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ORIGINAL ARTICLE

The expression of β_3-adrenoceptor and muscarinic type 3 receptor immuno-reactivity in the major pelvic ganglion of the rat

J. Eastham[1] · C. Stephenson[1] · K. Korstanje[2] · J. I. Gillespie[1]

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Abstract Bladder afferent outflow, linked to sensation, plays a critical role in bladder pathology: abnormal outflow results in altered sensation, leading to increased voiding frequency, urge and often incontinence. β_3-adrenoceptor agonists have been suggested to be beneficial in treating these symptoms. However, the absence of a significant sympathetic innervation of the detrusor and only a modest relaxation of bladder muscle by β_3 agonists has questioned the therapeutic site of action of β_3 agonists in the bladder. The present study was done to explore the possibility that β_3-adrenoceptors might be located in the pelvic plexus. Using the rat, where the pelvic plexus is located primarily within a single ganglion, the major pelvic ganglion (MPG), immuno-histochemical approaches were used to identify structures expressing β_3-adrenoceptor immuno-reactivity (β_3AR-IR). The only structures found to express β_3AR-IR were small-diameter tyrosine hydroxylase and vesicular mono-amine transporter immuno-reactive (TH-IR and vmat-IR) neurones. These neurones, found in clusters or singly on the periphery of the ganglion, or dispersed in smaller clumps throughout the MPG, are similar to the small intensely fluorescent (SIF) cells described previously. Not all small cells expressed β_3AR-IR. A population of the small cells were also immuno-reactive to the type 3 muscarinic receptor (M_3R-IR) and the P2X3 purinergic receptor (P2X3-IR). Clumps of small cells were associated with calcitonin gene-related peptide immuno-reactive (CGRP-IR) nerve fibres (putative sensory fibres) and a small number were contacted by putative cholinergic nerves expressing immuno-reactivity to vesicular acetylcholine transporter (vacht-IR). These observations are consistent with the idea that small cells are interneurons and one of the components making up complex neural circuits within the MPG. The precise physiological role of these neural elements in the MPG is unknown. However, as one therapeutic action of β_3-adrenoceptor agonists is to modulate sensation, it is possible that these neural circuits may be involved in the regulation of afferent outflow and sensation.

Keywords β_3-adrenoceptor · Immuno-reactivity · Major pelvic ganglion

✉ J. I. Gillespie
j.i.gillespie@ncl.ac.uk

[1] Uro-physiology Research Group, The Dental and Medical School, Newcastle University, Newcastle Upon Tyne NE2 4HH, England, UK

[2] Astellas Pharma Europe B.V., Elisabethhof 1, 2353 EW Leiderdorp, The Netherlands

Introduction

The site and specific mode of action of β_3-adrenoceptor agonists in the management of the over active bladder (OAB) has become the centre of considerable research interest. Originally conceived to relax the detrusor via a direct action on β_3-adrenoceptors at or near the bladder smooth muscle, there is now a growing body of data to suggest that other sites for β_3-adrenoceptors should be considered to explain the therapeutic actions of these agents. At therapeutic doses of 25 or 50 mg/day, the plasma concentrations of mirabegron are in the region of 30–75 nM (Krauwinkel et al. 2012). In this concentration range, little relaxation can be seen in isolated strips of human bladder (Svalø et al. 2013). Therefore, additional β_3-adrenoceptor-operated mechanisms associated with the lower urinary tract should be considered as potential targets for this drug.

In a rat model of detrusor overactivity (partial bladder outflow obstruction, pBOO), mirabegron, at similar doses to

those used in humans, was reported to reduce the non-voiding activity (NVA) observed throughout the filling phase during cystometry in conscious animals but not to affect the voiding contractions (Gillespie et al. 2012). It has been argued that the mechanisms underlying NVA might be an alternative therapeutic target for β_3-adrenoceptor agonists (Gillespie et al. 2012). NVA is thought to be the motor component of a motor-sensory system linked to bladder sensation (Sherrington 1892; Starling 1905; Iggo 1955; Vaughan and Satchell 1995; Gillespie 2004; Eastham and Gillespie 2013). Reducing NVA by β_3-adrenoceptor activation might reduce bladder sensation and alleviate the symptoms of OAB (Eastham and Gillespie 2013).

Exploration of isolated bladder tissues from the rat revealed that mirabegron, in concentrations comparable to those in plasma using therapeutic doses, had little effect on intrinsic micro-contractile activity, nerve-mediated contractions or agonist-induced contractions (Gillespie et al. 2015a, b). These studies also suggested that the inhibition of micro-contractile activity appears to be influenced by a β_1-adrenoceptor-dependent mechanism not β_3. Consequently, despite attempts to identify β_3-adrenoceptor mechanisms on contractile events within the rat bladder, no clear effect has yet been demonstrated. Therefore, there is a need to identify other elements within the LUT that might be a target for the therapeutic actions of β_3-adrenoceptor activation.

For many years, it has been known that the peripheral ganglia played a key role in bladder physiology and pathology (Langley and Anderson 1895a, b, 1896; Gabella 1990; De Groat and Booth 1993; Uvelius and Gabella 1995). In almost all species, the pelvic plexus consists of a large number of diffuse small ganglia located close to the pelvic organs (Langley and Anderson 1895a). Also, in some species, ganglia are found in the bladder wall (intra-mural ganglia: Gosling and Dixon 1974; (Gosling and Dixon 1974; Dixon et al. 1983; Gabella 1990; Smet et al. 1996; Zhou and Ling 1998; Gosling et al. 1999; Gillespie et al. 2006). Simplistically, they have been proposed to operate as a relay point for the parasympathetic nervous system (De Groat and Booth 1980, 1993). However, the diversity of nerve types within these ganglia (Keast et al. 1989; De Groat and Booth 1993), the possibilty of complex synaptic circuitry (Dail et al. 1975) and the presence of possible intra-ganglionic inter-neurones, small intensely fluorescent (SIF) cells (Dail et al. 1975; Eränkö 1976, 1978), has led to a growing realisation that there are likely to be, as yet, unrecognised physiological systems operating in the ganglia.

In rats, the pelvic plexus is not a distributed network of ganglia. Unusually, the peripheral neurones are collected into a single large ganglion, containing both sympathetic and parasympathetic neurones (approximately 50:50). In male rats, this ganglion is known as the major pelvic ganglion (MPG) and in the female equivalent is a paracervical ganglion (Frankenhauser's ganglion) (Langworthy 1965; El-Badawi and Schenk 1968; el-Badawi and Shenk 1968; Dail et al. 1975; Keast et al. 1989; De Groat and Booth 1993). Few, if any ganglia are found within the bladder wall of the rat (Uvelius and Gabella 1995). Thus, the MPG of the rat provides an opportunity to explore the peripheral neurological elements of the lower urinary tract.

Since evidence for functional β_3-adrenoceptor mechanisms within the bladder wall of the rat is tenuous, the present experiments were done to explore the possibility that β_3-adrenoceptor mechanisms might be operating within the MPG. In this study, immuno-histochemical techniques were employed to identify structures that might express β_3-adrenoceptor immuno-reactivity (β_3-IR) as well as immuno-reactivity to other signalling elements, e.g. the cholinergic, prostanoid and purinergic systems.

Methods

Tissue extraction and preparation

Wistar rats (male, $n=17$ weight 250–270 g) were killed by stunning and cervical dislocation (Approved UK Home Office: Scheduled 1 killing). MPG were surgically removed and immediately immersed in 4 % paraformaldehyde made up in phosphate buffer saline (PBS) for 120 min at 4 °C. Tissues were then washed in PBS and incubated in solutions with progressively higher sucrose solutions (10, 20 and 30 %) to act as cryo-preservant. Tissue was subsequently 'snap frozen' using isopentane cooled to freezing point with liquid nitrogen. Frozen tissue was then kept at −80 °C until needed. Tissue sections (7–8 μm) were cut at −25 °C and subsequently placed on poly-lysine-coated slides. Tissue morphology was examined using a light microscope to confirm that gross structural features were intact. Slides were labelled and stored at −80 °C until used.

