Identification of peripheral oxytocin-expressing cells using systemically applied cell-type specific adeno-associated viral vector

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AUTHOR CONTRIBUTIONS

M.L., L.P. conceived the study; V.G. produced and contributed the viral vector; A.A. L.P. performed surgery and collected tissue; L.P., M.Lo., A.A. performed immunofluorescence and acquired images; L.P., M.Lo., A.A., analysed images and data; L.P. wrote the manuscript; M.L. and V.G. edited and reviewed manuscript; M.L. secured research funding. All authors approved the final version.

ABSTRACT

Oxytocin is primarily synthesised in the brain and is widely known for its role in lactation and parturition after being released into the blood from the posterior pituitary gland. Nevertheless, peripheral tissues have also been reported to express oxytocin. Using systemic injection of a recombinant adeno-associated virus (rAAV) vector, we investigated the expression of the green fluorescent protein Venus under the control of the oxytocin promoter in the gastrointestinal tract, pancreas and testes of adult rats. Here we confirm that the vector infects oxytocin neurones of the enteric nervous system (ENS) in ganglia of the myenteric and submucosal plexuses. Venus was detected in 25–60% of the ganglia in the myenteric and submucosal plexuses identified by co-staining with the neuronal marker PGP9.5. Oxytocin expression was also detected in the islets of Langerhans in the pancreas and the Leydig cells of the testes. Our data illustrate that peripheral administration of the viral vector represents a powerful method to selectively label oxytocin-producing cells outside the brain.

INTRODUCTION

The pituitary hormone oxytocin is best known for its role in smooth muscle contraction after secretion from the pituitary gland into the bloodstream. In addition to its classical roles in parturition and milk ejection and its role in electrolyte homeostasis in rodents (1), oxytocin has emerged as a regulator of the brain-gut axis. Oxytocin is involved in energy homeostasis (2, 3), and hormonal signals released by the gastrointestinal tract in response to food intake (4), including cholecystokinin (CCK), secretin(5) and insulin(6), modulate the activity of oxytocin neurones.

Conversely, there is evidence that oxytocin is involved in regulating gut motility, enteric neuronal activity, mucosal homeostasis, intestinal permeability and inflammation (7). Oxytocin receptors are expressed in several cell types of the rat

intestine, including villi enterocytes and neurones of the myenteric and submucosal plexuses (8, 9). Oxytocin given systemically stimulates gastric emptying (10) and colon motility, and exogenous oxytocin can restore peristaltic movements in patients with gastric atony (11). Disruption of oxytocin signalling in oxytocin receptor-deficient mice results in increased excretion of faeces and faecal water content, and increased total bowel transit time (7). Finally, administration of the oxytocin receptor antagonist atosiban decreases gastric emptying in humans (12).

Oxytocin is also synthesised locally in peripheral organs including the gut. The enteric nervous system (ENS) contains more than 400 million neurones in humans. These neurones are organised in networks of interconnected ganglia located in the walls of the gastrointestinal tract, constituting both the myenteric (Auerbach) and submucosal (Meissner) plexuses. Immunohistochemical studies have reported oxytocin immunoreactivity throughout the rat gastrointestinal tract and, in colchicine-treated rats, immunoreactive oxytocin was found in neurones of the ENS (8). In the human, PCR and immunohistochemistry respectively showed expression of mRNA for the oxytocin precursor and of the peptide in different segments of gastrointestinal tract (13, 14).

In addition to the gut (13, 14), there is evidence that oxytocin is also synthesised in other peripheral organs, including the uterus (15), placenta, testis (16), pancreas (17, 18), and heart (19) along with its receptor expression in the same peripheral organs (13, 18, 20-22).

Several pieces of evidence also suggest that oxytocin can act directly at the pancreas in several species, stimulating insulin and glucagon secretion (23-25). In mice and humans, oxytocin has been shown to potentiate glucose-stimulated insulin secretion and also to stimulate beta-cell proliferation and prevent apoptosis (17). Oxytocin-receptor deficient mice showed an absence of oxytocin's protective effect against cytokine stress in isolated pancreatic islets, and impaired insulin secretion in response to a glucose tolerance test after exposure to a high-fat diet (18).

In human and rat testes extracts, immunoassays revealed a high concentration of oxytocin, suggesting either local peptide synthesis or uptake and accumulation by the tissue (26). Subsequent studies have revealed expression of the oxytocin gene in several species including human (27), bovine (28) and rat (29). In bovine and sheep testes, oxytocin mRNA appears to be expressed at a moderate level in Sertoli cells, but immunoreactive oxytocin and neurophysin 1 appear to be confined to the Leydig

cells, which contain no oxytocin mRNA detected by *in situ* hybridisation. Thus it seems that the Sertoli cells contain larger amounts of mRNA but express little or no oxytocin because of a post-translational block, but the Leydig cells may produce low levels of functional transcripts (30), although in monkeys oxytocin mRNA is expressed at highest levels in Leydig cells (31).

