCAGGCATCCAGCGT; 62 °C for 30 s.

2.3. Isolated sinoatrial node/right stellate ganglion/right vagus nerve preparation

The spontaneously beating atria with intact right stellate ganglion and right vagus nerve were isolated (n = 70 animals) and transferred to a preheated (37 ± 0.2 °C), continuously oxygenated, water-jacketed organ bath containing 60 ml Tyrode solution. The method for dissecting and measuring responses to nerve stimulation has been described previously [17]. Before starting each protocol, the mounted atria were allowed to equilibrate for 80 min until beating rate stabilized (±5

beats per minutes, bpm, over 20 min). The Tyrode's solution in the organ bath was replaced approximately every 30 min throughout each protocol. The vagus was stimulated at 1, 3, and 5 Hz, (20 V, 1 ms pulse duration for 30 s) before and after high level stimulation of the right stellate ganglion via ring electrodes placed around the ganglion itself (at 10 Hz, 20 V, 1 ms pulse duration for 2 min) and/or application of drugs to the organ bath. This level of sympathetic stimulation was chosen as it has previously been shown to reduce the heart rate response to peripheral vagal stimulation in-vivo and cause release of neuropeptide-Y [5,20]. These frequencies were chosen to produce physiological degrees of bradycardia from the baseline heart rate of up to 30–40%. A control experiment showed that the heart rate response to vagal nerve stimulation remained constant (±1 bpm at 5 Hz) over a two-hour period. We have previously shown that all heart rate changes from vagal nerve stimulation are completely abolished by hyoscine in this preparation and are therefore due to release of acetylcholine [30].

2.4. Measuring galanin and neuropeptide-Y release to sympathetic stimulation from isolated guinea pig right atrial preparations

The spontaneously beating right atrium was isolated (n = 6 animals) and transferred to a preheated (37 ± 0.2 °C), continuously oxygenated, water-jacketed organ bath containing 2 ml Tyrode solution where the atrium was pinned flat on a silver stimulating electrode. After an equilibration period of 30 min, the Tyrode solution was changed and the stellate stimulated at 10 Hz (20 V, 1 ms pulse duration for 2 min). 50 μl samples of perfusate were taken from the bath 5, 10, 15 and 20 min before and after sympathetic stimulation and neuropeptide-Y or galanin concentrations measured using commercially available ELISA based assays (S1346 neuropeptide-Y, and S1347 galanin, Penninsular Laboratories, Bachem) against serially diluted protein standards.

2.5. Measuring ^{3}H-acetylcholine release to field stimulation from isolated guinea pig right atrial preparations

The spontaneously beating right atrium was isolated (n = 5 animals) and transferred to a preheated (37 ± 0.2 °C), continuously oxygenated, water-jacketed organ bath containing 3 ml Tyrode solution where the atrium was pinned flat on a silver stimulating electrode. The method for determining the local release of ^{3}H-acetylcholine to field stimulation at 5 Hz (20 V, 1 ms pulse width, for 1 min) after incubation with ^{3}H-choline chloride (10 μCi, Amersham U.K.) and washing, was identical to that which we have previously described [17].

2.6. Solutions and drugs

The Tyrode solution contained (mM) NaCl 120, KCl 4, $MgCl_2$ 2, $NaHCO_3$ 25, $CaCl_2$ 2, Na_2HPO_4 0.1 and glucose 11. The solution was aerated with 95% O_2/5% CO_2 (pH 7.4) and its temperature was continuously monitored (Digitron 1408-K gauge) and kept at 37 ± 0.2 °C.

The effect of prolonged, high frequency right stellate stimulation was compared to that of bath applied ephrine (10 μM, Sigma) at a dose that produced a similar degree of tachycardia with or without beta-adrenergic blockade using metoprolol (10 μM, Sigma). Galanin (1–29, Bachem) was used in concentrations of up to 500 nM. The effect of sympathetic nerve stimulation or galanin on the heart rate response to vagal nerve stimulation was compared to those of the stable analogue of acetylcholine carbamylcholine (90 nM, Sigma) to determine whether the effects were pre or postsynaptic. This concentration was chosen to produce similar degrees of bradycardia to 5 Hz vagal nerve stimulation. M40 (1 μM, Tocris) was used as a selective galanin receptor antagonist and BIIE 0246 (1 μM, Tocris) as a selective antagonist of the Y_2 receptor. Protein kinase A was inhibited using H89 (0.5 μM, Calbiochem) and protein kinase C using Calphostin C (0.1 μM, Calbiochem). The concentrations chosen are above the reported Ki for the isolated enzymes (0.007 μM M40 for the $GalR_1$ receptor [31], 0.02 μM BIIE 0246 for the Y_2 receptor [32], 0.05 μM Calphostin C for PKC [33], and 0.05 μM H-89 for PKA [34]) but below those reported for non-specific actions of the drugs. They are the same as those previously used in similar preparations (e.g. [35] for M40, [17] for BIIE 0246, H89, and Calphostin C).

Drugs were dissolved in reagent grade water from an Elga purification system except Calphostin C, and H-89 that were dissolved in Dimethylsulfoxide (DMSO). A control experiment showed that DMSO at the concentrations used did not affect the heart rate response to vagal nerve stimulation at 1, 3 or 5 Hz.

2.7. Statistical analysis

Data are presented as mean ± S.E.M, and all data passed a normality test. The "n" number refers to the number of separate animals used and only one protocol was carried out on any preparation from a particular animal. One-way repeated measures ANOVA was used to evaluate the effect of an intervention. An unpaired student's *t*-test assuming unequal variance was used to evaluate significance between two independent experimental groups. Statistical significance was accepted at $p<0.05$ and was not affected by analyzing the data in terms of heart rate changes or change in pulse interval. Analyzing the data from the ^{3}H-acetylcholine release experiments in terms of the absolute increase in ^{3}H efflux to field stimulations S1 and S2, or the ratio between the two responses (S2/S1) did not change statistical significance.

3. Results

3.1. Galanin expression in stellate ganglia and galanin receptors on right atrial cholinergic neurons

RT-PCR from right and left stellate ganglia confirmed the presence of low levels of galanin mRNA. Immunohistochemistry of freshly fixed right and left stellate ganglia from 2 animals showed galanin staining in a small proportion (<5%) of tyrosine hydroxylase positive neurons (Fig. 1B). In these cells the fluorescence was brighter than background and granular in appearance. However, when the stellate neurons were dissociated and kept in culture for 3 days, nearly all the neurons exhibited immunoreactivity for both tyrosine hydroxylase (Fig. 1C left panel) and galanin (Fig. 1C right panel).

RT-PCR from intrinsic cardiac ganglia demonstrated the presence of mRNA for both the $GalR_1$ and $GalR_3$ receptors (Fig. 1D). However, immunohistochemistry showed staining for the $GalR_1$ receptor only in right atrial slices (Fig. 1E) localised in neuronal structures (see magnified inset). Co-staining with ChAT showed $GalR_1$ localised to cholinergic neurons only (Fig. 1F). This provides the anatomical basis for our hypothesis that galanin released from sympathetic neurons may alter vagal control of heart rate.