Staining procedures

Slides were removed from the freezer and maintained in a dry environment for 120 min. They were then washed in tris-buffer saline (TBS), tris-buffer saline tween (TBS-T) and TBS wash cycle for 5 min at each stage. Primary antibodies were diluted with 1× PBS or PBS with triton-X (1 %), pH 7.4. Combinations of primary antibodies (1°Abs) were then put on each slide and incubated overnight at 4 °C.

Primary polyclonal antibodies included against calcitonin gene-related peptide (CGRP) (1:500; Santa Cruz, Cat No. sc-57053), cyclooxygenase 1 (COX1) (1:100; Santa Cruz, Cat No. sc-1752), protein gene product 9.5 (PGP 9.5) (1:2,000;

Seratec, Cat No. 7863-0604), neuronal nitric oxide synthase (NOS) (1:500; Santa Cruz, Cat No. 648), β3-AR (1:200; Santa Cruz, Cat No. sc-1473), tyrosine hydroxylase (TH) (1:5,000; Santa Cruz, Cat No, 14007), purinergic receptor P2X3 (P2X3) (1:200; Santa Cruz, Cat No. 25694), neuro-filament H protein (NF-H) (1:1, 000; Abcam, Cat No. 9640796), muscarinic receptor type 3 (M_3) (1:100; Santa Cruz, Cat No. sc-9108) and vesicular mono-amine transporter (vmat) (1:100; Santa Cruz, Cat No. sc-7721). These antibodies were chosen since they have been characterised at the molecular level and used in papers published in the literature (see manufacturers data sheets for more information).

For two of two of the key antibodies used in this study, β_3-AR and muscarinic receptor type 3 (M_3), specific characterisations have been published that give confidence in their epitope specificity (Grol et al. 2009; Cernecka et al. 2014).

After overnight incubation, sections were washed in TBS, TBS-T, TBS wash cycles each for 20 min. Sections were then incubated with appropriate secondary fluorescent antibodies: mouse, goat and rabbit primary

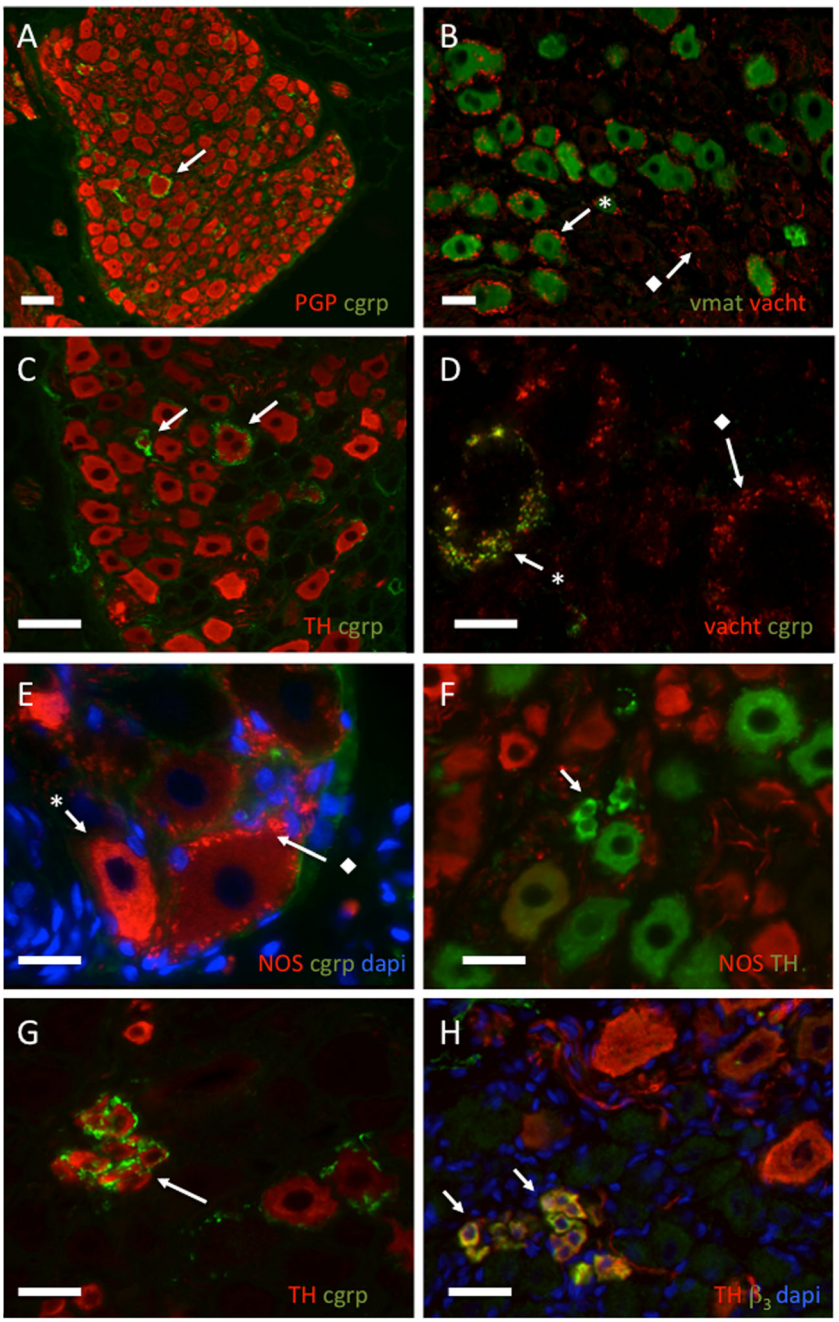

Fig. 1 Illustrations of the major cell types observable in the major pelvic ganglion (MPG) of the rat. The antibody combinations used were: **a**, *PGP* 9.5 (*red*) and calcitonin gene related peptide (*CGRP*: *green*); **b**, vesicular mono-amine transporter (*vmat*: *green*) and vesicular acetylcholine transporter (vacht: *red*); **c**, tyrosine hydroxylase (*TH*: *red*) and CGRP (*green*): **d**, vacht (*red*) and CGRP (*green*): **e**, neuronal nitric oxide synthase (NOS: *red*) and CGRP (*green*); **f**, NOS (*red*) and TH (*green*); **g**, TH (*red*) and CGRP (*green*); **h**, TH (*red*) and β_3-adrenoceptor (*green*). *Arrows and symbols* indicate specific features (see text). Sections **e** and **h** are also stained with the nuclear stain dapi (*blue*). Calibration *bars*: **a** and **c** 50 μm; **b**, **f**, **g** and **h** 30 μm; and **d** and **e** 20 μm

antibodies were visualised using donkey anti-mouse/goat/rabbit IgG antibody conjugate (Molecular Probes) Alexa Fluor 488 or 594.2°Abs were applied in PBS and used at 1:500 dilutions. The secondary antibodies were applied sequentially and were applied for 1 h at room temperature. After each incubation, slides were washed three times in TBS, TBS-T and TBS. After the final wash, sections were covered with Vectashield hard-set mounting medium with DAPI (nucleic acid molecular probe stain) and over-laid with a cover-glass (24×60 mm). Varnish was applied to the cover-glass to preserve slides.

Image and microscope analysis

Sections were viewed using an Olympus BX61 fluorescence microscope with × 10, ×20 and × 60 objectives. Images were captured using an Olympus XM10 monochrome camera in 16-bits digital format and examined further using Image J software (Java-based image processing program—National Institutes of Health (US)).