However, immunohistochemical studies may detect peptide that is taken up by tissues rather than synthesised there or may reflect cross-reaction of antibodies with other related antigens. Moreover, detection of mRNA by PCR does not necessarily imply the occurrence of any significant amount of synthesis. For example, the pregnant rat uterus expresses very large quantities of oxytocin mRNA – about 70 times the total hypothalamic content (15) – but the uterine mRNA differs from hypothalamic mRNA in having a truncated poly-A tail, and this appears to render it virtually inactive: the pregnant rat uterus contains just 2 ng of oxytocin per g net weight of tissue, compared to a total pituitary content of about 1.5 µg. These low levels of peptide may be accounted for binding of circulating oxytocin of pituitary origin to the highly abundant oxytocin receptors expressed in the pregnant rat uterus, and there is no evidence that significant amounts of oxytocin reach the circulation from the uterus.

Accordingly, we sought an alternative way to test whether oxytocin is expressed in the gastrointestinal tract, pancreas and testes. In the present study, we used systemic administration of cell-specific AAV driving Venus as a brightest version of GFP under the control of the oxytocin promoter to characterise the expression and distribution of oxytocin-producing cells in selected peripheral tissues.

METHODS

Animals

Adult male Sprague Dawley rats weighing 330 - 370 g were kept on a 12:12 h light/dark cycle (lights on at 07.00 h) at a room temperature of 20 - 21 °C, with *ad libitum* access to water and food. Five rats were injected intraperitoneally (i.p.) with the AAV, which expressed Venus under the control of the oxytocin promoter (32). Fifty microliters of the concentrated virus solution were added to 250 µl of sterile saline, and a total volume of 100 µl/kg was administered per rat. Based on previous pilot experiments, rats were then left for 6 weeks to allow transduction of target cells and the expression of Venus. All of the procedures were conducted in accordance with the

UK Home Office Animals Scientific Procedures Act 1986, and a project licence approved by the Ethical Committee of the University of Edinburgh.

Cloning of rAAV vector and production of rAAV

The rAAV vector was cloned as described previously (32). Briefly, for generating the rAAV with specific expression in oxytocin cells, we used the software BLAT from University of California, Santa Cruz (http://genome.ucsc.edu/cgi-bin/hgBlat) and selected a conserved 2.6 kb promoter directly upstream of the oxytocin gene exon 1. This DNA was amplified from an EcoRI-linearized BAC clone RP24-388N9 (RPCI-24 Mouse, BACPAC Resources) using a 5′ primer containing a NotI-restriction site (5′-ATTAGCGGCCGCAGATGAGCTGGTGAGCATGTGAAG ACATGC-3′) and a 3′ primer with a SalI-restriction site (5′-ATTAGTCGACGGCGA TGGTGCTCAGTCTGAGATCCGCTGT-3′), subcloned into pBlueScript SK and further cloned into the rAAV2 backbone, pAAV-αCaMKII-htTA, thereby substituting the αCaMKII-promoter. The resulting rAAV expression vector was used to exchange the htTA-gene for the gene of interest, Venus.

Production and purification of rAAV (Serotype 1/2) was as described (33). rAAV genomic titers were determined with QuickTiter AAV Quantitation Kit (Cell Biolabs) and RT-PCR using the ABI 7700 cycler (Applied Biosystems). rAAVs titers were $\sim 10^{10}$ genomic copies per µl.

Tissue collection

Six weeks after i.p. injection of the AAV, rats were euthanised with sodium pentobarbital (160 mg/kg) and were then transcardially perfused with 350 ml of heparinised (20 U/ml) 0.9 % saline solution to flush out blood. The gastrointestinal tract was then removed surgically, dissected into its different regions (i.e. stomach (antrum), duodenum, ileum, jejunum, colon) and the luminal content gently flushed out with 0.1 M phosphate buffer (PB) solution (pH 7.4), using an 18 G needle. The pancreas and testes were also removed and with the gut regions fixed by immersion in paraformaldehyde (PFA) 4 % in 0.1 M PB for 24 h which was then changed to increasing concentrations (10% every 24 h) of sucrose solution in 0.1M PB, to reach a final concentration of 30% 48 h after fixation. The tissue was kept at 4 °C in the 30% sucrose solution for at least 72 h.

Small pieces of tissue from each gut region were embedded in Cryo-M-Bed (Bright Instrument Co. Ltd. Huntingdon, UK) and frozen rapidly using dry ice, then 15-µm sections were cut transversally using a cryostat and mounted onto super frost slides. Other larger pieces of tissue were prepared for longitudinal sections Swiss roll technique, adapted from Williams et al. (34). Briefly, the pieces of fixed tissue were cut along their length with spring scissors and gently opened. One end was then gripped with self-closing forceps and the intestines gently rolled evenly around the forceps ensuring the rolls were not too tight and edges were kept flush. The completed roll was secured with a 30 G needle and the tissue frozen in Cryo-M-Bed and cut into 15-µm sections using a cryostat.