3.2. Vagal bradycardia post sympathetic stimulation and endogenous galanin and neuropeptide-Y release

After the equilibration period baseline heart rate in isolated sinoatrial node/dual autonomic innervation preparation was 190 ± 3 bpm (n = 70) and did not change significantly with the pharmacological interventions used unless stated. As can be seen in Fig. 2 (black lines), following 2 min of high frequency sympathetic stimulation in the presence of metoprolol, the heart rate response to vagal stimulation significantly reduced over the subsequent 20 min to 71 ± 5% of its starting value (baseline heart rate 187 ± 9 bpm, n = 6). This was not observed when norepinephrine (10 μM) was used rather than sympathetic stimulation where the heart rate response to vagal nerve stimulation remained at 114 ± 12% of its original starting value after 20 min (baseline heart rate: 187 ± 7 bpm, n = 5). In addition, the heart rate response to the stable analogue of acetylcholine, carbamylcholine (90 nM) which produces similar degrees of bradycardia as vagal nerve stimulation at

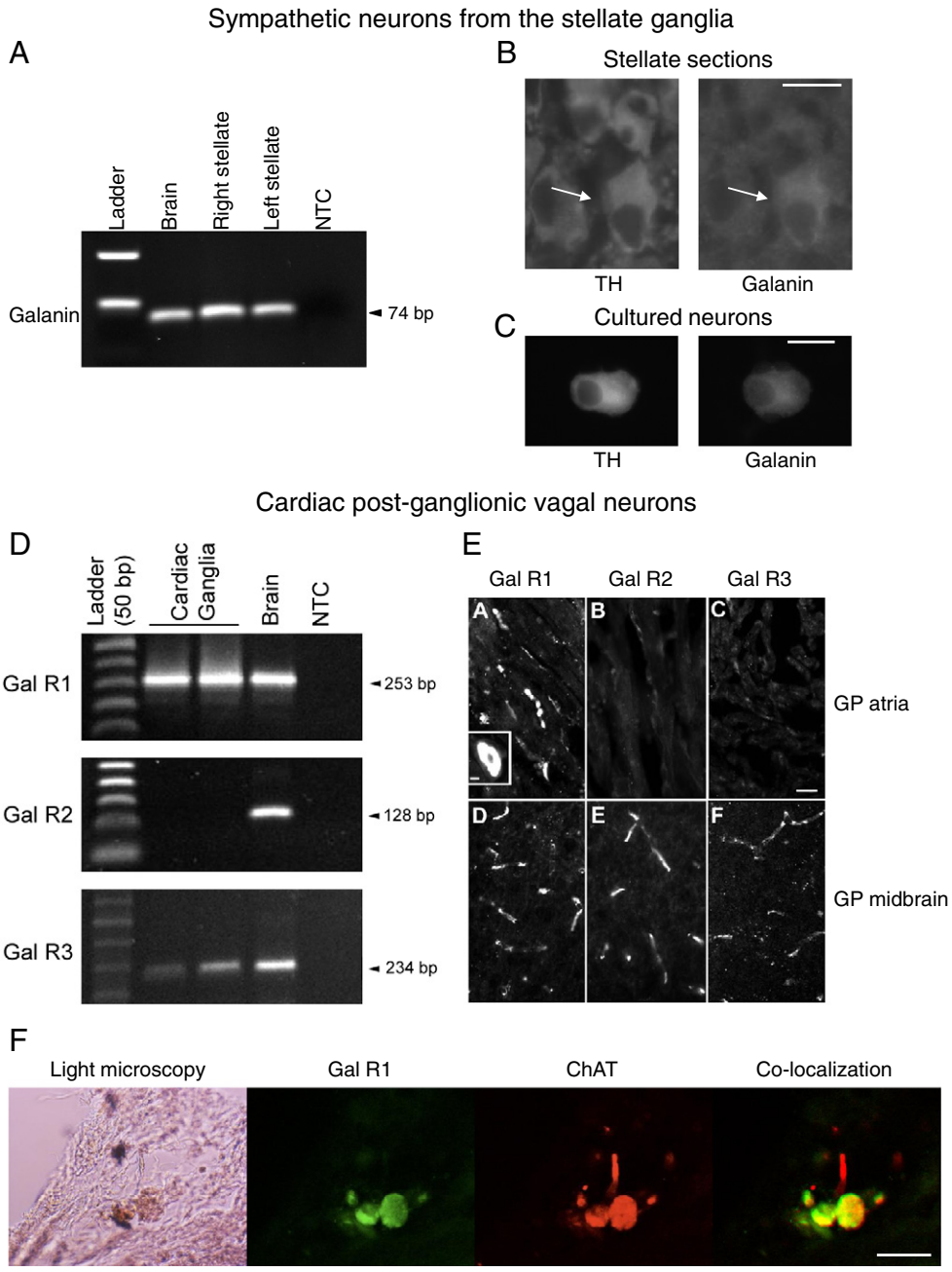

Fig. 1. Expression of galanin in stellate sympathetic neurons and $GalR_1$ on cardiac cholinergic neurons. (A) Reverse transcription-polymerase chain reaction (RT-PCR) products viewed on an ethidium bromide gel after amplification of freshly dissected stellate ganglia and brain mRNA. NTC, no template control, is a negative control assessing the absence of contamination. Galanin mRNA is present in both right and left stellate ganglia. (B) All stellate neurons from freshly dissected left and right stellate ganglia exhibit immunoreactivity for tyrosine hydroxylase (TH, left panel, 20 μm sections) but only occasionally do TH immunoreactive neurons also exhibit galanin immunoreactivity (<5%, right panel, calibration bar = 20 μm). (C) Dissociated stellate neurons cultured for 3-days exhibit immunoreactivity for TH (left hand panel) and galanin (right hand panel, calibration bar = 20 μm). (D) RT-PCR bands for the galanin receptors $GalR_1$ (253 base pairs) and $GalR_3$ (234 base pairs) from mRNA isolated from cardiac ganglia. Guinea pig brain was used as a positive control, and reaction mix without template was the negative control (NTC). (E) Immunohistochemistry of right atrial slices showing expression of $GalR_1$ receptor protein in neuronal structures only (see inset picture at higher magnification, calibration bar = 10 μm). $GalR_1$, $GalR_2$ and $GalR_3$ expression was demonstrated in guinea pig midbrain as a positive control (calibration bar = 20 μm). (F) Immunohistochemistry showing choline acetyltransferase (ChAT) staining with Texas Red in a neuron in right atrial slices also staining positive for the galanin $GalR_1$ receptor in green (fluorescein). Co-localization was demonstrated by overlap of staining in yellow (calibration bar = 20 μm).

5 Hz, was unaffected following high frequency sympathetic nerve stimulation remaining at 97 ± 6% of its starting value after 20 min (baseline heart rate 198 ± 8 bpm, n = 6). In each of these protocols the change in baseline heart rate during sympathetic stimulation or addition of norepinephrine in the presence of metoprolol was similar (sympathetic stimulation following by vagal nerve stimulation +14 ± 4%; norepinephrine followed by vagal nerve stimulation +15 ± 4%; sympathetic nerve stimulation followed by carbamylcholine +16 ± 8%). This suggests that released substances other than norepinephrine are acting pre-synaptically to alter vagal neurotransmission and cause a reduction in vagal bradycardia following high frequency sympathetic nerve stimulation.