Results

Figure 1 illustrates the cell types observed in the MPG. The general architecture, with large and small nerve cell bodies and cell processes, is seen in panel a, where all structures stain with the non-specific neuronal marker PGP (Wilson et al. 1988). It is noteworthy that a small number of neurones receive fibres that are immuno-reactive for CGRP (CGRP-IR: green, arrow) (see also panels c and d). In keeping with previous studies (Keast et al. 1989), approximately half of the neurones demonstrated tyrosine hydroxylase immuno-reactivity (TH-IR) or vesicular mono-amine transporter

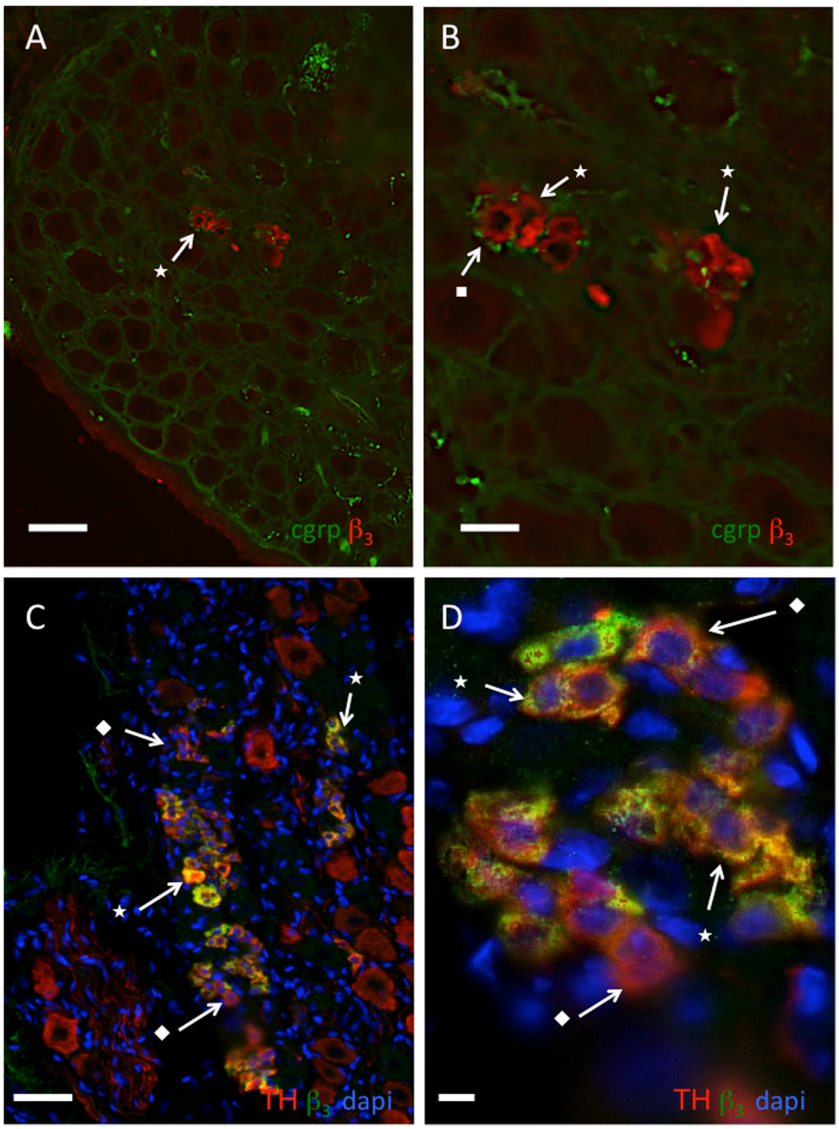

Fig. 2 Illustrations of staining associated with small-diameter SIF cells in the MPG. **a** demonstrates the observation that the only β_3-adrenoceptor immuno-reactive cells (β_3AR-IR: *red*) are SIF cells. Some β_3AR-IR cells are in close apposition to nerve fibres that are immuno-reactive for CGRP (CGRP-IR: *green*). **b** shows a section of the image in **a** at higher magnification. **c** and **d** show a collection of SIF cells stained for TH (*red*) and β_3 AR (*green*). Some cells are predominately TH-IR (*red*: *rhombus*) while there is a sub-population that are also β_3AR-IR (*green*: *star*). These cells appear yellow indicative of co-localisation. Sections in **c** and **d** are also stained with the nuclear stain dapi (*blue*). Calibration *bars*: **a** and **c** 30 μm; **b** 20 μm; **d** 10 μm

immuno-reactivity (vmat-IR) (panels b and c), suggesting that they are adrenergic. The remainder of the large neurones, showing weak immuno-reactivity to vacht, are likely to be cholinergic (see (Keast 1995). Many of the large adrenergic neurones are surrounded by vacht-IR fibres (panel b, (asterisk)). Other large neurones, weakly vacht-IR, are also surrounded by stronger vacht-IR fibres (panels b and d, (rhombus)), suggesting that these cells also are contacted by vacht-IR nerves. Large neurones, that are NOS-IR, are also found (panels e and f). Some of these neurones have NOS-IR terminals on their surface ((rhombus) panel e), suggesting the presence of nitrergic signalling between neurones. Panels f–h illustrate a further neuronal type found in the MPG. These are small-diameter TH-IR neurones (see arrowed structures in panels f, g and h). Based on the number and size, these are cells that have been previously described as the small intensely fluorescent cells (Dail et al. 1975; Eränkö 1976, 1978). Panel g shows that these cells are associated with CGRP-IR fibres. Importantly, panel h shows that these TH-IR small cells are also immuno-reactive to a β_3-adrenoceptor antibody (β_3AR-IR).

Figure 2a suggests that the only cells that demonstrate β_3AR-IR in the MPG are the SIF cells (star). No large neurones were observed with β_3AR-IR. Panel b shows a region of panel a demonstrating that the β_3AR-IR cells (star) are contacted by CGRP-IR fibres (square). Figure 2c, d shows that not all TH-IR small cells are β_3AR-IR suggesting that the population of small cells is heterogeneous. Figure 3 illustrates this heterogeneity again, but shows regional variation within the MPG. Collections of SIF cells are found with