This allowed us to examine the expression of oxytocin in both myenteric and submucous plexuses within different segments of the gastrointestinal tract, while preserving its morphology, and so one in every 8 sequentially cut sections (i.e. 120 µm) was mounted on the slide to avoid double counting of neural structures.

Pieces of pancreatic and testicular tissue were placed in Tissue Tek® Cryomold® moulds, embedded in Tissue Tek® OCT cryo-embedding compound, quickly frozen and cut into sections of 12 µm thickness. Sections were mounted onto gelatine-subbed slides and stored at -20°C prior to immunostaining.

Immunohistochemistry

The slides containing tissue sections were washed between steps in 0.1 M PB + 0.3 % Triton X-100 (PB-T; pH 7.4). Sections were incubated in blocking buffer solution containing 10% normal goat serum (NGS) and 1% bovine serum albumin (BSA) in 0.1 M PB-T (pH 7.4) for 1 h at room temperature, and were then incubated for 48 h at 4°C with primary antibodies (Table 1) diluted in blocking buffer. After incubation, the sections were washed and then incubated for 2 h at room temperature with the secondary antibody (Table 1) diluted in blocking buffer. After this, the slides were washed and incubated with 4′,6-diamidino-2-phenylindole (DAPI) diluted in 0.1 M PB (pH 7.4) to concentration 1:30,000 for 5 min. After washing with PBS, Vector® TrueVIEW® Autofluorescence quenching kit (SP-8400, Vector Laboratories) was applied for 5 minutes followed by an additional PBS wash. Slides were then cover-slipped using Permafluor mounting medium (cat #TA-030-FM; Fisher Scientific, Loughborough, UK). Incubations were carried out in a light-protected humidity chamber. To check the specificity of the immunoreactions, negative controls were

included in all immunofluorescence histochemistry experiments by omitting the addition of the primary antibody; no immunofluorescence signal was detected in these sections (e.g. Fig. 5A).

As the gastrointestinal tract tissue exhibited green auto-fluorescence, the expression of Venus was confirmed immunohistochemically (anti-GFP) and the immunoreaction visualised using Alexa Fluor 546 (orange) secondary antibody, then this signal was digitally transformed into green colour to make the labelling of Venus and protein gene product 9.5 (PGP9.5; Alexa Fluor 647 — red) more obvious.

Double immunochemistry was performed for Venus and oxytocin-neurophysin by incubating sections with the two primary antibodies simultaneously (Table 1). The immunoreactions were revealed following the same procedures as indicated above. For oxytocin-neurophysin immunostaining, sections were incubated in mouse anti-rat PS38-oxytocin-neurophysin monoclonal antibody kindly provided by Prof H. Gainer (NIH, Bethesda, MD, USA) diluted 1:5000. This antibody recognises the heterologous parts of oxytocin-neurophysin but not the nine-amino acid oxytocin peptide (35). Sections were incubated in 0.1 M PB for 1 h at room temperature in biotinylated horse anti-mouse IgG antibody (Vector Laboratories, Inc., Peterborough, UK). To visualise the oxytocin-neurophysin immunoreactivity, a solution containing 0.025% diaminobenzidine and 0.015% H_2O_2 in 0.1 M Tris was used.

Immunofluorescence histochemistry on brain sections was carried out with a similar protocol, but using free-floating sections. After staining, sections were mounted on gelatinised slides and cover-slipped using Permafluor mounting medium (cat #TA-030-FM; Fisher Scientific, Loughborough, UK).

Image acquisition and data analysis

Images were acquired using a Zeiss LSM800 confocal microscope. For each anatomical region of the gastrointestinal tract, four sections were analysed by capturing four randomly selected non-overlapping microscopic fields at 10x magnification (1277.8 x 1277.8µm) in each section. All the images captured contained PGP9.5 -labelled ganglia that were identified by visualising only the red channel, so as to avoid bias for selecting areas with Venus-labelled cells. Then, using a PC running Fiji version 1.52n, ganglia of enteric neurones were confirmed as positive and counted when they contained at least one nucleus stained with DAPI completely engulfed by PGP9.5 (red) fluorescence. Identified nerve structures were then analysed for

colocalization of Venus immunofluorescence. Three investigators independently analysed and quantified the number of ganglia containing neurones that expressed Venus.

The numbers of PGP/DAPI and PGP/DAPI/Venus-labelled ganglia were counted and the percentage of colocalization calculated in each image (microscopic field) and averaged for each section. The values in each section were averaged for the different gut regions in each rat, and the values are expressed as percentage of ganglia expressing Venus per microscopic field. Data are means ± SEM; only descriptive statistics were used in this study.

RESULTS

We used chimeric serotype 1/2 AAV, which permeates the blood-brain barrier poorly and thus has limited access to the brain after systemic injection. The hypothalamic magnocellular oxytocin neurones are the main source of oxytocin produced within the brain. There was little Venus expression in the hypothalamic paraventricular nucleus (data not shown) but several neurones in the rostral portion of the supraoptic nucleus (Fig. 1) were positive for Venus. All Venus-expressing neurones were double-labelled for oxytocin-neurophysin, indicating promotor-specific expression of Venus despite the low permeability of the AAV.