To test the hypothesis that such released substances may include the co-transmitters neuropeptide-Y or galanin, the heart rate response to vagal nerve stimulation post high frequency sympathetic stimulation was assessed in the presence of the neuropeptide-Y Y_2

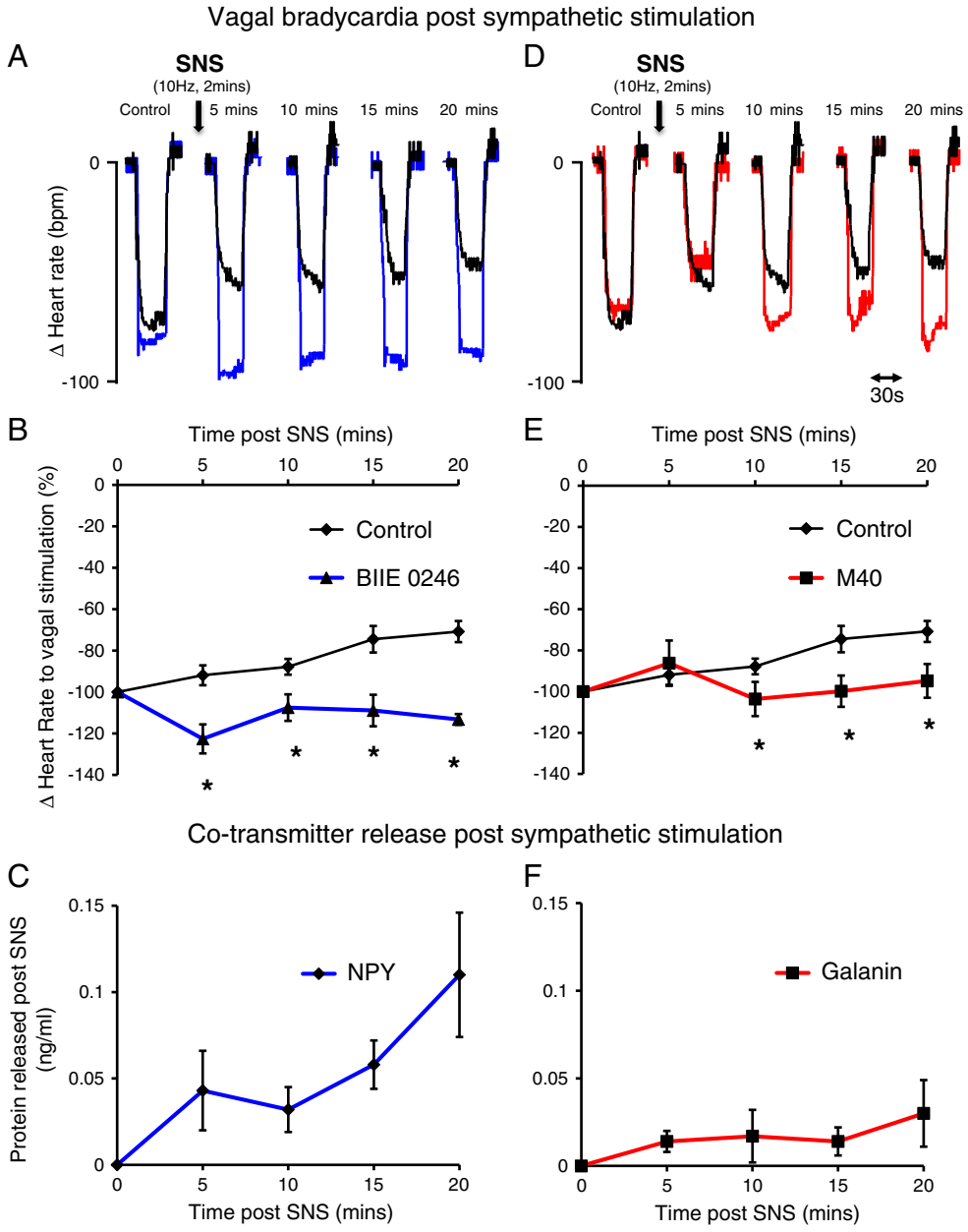

Fig. 2. Release of co-transmitters after high frequency sympathetic stimulation and the corresponding reduction in vagal bradycardia. Representative raw data traces (A and D) and group mean data (B and E) showing the heart rate response to vagal nerve stimulation (5 Hz, 30 s) before and during the 20 minute period after high frequency stimulation of the right stellate ganglion (SNS, 10 Hz, 2 min, in the presence of 10 μM metoprolol). A control experiment is shown in black (baseline heart rate 187 ± 9 bpm, n = 6), whilst in a separate set of experiments shown in blue (A and B), this was repeated in the presence of the neuropeptide-Y receptor antagonist BIIE 0246 (1 μM, baseline heart rate 180 ± 9 bpm, n = 6) and in a further set of experiments shown in red (D and E) this was repeated in the presence of the galanin $GalR_1$ receptor antagonist M40 (1 μM, baseline heart rate 183 ± pre-stimulation was not significantly different to that using sympathetic pre-stimulation in the presence of BIIE 0246 (data not shown). C and F show the organ bath concentrations of neuropeptide-Y (n = 6, blue) and galanin (n = 6, red) before and during the 20 min after high frequency sympathetic nerve stimulation (10 Hz, 2 min in the presence of metoprolol 10 μM) measured in 50 μl organ bath samples analysed using ELISA. (*$p < 0.05$ vs control).

receptor antagonist BIIE 0246, or the galanin receptor antagonist M40. As can be seen in Figs. 2A and B (blue traces), BIIE 0246 prevented the reduction in vagal bradycardia at all time points, mimicking the pattern of responses observed following norepinephrine pre-stimulation rather than sympathetic nerve stimulation (baseline heart rate 180 ± 9 bpm, n = 6). Figs. 2D and E demonstrates the effect of M40 (red traces). M40 only prevented the reduction in vagal bradycardia post sympathetic stimulation at 10 min and beyond (baseline heart rate 183 ± 13 bpm, n = 5). Release of both neuropeptide-Y and galanin could be detected in the perfusate surrounding the sinoatrial node/right stellate ganglion preparation at all time points following high frequency sympathetic stimulation (see Figs. 2C and F). Significantly more neuropeptide-Y was released compared to galanin after 15 and 20 min however. The maximum concentration of peptide released was 0.124 ± 0.03 ng/ml (0.03 ± 0.01 nM) neuropeptide-Y (n = 6), and 0.054 ± 0.012 ng/ml (0.02 ± 0.01 nM) galanin (n = 6) whereas the control peptide concentrations were below the detection level of the assays prior to sympathetic stimulation (<0.001 ng/ml).

We have previously shown that exogenous neuropeptide-Y can reduce vagal bradycardia and acetylcholine release at concentrations of 100 nM and above [17]. As can be seen in Fig. 3, exogenous galanin also significantly reduced vagal bradycardia at 50 nM at 1 Hz and at

all stimulation frequencies at 100 nM and above, although there was a trend to reduce vagal bradycardia even at 5–10 nM. The effect of galanin could be partially reversed with wash off of the peptide (baseline heart rate 192 ± 14 bpm, control 5 Hz −72 ± 6 bpm, 500 nM galanin −36 ± 10 bpm, wash off −53 ± 9 bpm, control 3 Hz −40 ± 6 bpm, 500 nM galanin −17 ± 5 bpm, wash off −34 ± 6, control 1 Hz −11 ± 1 bpm, 500 nM galanin −4 ± 1 bpm, wash off −9 ± 3, n = 6).

The GalR receptor antagonist M40 also prevented exogenous galanin (100 and 250 nM) from reducing the heart rate response to vagal nerve stimulation (baseline heart rate 200 ± 6 bpm, n = 5) whilst the neuropeptide-Y receptor antagonist BIIE 0246 did not prevent galanin from reducing vagal bradycardia at the same concentrations (baseline heart rate 169 ± 12 bpm, n = 5) as can be seen in Fig. 4. Conversely, the reduction in vagal bradycardia to exogenous neuropeptide-Y was not prevented by M40 (e.g. baseline heart rate 174 ± 1 bpm, 5 Hz control −64 ± 4 bpm, 1 μM M40 −52 ± 4 bpm, 250 nM neuropeptide-Y −40 ± 4 bpm, n = 4).