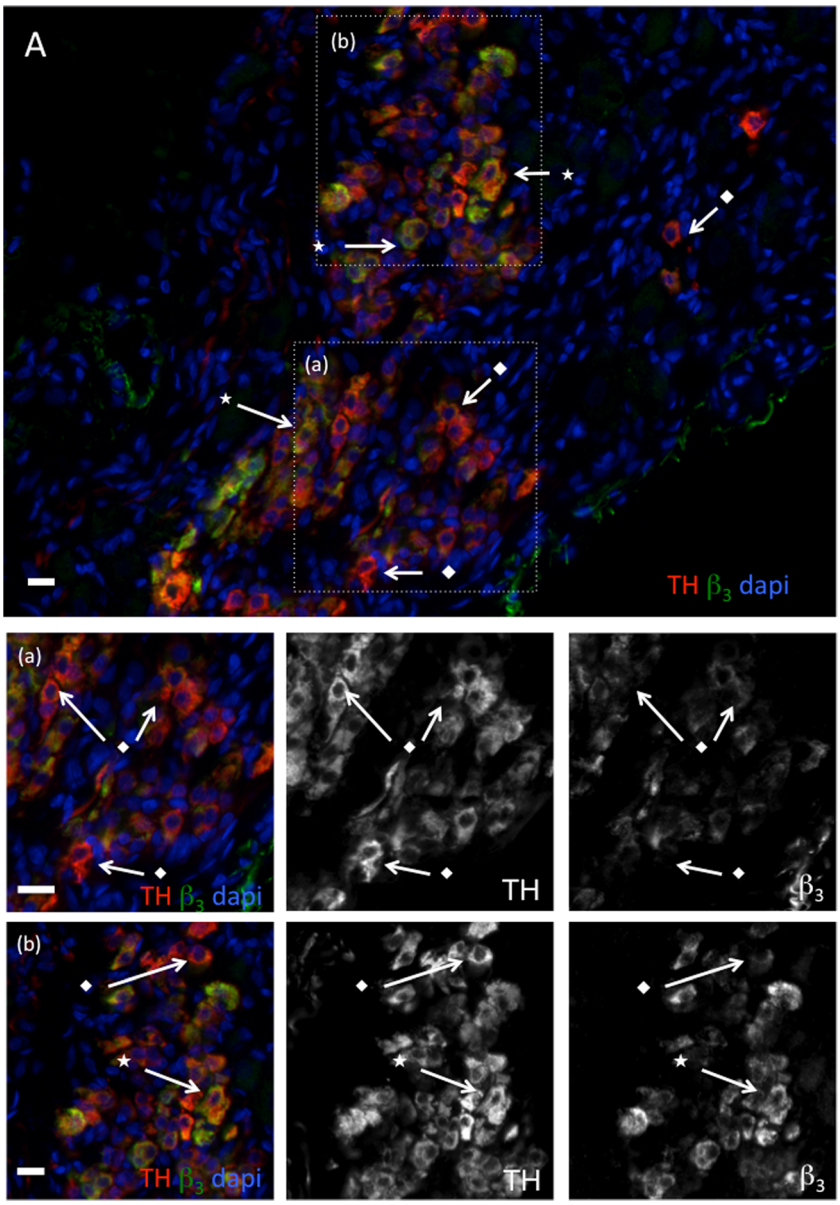

Fig. 3 Illustration of a regional variation within collections of SIF cells on the periphery of a MPG. **a** was stained to show tyrosine hydroxylase immuno-reactivity (TH-IR: *red*), β_3-adrenoceptor immuno-reactivity (β_3AR-IR: *green*) and dapi (*blue*). Predominantly TH-IR cells are indicated (*rhombus*) and cells with β_3AR-IR (*star*). Regions of the image (boxes: *a* and *b*) are illustrated in the panels below where the images are separated into the component images. Note that within these large clumps, all SIF cells are TH-IR, while only a sub-population are β_3AR-IR. *Arrows* indicate specific examples of cells or where they are present but demonstrate no immuno-reactivity. Calibration *bars* 15 μm

mixed TH-IR and β_3AR-IR (area (b)), while other areas (a) are predominantly TH-IR. The heterogeneity is emphasised in Fig. 4 that shows images at higher magnification, β_3AR-IR cells are seen in close contact to TH-IR only small cells (Fig. 4a). Also, in small clumps of SIF cells, single β_3AR-IR are often observed (Fig. 4b).

Using a characterised antibody to the type 3 muscarinic receptor (M_3R) (Grol et al. 2009), a sub-population of the SIF cells were seen to be immuno-reactive for the M_3 receptor (M_3-IR). Figure 5a illustrates a collection of small cells showing both TH-IR (red) and M_3R-IR (green). Clearly two populations are seen: TH-IR and M_3R-IR and TH-IR alone. The intensity of the M_3R-IR appears to vary from strong to weak. Part of the image is shown along with the separate images. It is clear that some small cells express TH-IR and M_3R-IR while others only express TH-IR.

It is likely that some of the small cells express both M_3R-IR and β_3AR-IR. Control experiments, using specific blocking peptides to these antibodies, were done to check this. By incubating each antibody with an excess of the specific peptide to which it was raised, then all specific antibody-binding sites are removed. Any residual staining is therefore non-specific. Figure 6 shows examples using serial sections to determine the extent of both M_3R-IR and β_3AR-IR with and without the respective blocking peptides. Figure 6a shows that the staining with the β_3 AR antibody is removed by the blocking peptide. Figure 6b shows that the staining with the M_3 antibody is also removed by blocking with its respective peptide. Thus, weight is given to the conclusion that the antibodies are detecting specifically M_3 receptors and β_3 AR on SIF cells.

Figure 7 examines in more detail the close apposition of CGRP-IR fibres on β_3AR-IR SIF cells. When present, these fibres give rise to multiple discrete foci of CGRP-IR. This pattern is seen on both β_3AR-IR and TH-IR small cells. Not all SIF cells receive CGRP-IR fibres (Fig. 7f) although CGRP-IR fibres may run close by.

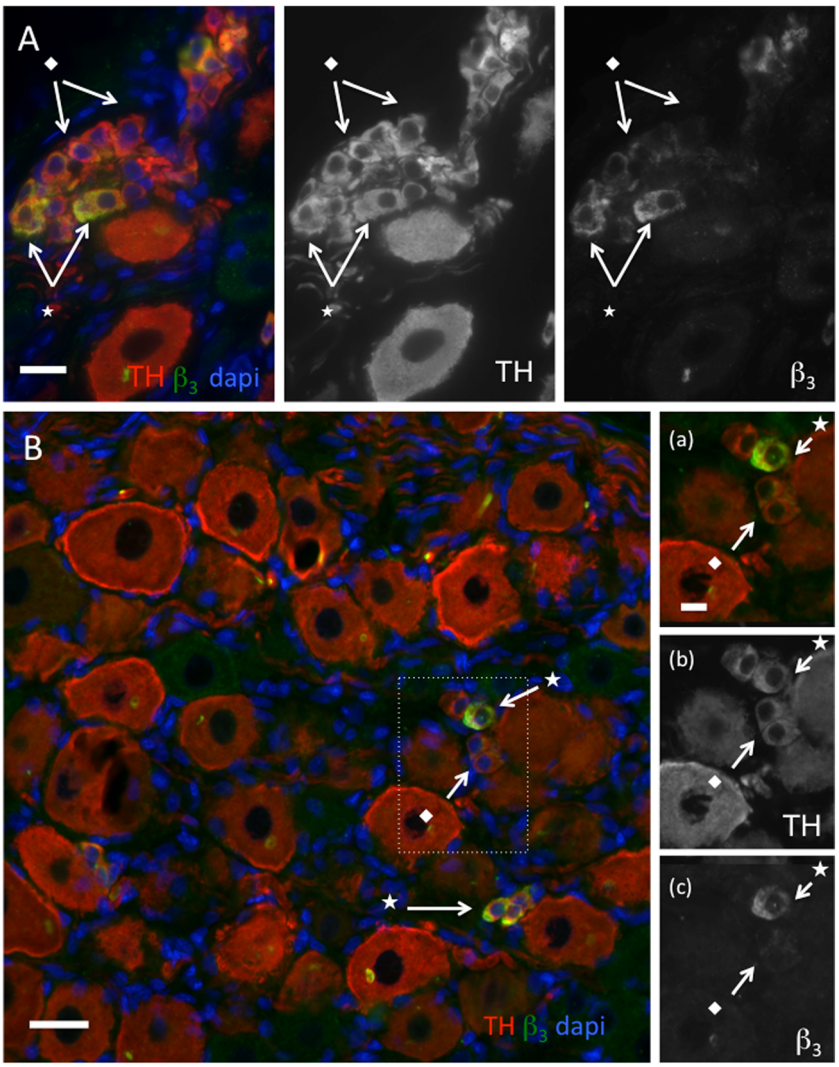

Fig. 4 Illustrations of the heterogeneity of small clumps of small SIF cells. **a** shows a section illustrating TH-IR (*red*) and β_3AR-IR (*green*) elements. Symbols and arrows indicate predominantly TH-IR (*rhombus*) and β_3AR-IR (*star*) cells. **b** shows a region of a section stained for TH (*red*) and β_3 AR (*green*). Part of this image (*dotted rectangle*) is shown in panel **a** with the component images (*b*) and (*c*). Note in (*b*) 4 TH-IR cells are seen (*rhombus*) but that only 1 cell is β_3AR-IR positive (*star*). Calibration *bars*: **a** and **b**, 20 μm (small panels 10 μm)

Since there appears to be a population of the small cells that express M_3R-IR, a series of experiments were done to see if cholinergic nerves were associated with small cells (Fig. 8). Using an antibody to vesicular acetylcholine transporter (vacht), vacht-IR could be detected in fibres within clumps of small cells (Fig. 8). Note that not all clumps of these cells show vacht-IR fibres.