Identification of nerve structures in the gut

To reveal the presence of nerve structures in sections of gastrointestinal tract we initially used NeuN and PGP9.5 antibodies as markers of mature neurones. Labelling by the NeuN antibody was limited and patchy and it was therefore not used subsequently. PGP9.5 is expressed in the cytoplasm of the diffuse neuroendocrine system (36) and strong labelling was found in the plexuses of the ENS, as reported previously (37-40). In our study the PGP9.5 antibody also labelled cells and nerve structures, and these were detected throughout the different regions of the gut (Fig. 2). As described previously (41), autofluorescence often occurs from inherent tissue components (collagen, elastin, lipofuscin, and red blood cells) and the extent and intensity of autofluorescence background made it difficult to distinguish the virally transduced cells in the tissues from the background signal (Fig. 2A-C). The use of antibody against GFP (Venus) for signal amplification and adopting an additional step

in the immunohistochemistry protocols to quench the autofluorescence drastically improved image quality in the later parts of the study (Fig. 2D,E).

Oxytocin-neurophysin expression in the rat gastrointestinal tract

In the myenteric and submucosal plexuses (Fig. 3A), Venus-positive cells were detected in ganglia and also in some single cells. In the sections analysed, no Venus-positive cells were found outside the submucosal layer and the boundary separating the circular and longitudinal muscular layers (Fig. 2).

In the myenteric plexus, Venus immunosignal was detected in 41±3% of the ganglia (range 35 - 46%; n = 5 rats); the highest number of Venus-positive ganglia was found in the duodenum and the lowest in the jejunum. In the submucosal plexus, the average number of ganglia Venus-immunopositive ganglia was 41±7 % (range 25 - 60%; n = 5 rats); the highest percentage of nerve structures containing Venus-positive cells was found in the colon, and the lowest was found in the jejunum (Fig. 3B). We also detected Venus-positive cells in the myenteric plexus of the stomach antrum in a small number of sections (not shown), but this was not quantified.

Notably, oxytocin-neurophysin immunosignal overlapped Venus immunoreactivity in all cell types of the gastrointestinal tract (Fig. 2E).

Oxytocin-neurophysin expression in the pancreas and testes

Venus-immunopositive cells in pancreas were restricted to its endocrine part and were exclusively detected in the islets of Langerhans (Fig. 4A-C), confined to the cells also labelled with an antibody against oxytocin (Fig. 4B). Furthermore, Venus and oxytocin immunosignals were colocalized with a few insulin immunoreactive cells in Langerhans islets (Fig. 4C,D).

The analysis of testes revealed that the Venus-immunopositive cells are located in the interstitial spaces (Fig. 5B), predominantly in the clusters of Leydig cells. Oxytocin expression in those cells was then confirmed immunohistochemically using oxytocin-neurophysin antibody (Fig. 5C).

DISCUSSION

Initially, our study aimed to investigate the expression of oxytocin in the nerve structures of the rat gastrointestinal tract. Accordingly, we found oxytocin-neurophysin expression in the myenteric and submucosal plexuses of the ENS in all the regions of

the gut analysed. These results confirm previous studies reporting the expression of oxytocin in the gastrointestinal tract in different species (8, 13, 14, 42).

In our study, we quantified ganglia containing labelled cells and found that Venus was expressed in approximately 40% of both myenteric and submucosal ganglia co-labelled with PGP9.5, the marker for enteric neurones. In the guinea pig ~15% of the myenteric and ~50% of the submucosal ganglion neurones have been reported to express immunoreactive oxytocin (42); in the myenteric plexus, immunoreactive oxytocin was expressed exclusively in the intrinsic enteric afferent neurones. In biopsies taken from the gastrointestinal tracts of elderly humans,10-30% of cells of the myenteric plexus were reported to express immunoreactive oxytocin, and in the submucosal plexus, immunoreactive oxytocin was reported in 30-50% of neurones in the ileum and 50-70 % of neurones in the colon (14).

Oxytocin-expressing neurones in the myenteric and submucosal ganglia are believed to project their nerve fibres to the inner circular muscle layer. In the guinea pig, a high density of oxytocin immunoreactive nerve fibres has also been described around the glands in the mucosa of the intestine and colon, and oxytocin here might act on the intestinal epithelium and gland epithelium to influence absorption and secretion (42).