3.3. Galanin attenuates vagal bradycardia via a pre-synaptic pathway to reduce acetylcholine release

Whilst galanin significantly reduced the heart rate response to vagus nerve stimulation at 5 Hz (see Fig. 5A at 500 nM, baseline heart rate 192 ± 14 bpm, n = 6), the heart rate response to 90 nM carbamylcholine, which produced similar degrees of bradycardia, was not affected by galanin (see Fig. 5B at 500 nM, baseline heart rate 190 ± 9 bpm, n = 6). This suggests that galanin acts pre-synaptically to modulate parasympathetic neurotransmission. To test this hypothesis directly, we demonstrated that the same concentration of galanin (500 nM) significantly reduced the release of ^{3}H-acetylcholine in response to field stimulation at 5 Hz in isolated atria (see Fig. 5C, n = 5). A time control experiment demonstrates no run down of ^{3}H-acetylcholine release over the two stimulations [17].

To investigate the second messenger pathway that couples galanin-induced inhibition of vagal bradycardia via $GalR_1$ to a reduction in acetylcholine release, we assessed the effect of galanin in the presence of the protein kinase A inhibitor H89 and the protein kinase C inhibitor calphostin C. Whilst galanin (at 100 and 250 nM) was still able to reduce vagal bradycardia in the presence of H89 (baseline heart rate 204 ± 9 bpm, n = 6), the same concentrations of galanin were unable to reduce vagal bradycardia in the presence of calphostin C (baseline heart rate 207 ± 3 bpm, n = 6) as can be seen in Fig. 6 suggesting that galanin is acting via a protein kinase C dependent pathway.

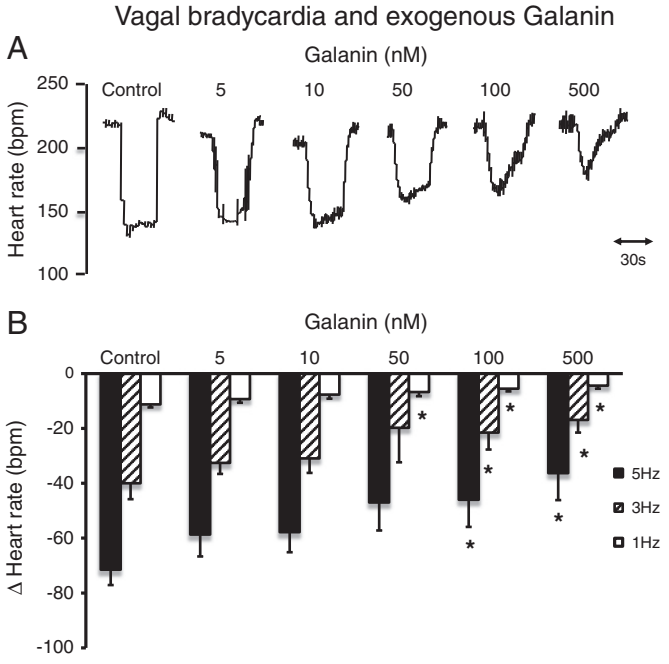

Fig. 3. Exogenous galanin reduces vagal bradycardia. Representative raw data traces (A at 5 Hz) and group mean data (B) showing the reduction in the magnitude of the bradycardia (beats per minute, bpm, baseline heart rate 192 ± 14 bpm, n = 6) to stimulation of the right vagus nerve (5, 3 or 1 Hz, for 30 s) in the presence of increasing concentrations of galanin (5–500 nM). (*p < 0.05 vs control).

4. Discussion

The main new findings of this study are as follows.

First, galanin is expressed in a small number of tyrosine hydroxylase positive neurons of the guinea pig stellate ganglion but can be found in most stellate neurons following tissue culture for 3 days, which may represent an acute injury response.

Secondly, galanin is released on high frequency sympathetic stimulation along with neuropeptide-Y, and the reduction in vagal bradycardia post high frequency sympathetic stimulation (in the presence of beta-blockade) is partly reversed by a galanin receptor antagonist and completely reversed by a neuropeptide-Y Y_2 receptor antagonist.

Thirdly, the $GalR_1$ receptor is localised on intrinsic cholinergic neurons at the sinoatrial node. Like neuropeptide-Y, exogenous galanin reduces vagal bradycardia via a pre-synaptic pathway involving a reduction in acetylcholine release that is $GalR_1$ receptor mediated and protein kinase C dependent.

Finally, there does not appear to be any cross reactivity between neuropeptide-Y and galanin in terms of their respective membrane receptors as Y_2 receptor inhibition does not alter the inhibitory action of galanin and $GalR_1$ inhibition does not alter the inhibitory action of neuropeptide-Y.

4.1. Sympathovagal cross-talk by adrenergic co-transmitters

We demonstrated that sympathetic neurons from freshly dissected left and right stellate ganglia contain mRNA for galanin although <5% of the total number of neurons express the protein. Others have also shown galanin immunoreactivity in intact stellate ganglia both in nerve fibres and a small number of small intensely fluorescent cells of the guinea pig stellate [36], although without co-staining for tyrosine hydroxylase. However, following dissociation and 3 days of culture, most sympathetic neurons from both ganglia express galanin. This is an interesting finding and may represent an injury response as local cardiac galanin expression is also increased in a rat model post myocardial infarction [37] driven by sympathetic hyperactivity [38]. Whether excess galanin production contributes to impaired vagal neurotransmission in this context remains to be shown.

We also demonstrated the release of both neuropeptide-Y and galanin into the tissue perfusate following sympathetic stimulation, however significantly more neuropeptide-Y was released than galanin. The peak perfusate concentrations (20–30 pM over 20 min post 2 minute sympathetic stimulation) were significantly less than the concentrations of exogenous peptide required to significantly reduce vagal bradycardia (50 nM and above, over a 10 min incubation period, although there was a trend for a reduction in vagal bradycardia even at the lowest 5 nM dose used) as is observed with many neurotransmitters and bioassays. Given localised metabolism, re-uptake and diffusion, neither are likely to accurately reflect the exact concentration of peptide released locally at the level of the neuron within the atrial tissue. Instead, the dose response curve experiments should be viewed as proof of principle that exogenous galanin can reduce vagal bradycardia.

Our perfusate concentrations were higher than those mean values observed in human plasma (e.g. galanin 4–6 pM [39], neuropeptide-Y 18 pM [40]) but lower than those of neuropeptide-Y collected from coronary venous sinus blood sampling immediately after cardiac