Figure 9 illustrates additional observations made on small cells in the MPG. Firstly, these cells stain for an antibody to the P_2X_3 receptor. P_2X_3-IR was observed on the majority of small cells. Also, using an antibody to the enzyme responsible for production of prostaglandin (cyclooxygenase: COX1) COX1-IR was also detected. Finally, using an antibody to neuro-filament (NF), it was noted that the small cells did not express NF-IR in contrast to the large neurones in the ganglion.

Discussion

The present study has identified a population of small-diameter neurones in the MPG of the rat that express β_3AR-IR and M_3R-IR. This conclusion relies on the specificity of the antibodies used. Since both antibodies have been characterised using a number of approaches (Grol et al. 2009; Cernecka et al. 2014) and here using specific blocking peptides, the observations described here may be looked upon with some confidence. If the detection of β_3AR and M_3R immuno-reactivity can be taken as evidence for the expression of functional receptors, then these observations support the idea that these cells are sensitive to both adrenergic and cholinergic agonists.

A population of small neurones within the MPG has been described previously (see De Groat and Booth 1993 for an overview). Because these cells were intensely fluorescent when viewed after formaldehyde fixation, they were described as 'small intensely fluorescent' cells. It is postulated that the small-diameter cells seen in this study, expressing β_3AR-IR and M_3R-IR, are SIF cells.

In early work, the presence of nerve fibres contacting SIF cells with small electron transparent vesicles was taken as indicative of a cholinergic input (Eränkö 1976). Also, using formaldehyde-induced fluorescence, adrenergic nerve terminals were proposed to surround SIF cells, suggestive of an adrenergic input. Similarly, catecholamines, derived from adjacent SIF cell processes or from the circulation, may also

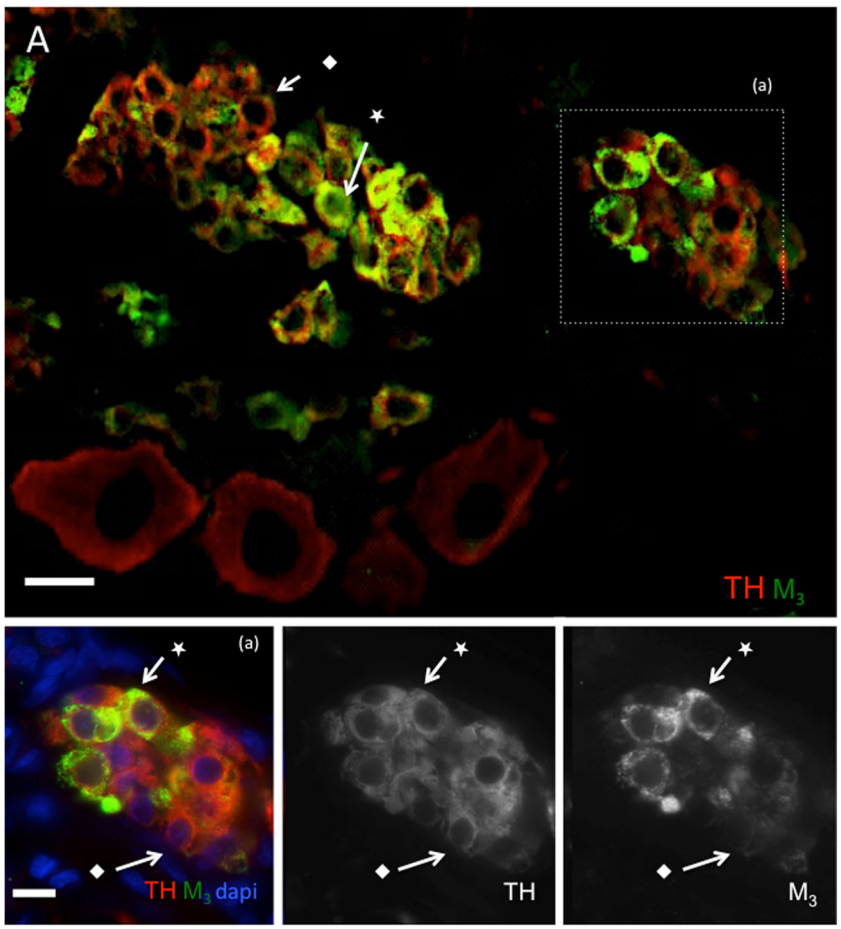

Fig. 5 Illustration of TH-IR and muscarinic type 3 receptor immuno-reactivity (M_3R-IR) in SIF cells in the MPG. **a** illustrates a large and small clump of SIF cells on the periphery of a ganglion: TH-IR (*rhombus*) and M_3 -IR (*star*). The region (*a*) is shown below (panel **a**) illustrating the combined and component images. M_3R-IR and TH-IR positive cells (*star*: *yellow*) and TH-IR only cells (*rhombus*, *red*) cells can be identified. Calibration *bars*: **a** 25 μm and (*a*) 15 μm

provide an input to SIF cells (Eränkö 1976). The present observations support these observations, showing nerve fibres in close apposition to SIF cells containing vacht, indicative of cholinergic transmission, and the presence of M_3 receptors suggesting a responsiveness to acetylcholine. The presence of β_3-adrenoceptors supports the idea that SIF cells can respond to catecholamines. Thus, there is now compelling evidence for a cholinergic and adrenergic signalling system associated with SIF cells of the MPG. What is important from this study is the identification of specific β_3AR and M_3R sub-types. It is noteworthy that not all SIF cells co-express β_3AR-IR and M_3R-IR, suggesting that there might be different populations of these cells. Indeed, two populations of SIF cells has already been suggested, type I and type II: type I have been suggested to be interneurons, while type II have properties more