Our anatomical findings may offer the technical prospect of studying the effects of oxytocin on gut motility and transit (21, 43). These effects might be mediated by release of oxytocin from the posterior pituitary gland, and some gut-related peptides, such as CCK, secretin and insulin, stimulate oxytocin release into the bloodstream (5, 6, 44). However, it is possible that oxytocin synthesised within the gastrointestinal tract modulates functions in an autocrine/paracrine manner. Supporting this notion, oxytocin been shown to induce relaxation of the colon (45, 46), and to reduce the sensitivity of mesenteric afferents (47) *in vitro* by a mechanism that involves the release of nitric oxide by enteric cells expressing oxytocin receptors. Furthermore, disruption of oxytocin signalling has been associated with an increased myenteric neuronal response to cholera toxin *in vitro*, and reduced villus height and crypt depth *in vivo* (7). In line with this, oxytocin application attenuates the expression of transcripts encoding inflammatory pathway genes in a necrotising enterocolitis mice model (48), and also the activation of intracellular signalling pathways related to inflammatory response following lipopolysaccharide application to Caco2BB gut cells (49). However, the physiological significance of endogenous oxytocin in the gastrointestinal

tract in health and disease needs to be further evaluated by available viral vector means.

Exploring our work to other tissues we surprisingly also found expression of oxytocin/Venus-positive cells in rat pancreatic islets. Detection and quantification of a higher content of oxytocin in human and rat pancreatic extracts than that present in plasma has led to the notion of locally synthesised oxytocin (50). However, Watanabe et al. (18) found abundant oxytocin receptor mRNA in the mouse pancreatic islets, they found only trace levels of oxytocin mRNA. Nevertheless, Mohan et al. (17) reported oxytocin immunostaining in cells of isolated mouse islets, mostly in insulin-containing cells; in the present study, we detected the immunohistochemical expression of both Venus and oxytocin in cells of the rat pancreatic islets, suggesting that they express functional oxytocin transcripts, but we found relatively little co-localisation of insulin and oxytocin.

Furthermore, the analysis of testes revealed oxytocin/Venus immunosignal in Leydig cells, in line with previous studies reporting the expression of oxytocin mRNA and peptide in rat and bovine testes (30, 51, 52). Oxytocin in the testis is thought to have a role in regulating reproductive and endocrine functions, including modulation of steroid metabolism, contractility of the seminiferous tubules, and seasonal changes in the reproductive system in some species (53-55).

Recombinant adeno-associated viruses (rAAV) are normally delivered directly into the brain to study central neuronal systems, which circumvent the blood-brain barrier that restricts the access of several molecules including most of the rAAV serotypes (56, 57). In accordance, we detected only few Venus/oxytocin positive neurons in the rostral part of the supraoptic nucleus and the PVN.

In the hypothalamus, oxytocin is translated from a three-exon gene into a large precursor consisting of a pre-pro-hormone of 125 amino acid residues: exon A encodes a 19-amino acid signal peptide, the 9-amino acid biologically active oxytocin peptide, and the first 9 amino acids of its associated neurophysin carrier; the core section of the neurophysin and its COOH-terminal 16 amino acid residues are encoded by exons B and C, respectively (58). In the cell bodies of oxytocin neurones, this precursor molecule is packaged into large dense-core vesicles, each containing about 80,000 molecules of the precursor. These vesicles are transported down the axons to the posterior pituitary from where they will be secreted into the systemic circulation, and *en route* the precursor molecule is processed enzymatically to yield

various fragments including oxytocin itself and its associated neurophysin (59, 60). Thus in oxytocin-expressing cells oxytocin is present at equimolar concentrations with its associated neurophysin. However the (much larger) neurophysin molecule is much easier to detect immunocytochemically (35).

The immunohistochemical detection of oxytocin itself in non-hypothalamic sources (where the peptide is synthesised at low rates) and axonal pathways has been difficult and commonly requires colchicine treatment to accumulate the peptide vesicles within the cell bodies (61), which in the rat gut enhances oxytocin punctuate staining in the neuropile of myenteric ganglia and some cell bodies (8). Otherwise, Venus is ubiquitously distributed throughout the cytoplasm of infected oxytocin cells, and has previously been shown to be a sensitive and specific method for identifying neuronal projections that have escaped conventional immunohistochemical detection (32, 62). Whether expression of the oxytocin gene, as reflected by Venus expression, is consistently accompanied by the expression of biologically active oxytocin remains to be determined as the antibody against the oxytocin-associated neurophysin used in this study recognises both the precursor and mature forms of neurophysin (35). Further studies should consider the use of specific antibodies targeting the (bioactive) oxytocin nonapeptide as reported recently (63), and the use of oxytocin-deficient animals as an additional (negative) control for Venus and oxytocin-neurophysin labelling in peripheral tissues.

The results presented here demonstrate that viral vector-based systems are powerful tools to label peripheral peptide-synthesising cells, which overcomes problems of detection of peptides of interest using conventional immunohistochemistry. Future studies using genetic expression of fluorescence markers and manipulation of activity of virally-infected cells by chemo- and optogenetic means will allow more thorough interrogation of the function of these cells in physiological and pathological conditions both *in vitro* and *in vivo*.