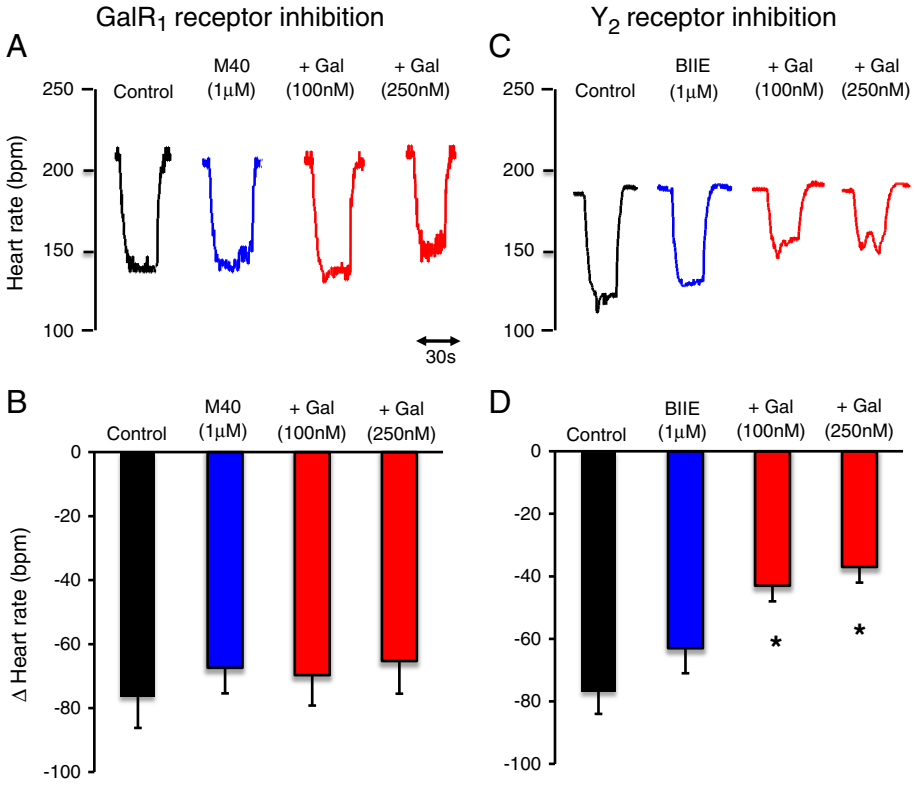

Fig. 4. Galanin acts via the $GalR_1$ receptor but not via the neuropeptide-Y Y_2 receptor. Representative raw data traces (A and C) and group mean data (B and D) showing the effect of galanin (100 and 250 nM) on the heart rate response (beats per minute, bpm) to vagal nerve stimulation (5 Hz, 30 s) in the presence of antagonists of the $GalR_1$ receptor (M40 1 μM, baseline heart rate 200 ± 6 bpm, n = 5, A and B) or the neuropeptide-Y Y_2 receptor (BIIE 0246 1 μM, baseline heart rate 169 ± 12 bpm, n = 5, C and D). (*$p<0.05$ vs control and BIIE 0246).

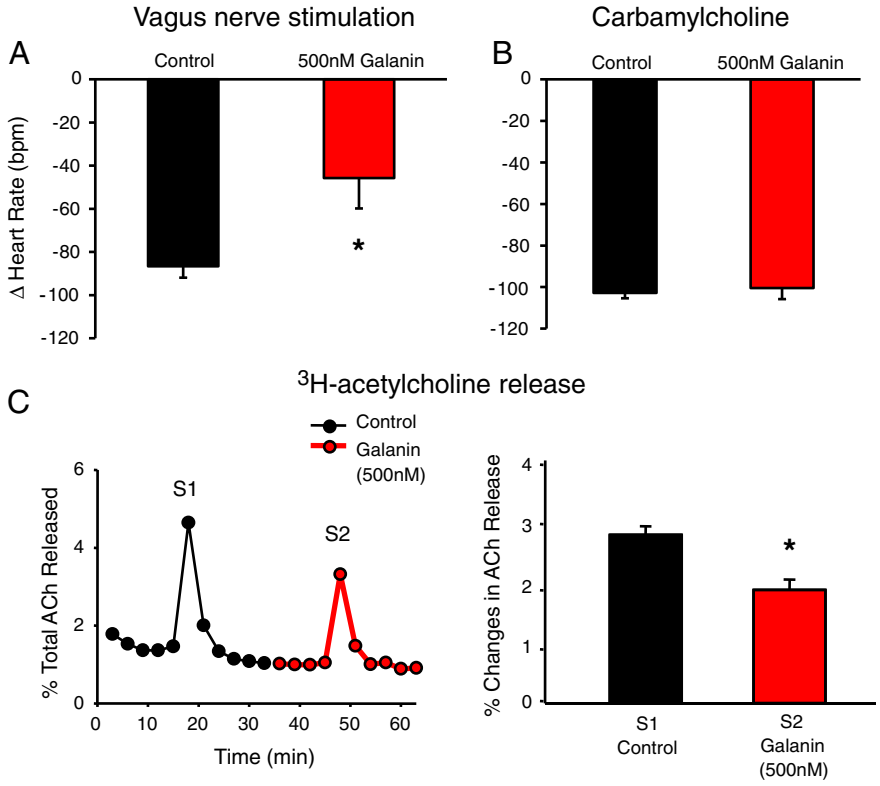

Fig. 5. A pre-synaptic action of galanin reduces vagal bradycardia and acetylcholine release. Galanin (500 nM) significantly reduced (*$p<0.05$) the heart rate response (beats per minute, bpm) to vagal nerve stimulation at 5 Hz (baseline heart rate 192 ± 14 bpm, n = 6, A), but did not affect the magnitude of the bradycardia in response to the stable analogue of acetylcholine, carbamylcholine (90 nM, baseline heart rate 174 ± 1 bpm, n = 6, B). This suggests that galanin is acting via a presynaptic pathway. Galanin also significantly reduced the release of ^{3}H-acetylcholine to field stimulation (5 Hz, S1 vs S2, n = 5) as demonstrated by the representative raw data trace (C, left panel) and group mean data (C, right panel).

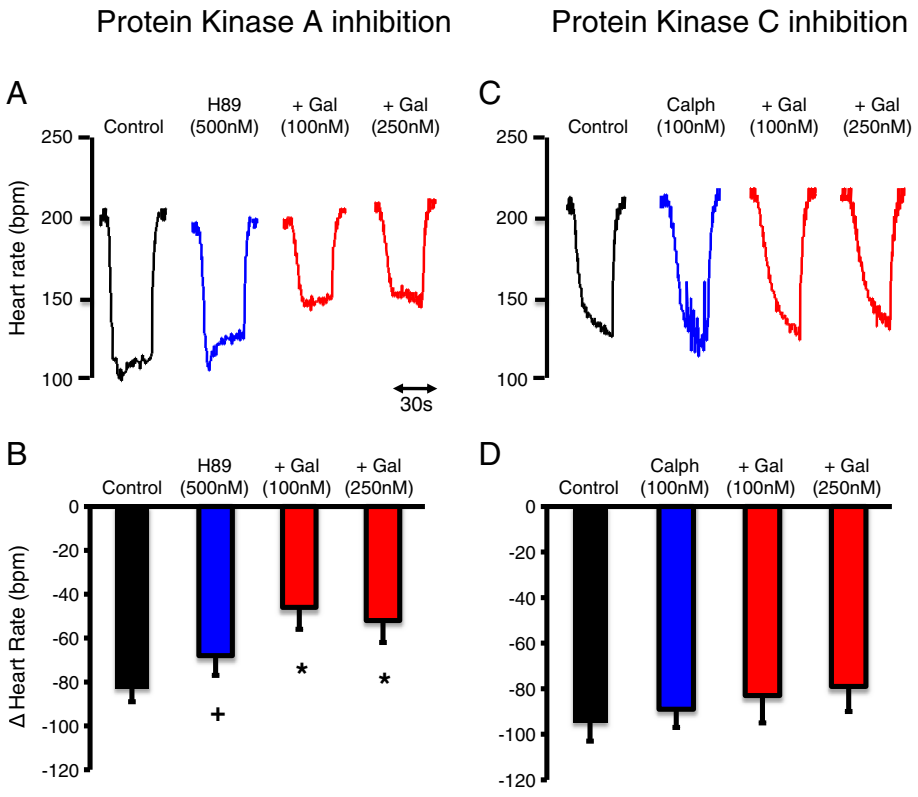

Fig. 6. Inhibition of protein kinase C, but not protein kinase A prevents the reduction in vagal bradycardia to galanin. Inhibition of protein kinase A (H89 500 nM, baseline heart rate 204 ± 9 bpm, n = 6) significantly reduced (+p<0.05) the heart rate response (beats per minute, bpm) to right vagal nerve stimulation (5 Hz, 30 s). However, galanin (100 and 250 nM) was able to further reduce the magnitude of the vagal bradycardia (*p<0.05) in the presence of protein kinase A inhibition (A, raw data trace, B, mean data). Inhibition of protein kinase C (calphostin C 100 nM, baseline heart rate 207 ± 3 bpm, n = 6) prevented the reduction in vagal bradycardia to galanin (100 and 250 nM) suggesting that galanin was acting via a protein kinase C rather than a protein kinase A dependent pathway.

sympathetic stimulation (peaking at 1 nM after 3 min of sympathetic stimulation at 10 Hz [20]). Perfusate protein levels unsurprisingly steadily rose in our static organ bath preparation over a 20-minute period. The dynamics of release and vagal responsiveness in our preparation are unlikely to accurately reflect the exact time course observed in-vivo. For example, coronary venous sinus levels of neuropeptide-Y and vagal inhibition post sympathetic stimulation peaked inside 10 min and then were observed to fall in the hour following sympathetic stimulation in-vivo [20]. Importantly we demonstrated that the reduction in vagal bradycardia post sympathetic stimulation was reversed at all time points by a Y_2 receptor antagonist and at 10 min and beyond by a $GalR_1$ receptor antagonist. Moreover we have demonstrated that these receptor antagonists inhibit the effects of their corresponding peptide whilst not affecting the response to the other peptide. This provides convincing evidence that neuropeptide-Y and galanin contribute to the reduction in vagal bradycardia post sympathetic stimulation although the action of both peptides is not essential for this to occur.