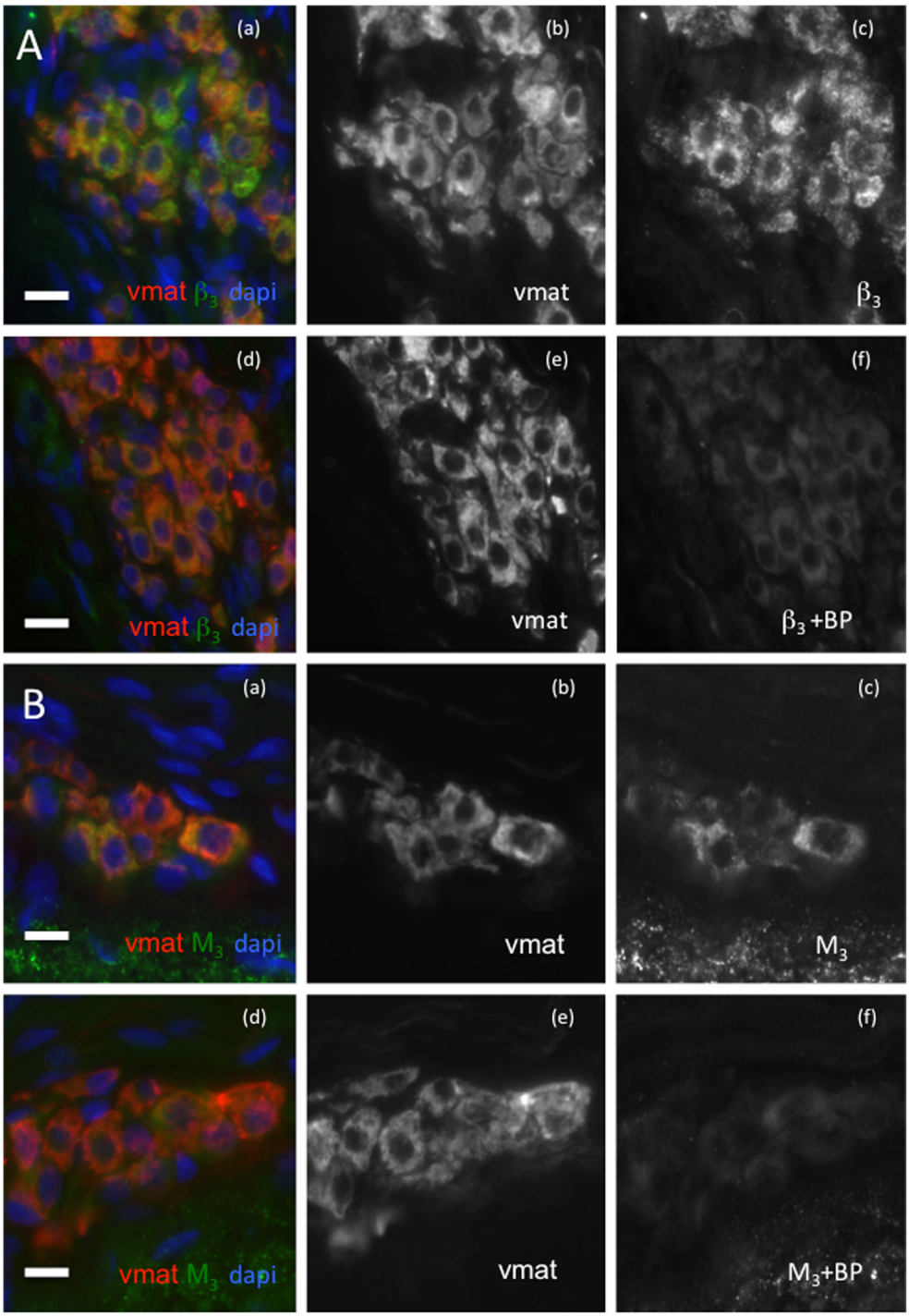

Fig. 6 Illustration of the effects of pre-incubation of the β_3 AR and M_3R antibodies with their respective blocking peptides on the detection of β_3AR-IR and M_3R-IR in small cells of the MPG. **a** (*a*) shows an image from a section stained showing vmat-IR (*red*) and β_3AR-IR (*green*) (co-localising cells appear yellow). Individual stainings are shown in (*b*) and (*c*). Panels (*d–e*) show the same region in the next section. Here, the tissue was incubated with the same vmat antibody but now in conjunction with the β_3 AR antibody plus blocking peptide. Images (*c*) and (*e*) were taken with the same exposure. **b** shows a similar experiment on serial sections examining the effects of the M_3R blocking peptide. Note, comparison of panels (*c*) and (*e*) show the loss of M_3R-IR in the presence of blocking peptide. Calibration *bars*: **a** 25 μm, **b** 20 μm

akin to neuro-endocrine cells (Owman et al. 1983; Taxi et al. 1983; De Groat and Booth 1993).

The present observations also show that SIF cells, in large and small clumps, are in close contact with CGRP-IR nerves. When associated with SIF cells, these CGRP-IR fibres appear as diffuse collections of small varicose structures, reminiscent of synaptic contacts. It is generally accepted that CGRP is found in afferent nerves. Afferent nerves from the bladder pass through the MPG en route to the dorsal root ganglia and the central nervous system. The CGRP-IR fibres, observed to contact SIF cells and also a population of large cholinergic and adrenergic neurones, are suggestive of a sensory input into neurones of the ganglia. This may constitute part of a reflex arc within the ganglion. Alternatively, the possibility exists that these CGRP fibres are not afferent and that they might originate from elsewhere, for example, the spinal cord. Evidence for this possibility remains to be obtained.

The complexity of the MPG and other peripheral ganglia has been recognised and has led to the suggestion that they are more than simple relay stations, having the machinery and capacity for information processing and modulated output. In relation to bladder function, this possibilty of complex circuitry has been explored primarily in relation to the control of functions associated with voiding (de Groat and Booth 1980; De Groat and Booth 1993). This is questionable. Although there is some evidence to suggest that there may be some modulation of efferent pathways, this does not appear to be a major site to modulate voiding.

There is evidence for information processing in other peripheral ganglia involved in motor-sensory systems in the gut, for example in the prevertebral ganglion (PVG). Here, systems modulating motility are linked to sensory processing. Mechano-sensory afferents fibres, passing through PVG send collaterals to sympathetic neurones. These peripheral inputs are then integrated with central inputs in a complex reflex system modulating gut motility and so indirectly influencing afferent outflow from the gut (Crowcroft et al. 1971; Szurszewski 1981; Szurszewski et al. 2002). The present study leads to speculation that similar reflex interactions within the MPG might modulate bladder systems that generate and

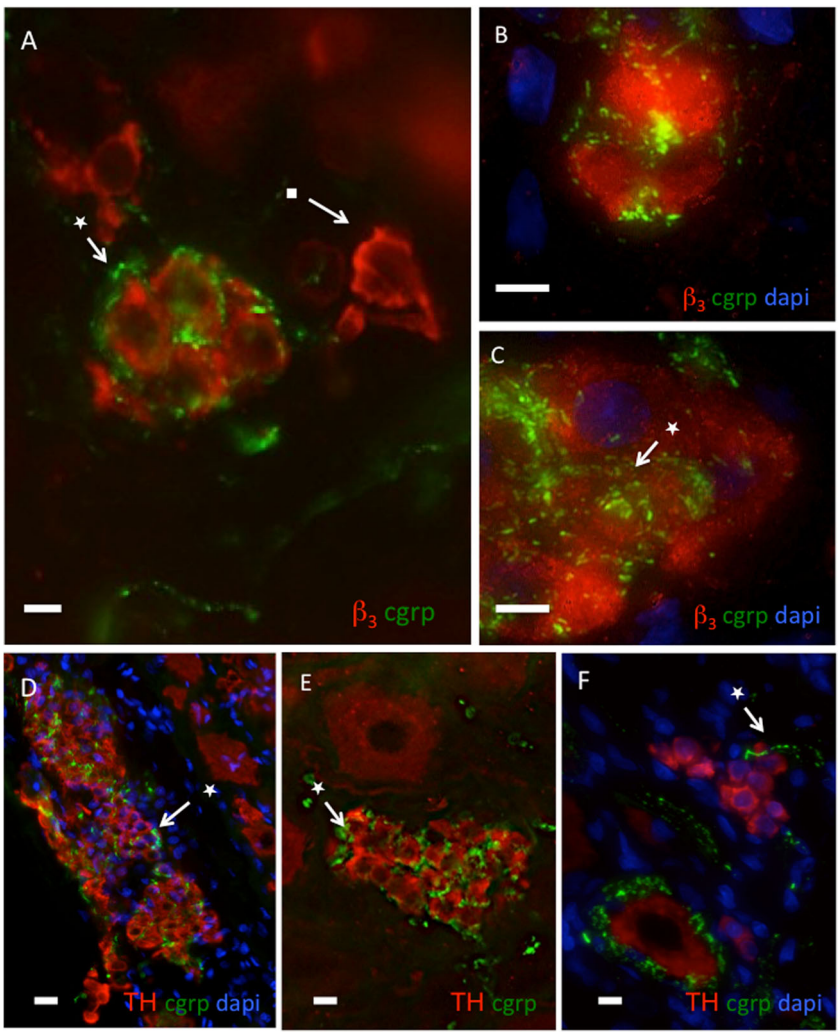

Fig. 7 Examples of the close apposition of CGRP-IR processes to β_3AR-IR cells (**a**–**c**) and TH-IR (**d**–**f**) SIF cells. A shows a small clump in which all cells are surrounded by CGRP-IR nerve processes. Adjacent to this is a single SIF cell with no CGRP-IR processes (*square*). **b** and **c** show magnified images illustrating the nature of the CGRP-IR processes on the SIF cells. Discrete punctate collections of CGRP-IR are readily seen. **d** and **e** illustrate TH-IR SIF cells in close apposition to fibres that are CGRP-IR. In **f**, a small clump of TH-IR cells is seen with no associated CGRP-IR structures. However, note the CGRP-IR fibres surrounding an adjacent large TH-IR positive neuron. Calibration bars: **a** 20 μm; **b** and **c** 10 μm; **d**, **e**, **f** and **e** 10 μm

modulate motor systems linked to afferent outflow, involved in motor control (Sugaya and de Groat 2007; Sugaya and De Groat 2009) and possibly bladder sensation (Eastham and Gillespie 2013) (see below).