TABLE AND FIGURE LEGENDS

Table 1: Primary and secondary antibodies used for immunohistochemistry

Antibody	Dilution	Catalogue #	Manufacturer
Primary			
Rabbit anti-PGP9.5	1:4000	Z511601-2	Agilent Technologies, Cheshire, UK
Chicken anti-GFP	1:5000	AB13970	Abcam, Cambridge, UK
Mouse anti-Oxytocin-neurophysin	1:5000	PS-38	Prof H. Gainer (NIH, Bethesda, USA)
Guinea pig anti-Oxytocin	1:200	AB51637	Abcam, Cambridge, UK
Rabbit monoclonal anti-Insulin	1:500	EPR17539 (AB181547)	Abcam, Cambridge, UK
Secondary			
Alexa Fluor 546 goat anti-mouse	1:500	A-11003	Invitrogen, Paisley, UK
Alexa Fluor 546 goat anti-chicken	1:500	A-11040	
Alexa Fluor 647 goat anti-rabbit	1:500	A-21245	
Alexa Fluor 488 goat anti-chicken	1:500	A-11039	
Alexa Fluor 546 goat anti-rabbit	1:500	A-11035	
Alexa Fluor 546 goat anti-guinea pig	1:500	A-1107	
Alexa Fluor 647 donkey anti-rabbit	1:500	711-605-152-JIR	Stratech Scientific, Ely, UK

Figure 1: Cell-type expression of Venus in virally-infected oxytocin neurones. A) Oxytocin neurones of the rostral supraoptic nucleus expressing Venus under the control of the oxytocin promoter (green) 6 weeks after intraperitoneal administration of the adeno-associated virus. **B)** Immunolabelling for oxytocin-neurophysin (red) and **C)** overlay (yellow). OC, optic chiasm.

Figure 2: Immunohistochemistry of Venus and oxytocin expression in the adult rat gut. A, B) Examples of ganglia (red) expressing Venus (green, yellow in merged images, white arrows) in the **A)** the myenteric plexus of the ileum, and **B)** the submucosal plexus of the duodenum. **C,D)** Example of ganglia that do not express Venus (yellow arrow) in the myenteric plexus of the colon. **E)** Example of a viral-transfected cell (green) in the colon that is also stained by fluorescence histochemistry for oxytocin (red). Nuclear marker (DAPI) in blue.

Figure 3: Oxytocin (Venus) expression in the adult rat gut. **A)** Haematoxylin and eosin staining of a section from the duodenum. **B)** Percentage of PGP9.5-positive nerve structures (ganglia) expressing Venus in the myenteric (blue bars) and submucosal (orange bars) plexuses of the enteric nervous system in different regions of the gut. Each dot represents the (average) value per animal analysed; n=5, mean ± SEM.

Figure 4: Immunohistochemistry of Venus, oxytocin and insulin expression in the adult rat pancreas. A) Examples of islet of Langerhans expressing Venus (green) and after signal amplification with antibody against Venus (red), yellow in merged images. **B)** Example of a viral-transfected islet (green) which is also stained by fluorescence histochemistry for oxytocin (red). **C)** Example of a viral-transfected islet (green) co-stained for insulin. **D)** Example for double fluorescence histochemistry for insulin (green) and oxytocin (red). Nuclear marker (DAPI) in blue.

Figure 5: Immunohistochemistry of Venus and oxytocin expression in the adult rat testes. A) Haematoxylin and eosin staining of a section from the testes **B)** Examples of testes tissue expressing Venus (green) and negative control without primary antibody treatment. **C)** Example of a virally-transfected tissue (green) which is

also stained by fluorescence histochemistry for oxytocin (red). The overlay shows oxytocin expression in the Leydig cells. Nuclear marker (DAPI) in blue.

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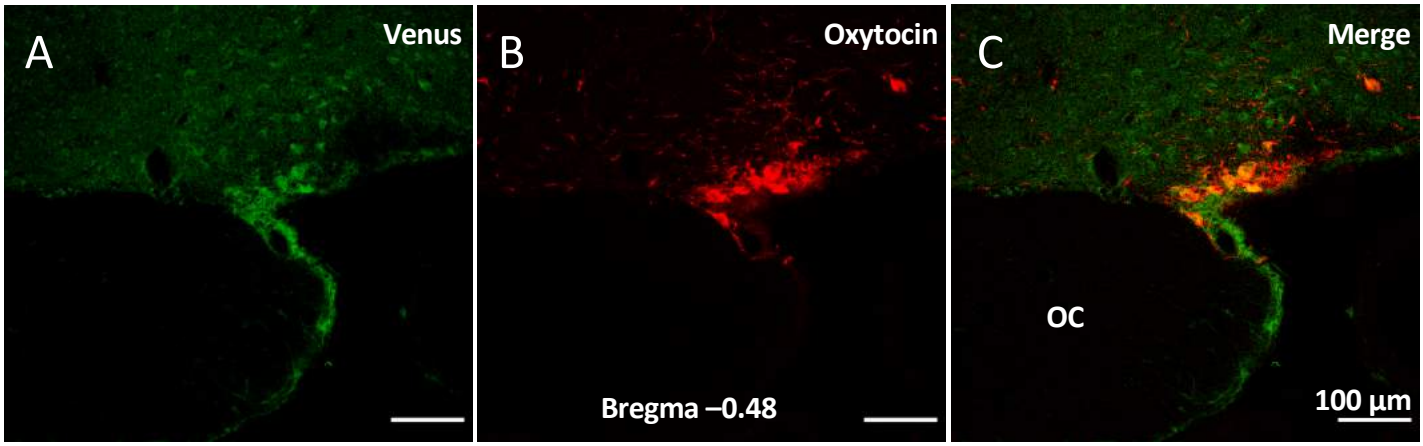