Exogenous galanin may reduce the heart rate response to peripheral stimulation of the right vagus nerve in the mouse [4,18] and cat [41] in-vivo, but this was not observed in the rat [42] or dog [43]. Systemic infusion of neuropeptides in-vivo is also complicated by potential changes in haemodynamics and other circulating factors complicating the interpretation of data. Interestingly, galanin has been identified in human plasma and its levels rise following exercise [44] and during head up tilt table testing of patients with vasovagal syncopy as blood pressure and heart rate increase. Indeed intravenous administration of galanin increases resting heart rate in humans [45,46] associated with the abolition of sinus arrhythmia [45]. Galanin infusion also prevents the bradycardia secondary to the cholinesterase inhibitor pyridostigmine in humans [47] suggesting a vagal site of action, but to our knowledge this is the first direct evidence that galanin is released during cardiac sympathetic hyper-activity and that it directly inhibits cholinergic neurotransmission.

We also observed a small increase in the magnitude of the vagal bradycardia after 5 min in the presence of norepinephrine (to 122 ± 13% of control), or following sympathetic nerve stimulation following Y_2 receptor inhibition (to 123 ± 7% of control). This was likely to be related to the small increase in baseline heart rate despite beta-blockade and post-synaptic accentuated antagonism. For example the heart rate response to vagal nerve stimulation (5 Hz) post sympathetic stimulation (10 Hz for 2 min) in the absence of a beta-blocker increased to 168 ± 21% from control where baseline heart rate increased by 70 ± 11% (n = 4). Similar observations were made using norepinephrine and carbamylcholine.

4.2. Mechanism of action of galanin on vagal function

We clearly demonstrated that exogenous galanin inhibits the heart rate response to peripheral vagal nerve stimulation via a pre-synaptic mechanism. Moreover, galanin inhibits the evoked release of ^{3}H-acetylcholine in response to field stimulation at the same frequency of stimulation and at the same dose. After loading right atrial neuronal stores with ^{3}H-choline, measuring the increase in radioactivity efflux to field stimulation has been taken to represent acetylcholine release arising mainly from post-ganglionic vagal neurons. The increase in organ bath radioactivity during field stimulation is prevented by removal of extracellular calcium and can be inhibited by N-type calcium channel blockers [48,49]. However, it is not clear how much ^{3}H-acetylcholine release arises from the pre-ganglionic–post-ganglionic synapse. We have tried to stimulate post-ganglionic neuronal nicotinic receptors using a variety of nicotinic agonists but they produced very small degrees of bradycardia (even in the

presence of β blockade), which were not comparable to peripheral vagus stimulation or muscarinic agonists. Further attenuation of such small responses would be difficult to detect making it hard to draw clear conclusions regarding the exact site of action of galanin by this method. However, the immunochemical localisation of $GalR_1$ to post-ganglionic cholinergic neurons strongly suggests that this is the pre-synaptic site of action of galanin in our preparation.

We also demonstrated that protein kinase C inhibition prevents the action of galanin of vagal bradycardia whilst protein kinase A inhibition had no effect. Neuromodulators such as neuronal nitric oxide [50] and natriuretic peptides [51] increase acetylcholine release and vagal bradycardia, and that this can be prevented by several different protein kinase A inhibitors (including 0.5 μM H89). As observed previously, H89 and other protein kinase A inhibitors alone produce a small but significant reduction in vagal bradycardia [50] whilst phosphodiesterase inhibition with milrinone increased acetylcholine release and vagal bradycardia [50]. This suggests that there is a baseline level of neuronal cAMP production increasing the gain of acetylcholine release. We have also previously demonstrated that the inhibitory action of neuropeptide-Y on acetylcholine release and vagal bradycardia was unaffected by H89, but prevented by several different protein kinase C inhibitors (including 0.1 μM calphostin C) [17]. In keeping with this hypothesis, the protein kinase C activator PMA can mimic the effect of both neuropeptide-Y and galanin by inhibiting acetylcholine release [17]. Importantly we also demonstrated that inhibition of the Y_2 receptor with BIIE 0246 did not prevent the action of galanin, whilst inhibition of $GalR_1$ with M40 does not prevent the action of neuropeptide-Y. This supports the notion that the two co-transmitter signalling pathways integrate at the level of the second messenger rather than by sharing similar membrane receptors.

Neuropeptide-Y and galanin may signal directly through the IP3/DAG–protein kinase C pathway, although both the Y_2 [52] and $GalR_1$ receptor [53] are traditionally thought to couple to Gαi. It is also possible that protein kinase C may be involved indirectly, for example following a change in intracellular calcium handling to alter neuronal excitability or the vesicular release mechanism itself. For example, neuropeptide-Y increases outward potassium current in ventricular myocytes via a pathway sensitive to both pertussis toxin and calphostin C [54]. Conversely galanin has been shown to inhibit N-type calcium currents in mudpuppy cholinergic neurons [55]. It is difficult to investigate the role of G proteins experimentally in the preparation used here as treatment with pertussis toxin ablates cholinergic mediated bradycardia via a post-synaptic mechanism [56] (Fig. 7).

4.3. Perspectives

Excessive adrenergic activity is a negative prognostic indicator post myocardial infarction [8,9] and during congestive cardiac failure [7,10], and has been implicated in both the aetiology and progression of hypertension [57]. Under normal conditions the cardiac vagus nerve acts as nature's beta-blocker, preventing intracellular calcium overload and slowing heart rate. It can directly raise the threshold for induction of ventricular fibrillation [58]. Conditions that promote high vagal tone [e.g. exercise training [59–61] and nNOS vagal gene transfer [24]] protect against mortality. It is possible that during myocardial infarction and congestive heart failure where neuropeptide-Y plasma levels are known to be elevated, both sympathetic co-transmitters are at least partly responsible for reducing vagal activity. Local cardiac galanin expression is also increased in a rat model post myocardial infarction [37], and this is driven by sympathetic hyperactivity [38]. If these co-transmitters are responsible for impairing vagal function in pathological conditions associated with high sympathetic drive, drugs targeted at their receptors may provide additional benefit when combined with beta-blockers and ACE inhibitors, which target the sympatho-adrenal axis alone.