It has been proposed that β_3 agonists are effective in relieving the symptoms of OAB (Sacco and Bientinesi 2012; Sacco 2014). Specific β_3-adrenoceptor agonists such as mirabegron were developed with the idea that they mimicked the effects of the sympathetic system in the bladder wall, to cause a relaxation of the detrusor and so reduce or remove activity associated with OAB. Indeed, there is evidence demonstrating the presence of β_3-adrenoceptors in the bladder of both humans and rats (Seguchi et al. 1998; Fujimura et al. 1999). Also, that these receptors are coupled to adenylyl cyclase and that this system can relax pre-contracted strips of human and rat bladder (Igawa et al. 1998; Seguchi et al. 1998; Fujimura et al. 1999). Thus, a possible therapeutic mode of action of this class of drug was put forward. However, there is a difficulty with this proposed therapeutic mode of action of the β_3-adrenoceptor agonists. In both humans and rats, the adrenergic innervation of the detrusor is sparse with large areas of the muscle having no adrenergic nerves (Watanabe and Yamamoto 1979; Dixon et al. 1992). Therefore, a function of physiological sympathetic regulation of the detrusor in the main body of the bladder wall is open for discussion.

In the rat, it has been shown that β_3-adrenoceptor agonists are capable of modulating storage function. In one model of bladder over activity, intra-vesical infusion of PGE_2, it was shown that the β_3-adrenoceptor agonist CL316,243 prolonged bladder filling time and so reduced voiding frequency (Takeda

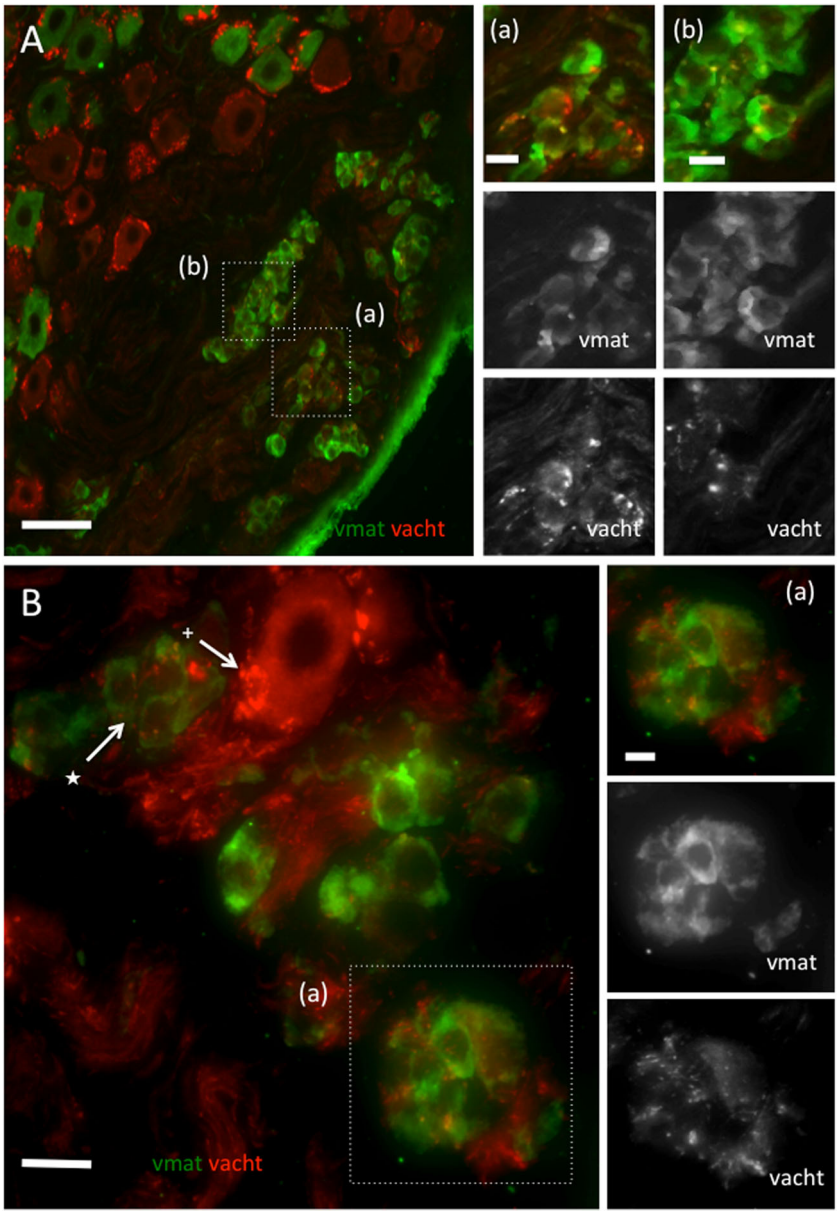

Fig. 8 Example of further complexities of the signalling elements that might impinge on SIF cells. **a** shows vesicular acetylcholine immuno-reactive (*vacht-IR*) nerve fibres in close apposition to SIF cells. vmat-IR large neurones (*square*: *green*) are seen to be surrounded by vacht-IR fibres (*red*). vmat-IR negative large neurones (putative cholinergic neurones) are also contacted by vacht-IR fibres (*star*, *red*). The *boxes* delineated by (*a*) and (*b*) are shown at higher magnification. vmat-IR positive SIF cells (*green*) are seen to be in close proximity to vacht-IR fibres (*red*). Only a small number of SIF cell clumps exhibit vacht-IR fibres. **b** shows further examples of small-diameter SIF cells that are not contacted by vacht-IR fibres and clumps of cells that are. Calibration bars: **a** 50 μm (insets 10 μm), **b** 20 μm (insets 10 μm)

et al. 2002). Also, in a decerebrate rat model, to study bladder function, plasma concentrations of mirabegron of 100 nM were sufficient to modulate bladder function (Sadananda et al. 2013). Therefore, in the rat, as in human, there is evidence for actions of β_3-adrenoceptor agonists at low concentrations that are not mediated via sympathetic supply to the bladder wall or relaxation of the detrusor.

In a study using a different model of bladder over activity, cystometry in conscious rats with partial bladder outflow obstruction, it was shown that mirabegron and the M_3R antagonist tolterodine had effects on the NVA observed during bladder filling: both mirabegron and tolterodine reducing NVA (Gillespie et al. 2012). It has been argued that NVA is part of a motor-sensory system operating in the bladder: the motor activity being coupled to afferent nerve discharge (Iggo 1955; Gillespie 2004). As the bladder fills, the motor activity increases, afferent outflow increases and, possibly, sensation is increased (Starling 1905; Iggo 1955; Vaughan and Satchell 1995; Gillespie 2004; Eastham and Gillespie 2013). Based on these observations, it was proposed that NVA and sensation were modulated by cholinergic excitatory and β adrenergic inhibitory inputs. However, where this regulation takes place is unknown.