Figure 1: Cell-type expression of Venus in virally-infected oxytocin neurones . A) Oxytocin neurones of the rostral SON expressing Venus under the control of the oxytocin promoter (green) 6 weeks after of intraperitoneal administration of the adeno-associated virus. **B)** Immunolabelling for oxytocin-neurophysin (red) and **C)** overlay (yellow). OC, optic chiasm.

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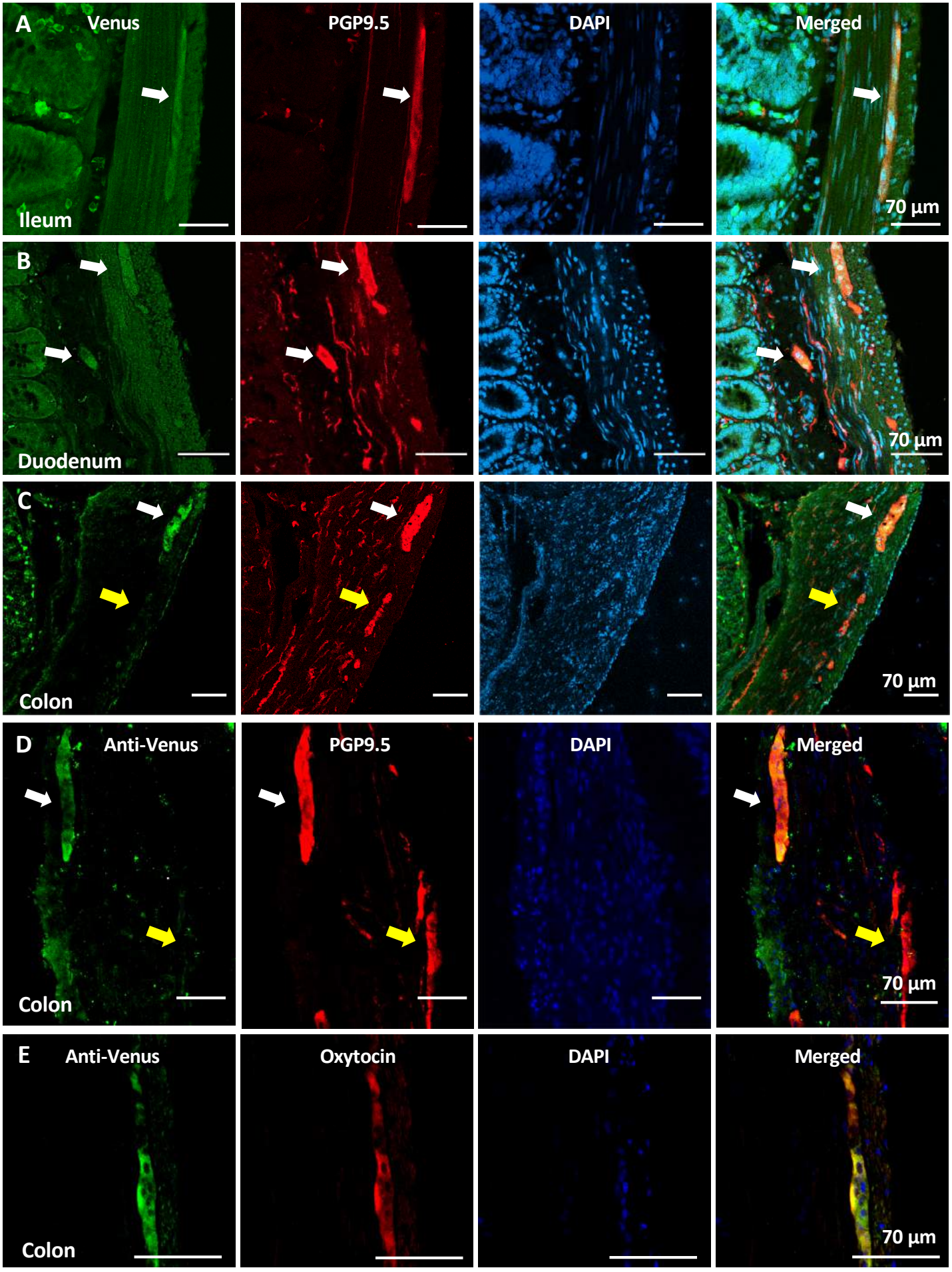

Figure 2: Immunohistochemistry of Venus and oxytocin expression in the adult rat gut. A, B) Examples of ganglia (red) expressing Venus (green, yellow in merged images, white arrows) in the **A)** myenteric plexus of the ileum, and **B)** the submucosal plexus of the duodenum. **C,D)** Example of ganglia which do not express Venus (yellow arrow) in the myenteric plexus of the colon. **E)** Example of a viral-transfected cell (green) in the colon which is also stained by fluorescence histochemistry for oxytocin (red). Nuclear marker (DAPI) in blue.