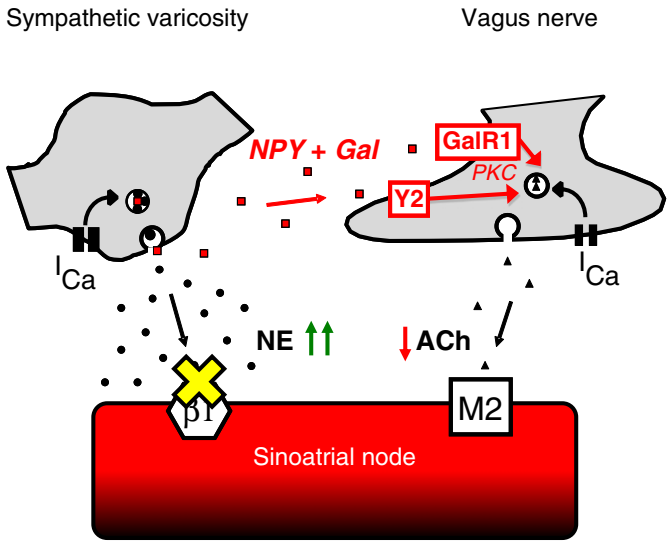

Fig. 7. Cardiac sympatho-vagal crosstalk by adrenergic co-transmitters. A diagram hypothesizing the mechanism by which the adrenergic co-transmitters neuropeptide-Y (NPY, red square) and galanin (Gal, red square) are released during high frequency sympathetic stimulation in the presence of beta blockade (norepinephrine, NE, black circle, and metoprolol, yellow cross). Neuropeptide-Y and galanin act via the Y_2 and $GalR_1$ receptors respectively to reduce acetylcholine (ACh, black triangle) release and subsequent vagal bradycardia at the sinoatrial node. Both $GalR_1$ and Y_2 receptor signalling directly or indirectly involves protein kinase C (PKC).

Funding

NH acknowledges support from the BHF Centre of Research Excellence (RE/08/004), Oxford. This work was supported by a British Heart Foundation project grant (PG/11/6/28660), an Academy of Medical Sciences Clinical Lecturer Starter grant and National Institutes of Health grants HL068231 and P20RR-016435. NH is a Clinical Lecturer and BHF CRE Intermediate Fellow at the University of Oxford and Specialist Registrar in Cardiology at the Oxford University Hospitals NHS Trust. JC holds a scholarship at Lincoln College, Oxford.

Disclosure statement

None.

References

[1] Kummer W. Galanin- and neuropeptide Y-like immunoreactivities coexist in paravertebral sympathetic neurones of the cat. Neurosci Lett 1987;78:127–31.
[2] Warner MR, Levy MN. Sinus and atrioventricular nodal distribution of sympathetic fibers that contain neuropeptide Y. Circ Res 1990;67:713–21.
[3] Burnstock G. Autonomic neurotransmission: 60 years since Sir Henry Dale. Annu Rev Pharmacol Toxicol 2009;49:1–30.
[4] Potter EK, Smith-White MA. Galanin modulates cholinergic neurotransmission in the heart. Neuropeptides 2005;39:345–8.
[5] Warner MR, Levy MN. Neuropeptide Y as a putative modulator of the vagal effects on heart rate. Circ Res 1989;64:882–9.
[6] Herring N, Paterson DJ. Neuromodulators of peripheral cardiac sympatho-vagal balance. Exp Physiol 2009;94:46–53.
[7] Cohn JN, Levine TB, Olivari MT, Garberg V, Lura D, Francis GS, et al. Plasma norepinephrine as a guide to prognosis in patients with chronic congestive heart failure. N Engl J Med 1984;311:819–23.
[8] Kleiger RE, Miller JP, Bigger Jr JT, Moss AJ. Decreased heart rate variability and its association with increased mortality after acute myocardial infarction. Am J Cardiol 1987;59:256–62.
[9] La Rovere MT, Bigger Jr JT, Marcus FI, A Mortara, PJ Schwartz. Baroreflex sensitivity and heart-rate variability in prediction of total cardiac mortality after myocardial infarction. ATRAMI (Autonomic Tone and Reflexes After Myocardial Infarction) Investigators. Lancet 1998;351:478–84.
[10] Nolan J, Batin PD, Andrews R, Lindsay SJ, Brooksby P, Mullen M, et al. Prospective study of heart rate variability and mortality in chronic heart failure: results of the