The observations presented here, that SIF cells possess both β_3AR-IR and M_3R-IR, might provide some further insight. The SIF cells of the MPG appear to be a primary site for the expression of β_3-adrenoceptors in structures associated with the organs of the lower pelvis. Since no major effects of β_3-adrenoceptor agonists or M_3 receptor antagonists have been noted on gut, prostate and penile function (Sacco and Bientinesi 2012; Chapple 2014; Chapple et al. 2014; Sacco 2014), it seems plausible that the β adrenergic system in which they operate is the bladder. Therefore, SIF cells may be a key element in the control of bladder function, possibly in the control of afferent outflow and sensation. As SIF cells express both β_3AR-IR and M_3R-IR, it is possible that these collections of cells in the pelvic ganglion are the site for the physiological integration of both cholinergic and adrenergic inputs. The SIF cells are potentially part of a greater neural network, consisting of afferent inputs from the bladder wall and CNS that has the capability to influence the generation and regulation of NVA. The reflex pathways and the intra-ganglionic elements possibly involved in

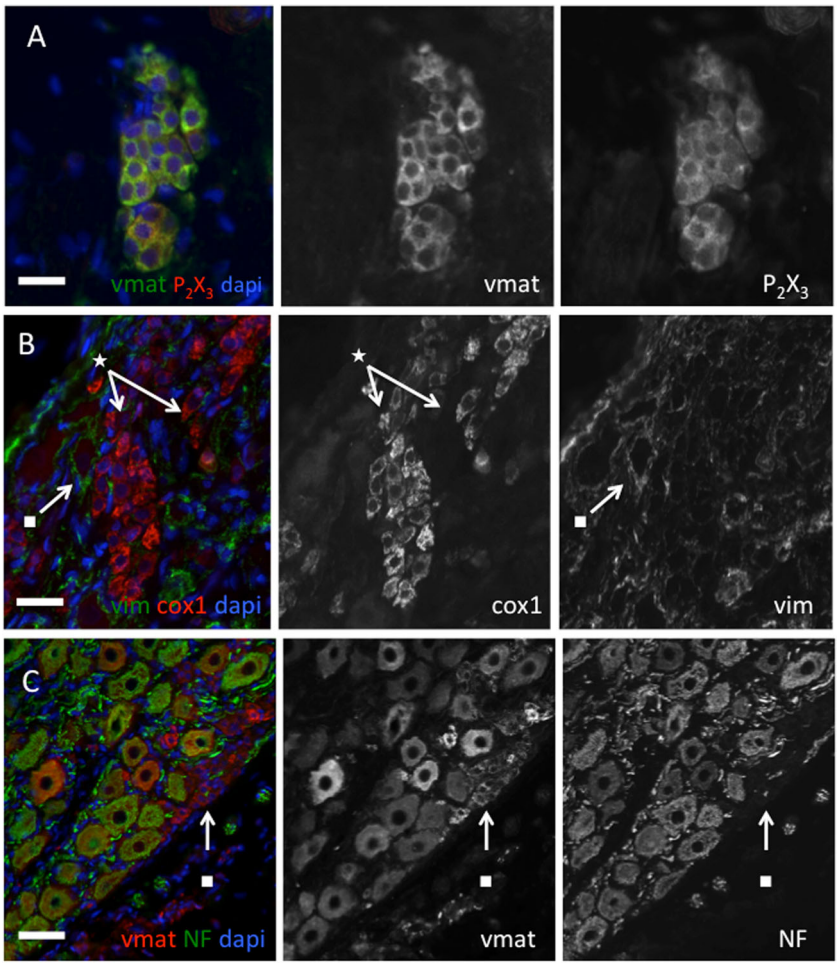

Fig. 9 Examples of additional immuno-reactivity of small cells in the MPG. **a**, **b** and **c** shows respectively staining with antibodies to the purinergic receptor P_2X_3, the enzyme cyclooxygenase (*COX1*) and to neuro-filament (*NF*). Note that the small cells do not stain with the NF antibody. Calibration *bars*: 50 μm

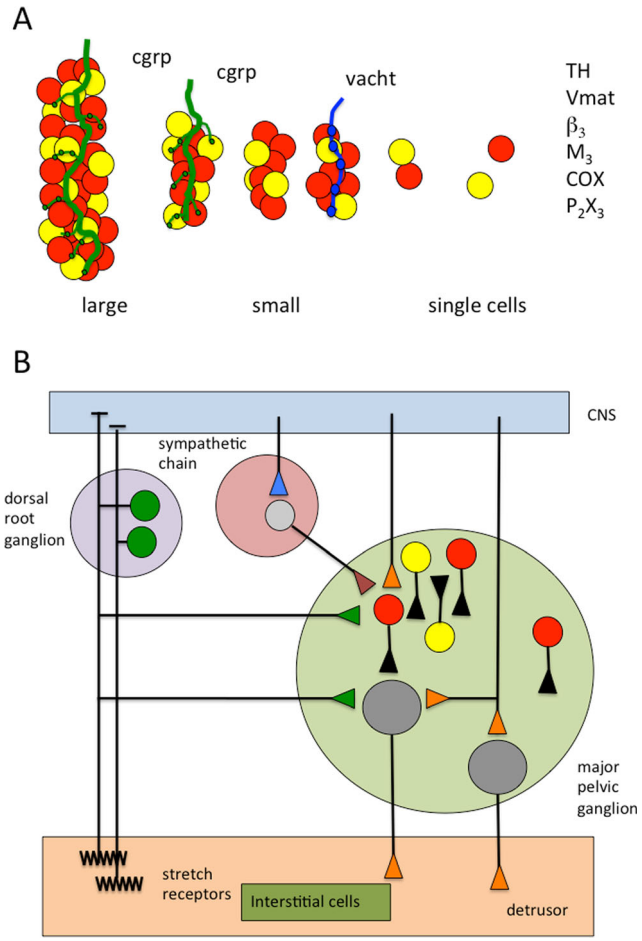

Fig. 10 Cartoons summarising the key observations. **d** shows that clumps of SIF cells of different sizes are readily found within all regions of the MPG. All cells are TH-IR and vmat-IR. There is the indication that all cells may also be COX1-IR and P_2X_3-IR. In addition, there appear to be sub-populations that are β_3AR-IR or M_3R-IR or may express both β_3AR-IR and M_3R-IR. The largest clumps and the majority of smaller clumps are associated with fibres showing CGRP-IR. Some clumps show no CGRP-IR. A small number of small clumps also appear to have fibres that are vacht-IR, suggesting the presence of cholinergic nerve fibres. Single, isolated SIF cells are also found. **b** suggests (1) possible complex interactions that might occur within the MPG and between the different components and (2) possible physiological systems that such interactions and neural circuitry might involve. Such circuitry is similar to that described in the inferior mesenteric ganglion (Szurszewski et al. 2002). SIF cells are likely to have inputs from sympathetic adrenergic neurones [ref] and cholinergic fibres (vacht-IR). These fibres may be involved in the activation of β_3 AR and M_3R on sub-populations of SIF cells. They also have inputs from afferent fibres (CGRP-IR). As SIF cells also express TH-IR and vmat-IR, there is the likelihood that they can release catecholamines, suggesting that they can act in a paracrine manner on adjacent cells within the clumps. Based on observations on isolated tissues and the whole animals using β_3 AR agonists (Gillespie et al. 2012; Gillespie et al. 2015b), it is proposed that this system may function to modulate motor activity during the filling phase: non-voiding activity. This system is in addition to but different from that involved in activation of the detrusor during voiding

this motor-sensory regulation are illustrated in Fig. 10b. Thus, it can be hypothesised that the site of peripheral and central modulation of the motor system involved in generating afferent outflow from the bladder in the rat resides in the MPG.

If this analysis for the situation we observed in rats, in spite of the anatomical differences brought up earlier in this paper, is translatable to the human situation, it implies a concept that there are mechanisms located in the periphery, capable of modulating bladder afferent outflow. The possibility of having both excitatory and inhibitory inputs suggests that it could function to increase or decrease afferent outflow from the bladder. If so, then this represents a possible mechanism that would allow a degree of control of bladder sensation. It is part of everyday experience that sensations of bladder fullness can increase and decrease, despite there being no change in bladder volume. Almost certainly, such modulation will have a central component. However, the modulation of sensation by β_3-adrenoceptor agonists and specific M_3 cholinergic antagonists, the identification of β_3AR-IR and M_3R-IR on SIF cells and the possibility that these elements are involved in regulating a motor-sensory system suggest that there are also mechanisms in the periphery involved in the modulation of bladder sensation (Eastham and Gillespie 2013).

Compliance with ethical standards This manuscript has been prepared to comply with all required ethical standards. There are no conflicts of interest to be declared.

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