United Kingdom heart failure evaluation and assessment of risk trial (UK-heart). Circulation 1998;98:1510–6.
[11] Maisel AS, Scott NA, Motulsky HJ, Michel MC, Boublik JH, Rivier JE, et al. Elevation of plasma neuropeptide Y levels in congestive heart failure. Am J Med 1989;86: 43–8.
[12] Hulting J, Sollevi A, Ullman B, Franco-Cereceda A, Lundberg JM. Plasma neuropeptide Y on admission to a coronary care unit: raised levels in patients with left heart failure. Cardiovasc Res 1990;24:102–8.
[13] Ullman B, Hulting J, Lundberg JM. Prognostic value of plasma neuropeptide-Y in coronary care unit patients with and without acute myocardial infarction. Eur Heart J 1994;15:454–61.
[14] Schwertfeger E, Klein T, Vonend O, Oberhauser V, Stegbauer J, Rump LC. Neuropeptide Y inhibits acetylcholine release in human heart atrium by activation of Y2-receptors. Naunyn Schmiedebergs Arch Pharmacol 2004;369:455–61.
[15] Manabe N, Foldes FF, Torocsik A, Nagashima H, Goldiner PL, Vizi ES. Presynaptic interaction between vagal and sympathetic innervation in the heart: modulation of acetylcholine and noradrenaline release. J Auton Nerv Syst 1991;32:233–42.
[16] Nakatsuka H, Nagano O, Foldes FF, Nagashima H, Vizi ES. Effects of adenosine on norepinephrine and acetylcholine release from guinea pig right atrium: role of A1-receptors. Neurochem Int 1995;27:345–53.
[17] Herring N, Lokale MN, Danson EJ, Heaton DA, Paterson DJ. Neuropeptide Y reduces acetylcholine release and vagal bradycardia via a Y2 receptor-mediated, protein kinase C-dependent pathway. J Mol Cell Cardiol 2008;44:477–85.
[18] Smith-White MA, Iismaa TP, Potter EK. Galanin and neuropeptide Y reduce cholinergic transmission in the heart of the anaesthetised mouse. Br J Pharmacol 2003;140:170–8.
[19] Ilebekk A, Bjorkman JA, Nordlander M. Influence of endogenous neuropeptide Y (NPY) on the sympathetic–parasympathetic interaction in the canine heart. J Cardiovasc Pharmacol 2005;46:474–80.
[20] Warner MR, Senanayake PD, Ferrario CM, Levy MN. Sympathetic stimulation-evoked overflow of norepinephrine and neuropeptide Y from the heart. Circ Res 1991;69:455–65.
[21] Smith-White MA, Herzog H, Potter EK. Role of neuropeptide Y Y(2) receptors in modulation of cardiac parasympathetic neurotransmission. Regul Pept 2002;103:105–11.
[22] Diaz-Cabiale Z, Parrado C, Narvaez M, Millon C, Puigcerver A, Fuxe K, et al. Neurochemical modulation of central cardiovascular control: the integrative role of galanin. EXS 2010;102:113–31.
[23] Calupca MA, Vizzard MA, Parsons RL. Origin of pituitary adenylate cyclase-activating polypeptide (PACAP)-immunoreactive fibers innervating guinea pig parasympathetic cardiac ganglia. J Comp Neurol 2000;423:26–39.
[24] Dawson TA, Li D, Woodward T, Barber Z, Wang L, Paterson DJ. Cardiac cholinergic NO-cGMP signaling following acute myocardial infarction and nNOS gene transfer. Am J Physiol Heart Circ Physiol 2008;295:H990–8.
[25] Tompkins JD, Parsons RL. Exocytotic release of ATP and activation of P2X receptors in dissociated guinea pig stellate neurons. Am J Physiol Cell Physiol 2006;291:C1062–71.
[26] Merriam LA, Locknar SA, Girard BM, Parsons RL. Somatic ATP release from guinea pig sympathetic neurons does not require calcium-induced calcium release from internal stores. Am J Physiol Cell Physiol 2010;299:C836–43.
[27] Wang L, Li D, Plested CP, Dawson T, Teschemacher AG, Paterson DJ. Noradrenergic neuron-specific overexpression of nNOS in cardiac sympathetic nerves decreases neurotransmission. J Mol Cell Cardiol 2006;41:364–70.
[28] Zvarova K, Vizzard MA. Changes in galanin immunoreactivity in rat micturition reflex pathways after cyclophosphamide-induced cystitis. Cell Tissue Res 2006;324:213–24.
[29] Young BA, Girard BM, Parsons RL. Neurturin suppresses injury-induced neuronal activating transcription factor 3 expression in cultured guinea pig cardiac ganglia. J Comp Neurol 2008;508:795–805.
[30] Sears CE, Choate JK, Paterson DJ. Inhibition of nitric oxide synthase slows heart rate recovery from cholinergic activation. J Appl Physiol 1998;84:1596–603.
[31] Smith KE, Walker MW, Artymyshyn R, Bard J, Borowsky B, Tamm JA, et al. Cloned human and rat galanin GALR3 receptors. Pharmacology and activation of G-protein inwardly rectifying K+ channels. J Biol Chem 1998;273:23321–6.
[32] Dumont Y, Cadieux A, Doods H, Pheng LH, Abounader R, Hamel E, et al. BIIE0246, a potent and highly selective non-peptide neuropeptide Y Y(2) receptor antagonist. Br J Pharmacol 2000;129:1075–88.
[33] Kobayashi E, Nakano H, Morimoto M, Tamaoki T. Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. Biochem Biophys Res Commun 1989;159:548–53.
[34] Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, et al. Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells. J Biol Chem 1990;265:5267–72.
[35] Bartfai T, Langel U, Bedecs K, Andell S, Land T, Gregersen S, et al. Galanin-receptor ligand M40 peptide distinguishes between putative galanin-receptor subtypes. Proc Natl Acad Sci U S A 1993;90:11287–91.
[36] Elfvin LG, Hokfelt T, Bartfai T, Bedecs K. Immunohistochemical demonstration of galanin-, and galanin message-associated peptide-like immunoreactivities in sympathetic ganglia and adrenal gland of the guinea pig. Microsc Res Tech 1994;29:131–42.
[37] Habecker BA, Gritman KR, Willison BD, Van Winkle DM. Myocardial infarction stimulates galanin expression in cardiac sympathetic neurons. Neuropeptides 2005;39:89–95.
[38] Ewert TJ, Gritman KR, Bader M, Habecker BA. Post-infarct cardiac sympathetic hyperactivity regulates galanin expression. Neurosci Lett 2008;436:163–6.
[39] Grenback E, Hulting AL, Bucht E. Galanin in human plasma. Neuropeptides 2005;39:337–40.
[40] Pernow J, Lundberg JM, Kaijser L, Hjemdahl P, Theodorsson-Norheim E, Martinsson A, et al. Plasma neuropeptide Y-like immunoreactivity and catecholamines during various degrees of sympathetic activation in man. Clin Physiol 1986;6:561–78.
[41] Ulman LG, Potter EK, McCloskey DI. Effects of sympathetic activity and galanin on cardiac vagal action in anaesthetized cats. J Physiol 1992;448:225–35.
[42] Smith-White MA, Wallace D, Potter EK. Sympathetic–parasympathetic interactions at the heart in the anaesthetised rat. J Auton Nerv Syst 1999;75: 171–5.
[43] Ulman LG, Moriarty M, Potter EK, McCloskey DI. Galanin antagonist effects on cardiac vagal inhibitory actions of sympathetic stimulation in anaesthetized cats and dogs. J Physiol 1993;464:491–9.
[44] Legakis IN, Mantzouridis T, Saramantis A, Phenekos C, Tzioras C, Mountokalakis T. Human galanin secretion is increased upon normal exercise test in middle-age individuals. Endocr Res 2000;26:357–64.
[45] Carey DG, Iismaa TP, Ho KY, Rajkovic IA, Kelly J, Kraegen EW, et al. Potent effects of human galanin in man: growth hormone secretion and vagal blockade. J Clin Endocrinol Metab 1993;77:90–3.
[46] degli Uberti EC, Ambrosio MR, Bondanelli M, Trasforini G, Margutti A, Valentini A, et al. Human galanin reduces plasma norepinephrine levels in man. J Clin Endocrinol Metab 1995;80:1894–8.
[47] degli Uberti EC, Bondanelli M, Margutti A, Ambrosio MR, Valentini A, Campo M, et al. Acute administration of human galanin in normal subjects reduces the potentiating effect of pyridostigmine-induced cholinergic enhancement on release of norepinephrine and pancreatic polypeptide. Neuroendocrinology 1996;64: 398–404.
[48] Wetzel GT, Brown JH. Relationships between choline uptake, acetylcholine synthesis and acetylcholine release in isolated rat atria. J Pharmacol Exp Ther 1983;226:343–8.
[49] Seebeck J, Schmidt WE, Kilbinger H, Neumann J, Zimmermann N, Herzig S. PACAP induces bradycardia in guinea-pig heart by stimulation of atrial cholinergic neurones. Naunyn Schmiedebergs Arch Pharmacol 1996;354:424–30.
[50] Herring N, Paterson DJ. Nitric oxide-cGMP pathway facilitates acetylcholine release and bradycardia during vagal nerve stimulation in the guinea-pig in vitro. J Physiol 2001;535:507–18.
[51] Herring N, Zaman JA, Paterson DJ. Natriuretic peptides like NO facilitate cardiac vagal neurotransmission and bradycardia via a cGMP pathway. Am J Physiol Heart Circ Physiol 2001;281:H2318–27.
[52] Balasubramaniam AA. Neuropeptide Y family of hormones: receptor subtypes and antagonists. Peptides 1997;18:445–57.
[53] Wang S, Hashemi T, Fried S, Clemmons AL, Hawes BE. Differential intracellular signaling of the GalR1 and GalR2 galanin receptor subtypes. Biochemistry 1998;37:6711–7.
[54] Heredia MP, Fernandez-Velasco M, Benito G, Delgado C. Neuropeptide Y increases 4-aminopyridine-sensitive transient outward potassium current in rat ventricular myocytes. Br J Pharmacol 2002;135:1701–6.
[55] Merriam LA, Parsons RL. Neuropeptide galanin inhibits omega-conotoxin GVIA-sensitive calcium channels in parasympathetic neurons. J Neurophysiol 1995;73:1374–82.
[56] Danson EJ, Zhang YH, Sears CE, Edwards AR, Casadei B, Paterson DJ. Disruption of inhibitory G-proteins mediates a reduction in atrial beta-adrenergic signaling by enhancing eNOS expression. Cardiovasc Res 2005;67:613–23.
[57] Grassi G. Assessment of sympathetic cardiovascular drive in human hypertension: achievements and perspectives. Hypertension 2009;54:690–7.
[58] Ng GA, Brack KE, Patel VH, Coote JH. Autonomic modulation of electrical restitution, alternans and ventricular fibrillation initiation in the isolated heart. Cardiovasc Res 2007;73:750–60.
[59] Danson EJ, Paterson DJ. Enhanced neuronal nitric oxide synthase expression is central to cardiac vagal phenotype in exercise-trained mice. J Physiol 2003;546: 225–32.
[60] Danson EJ, Mankia KS, Golding S, Dawson T, Everatt L, Cai S, et al. Impaired regulation of neuronal nitric oxide synthase and heart rate during exercise in mice lacking one nNOS allele. J Physiol 2004;558:963–74.
[61] Cole CR, Blackstone EH, Pashkow FJ, Snader CE, Lauer MS. Heart-rate recovery immediately after exercise as a predictor of mortality. N Engl J Med 1999;341: 1351–7.