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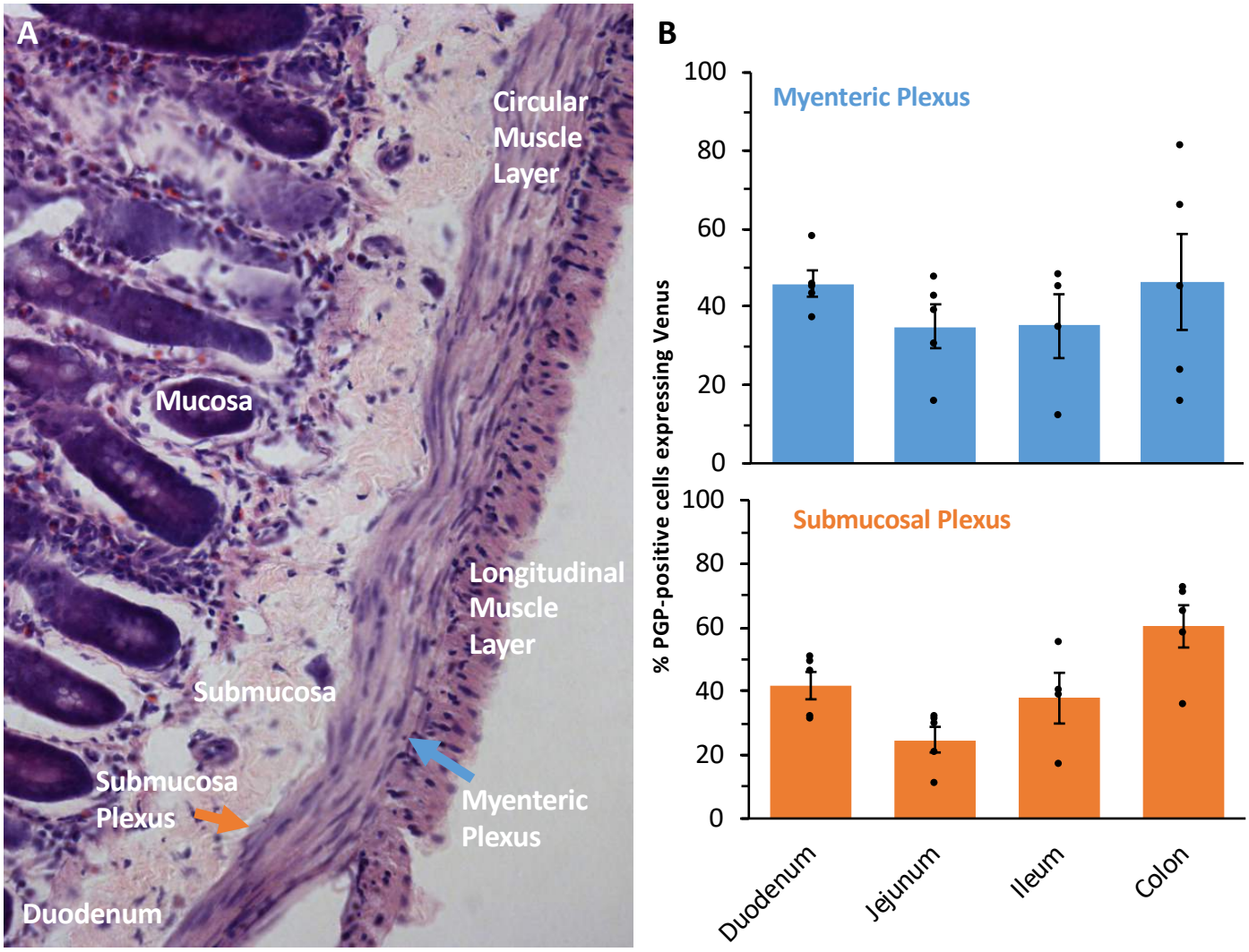

Figure 3: Oxytocin (Venus) expression in the adult rat gut. **A)** Haematoxylin and eosin staining of a section from the duodenum. **B)** Percentage of PGP9.5-positive nerve structures (ganglia) expressing Venus in the myenteric (blue bars) and submucosal (orange bars) plexuses of the enteric nervous system in different regions of the gut. Each dot represents the (average) value per animal analysed; n=5, mean ± SEM.

Paiva et al., Fig. 3

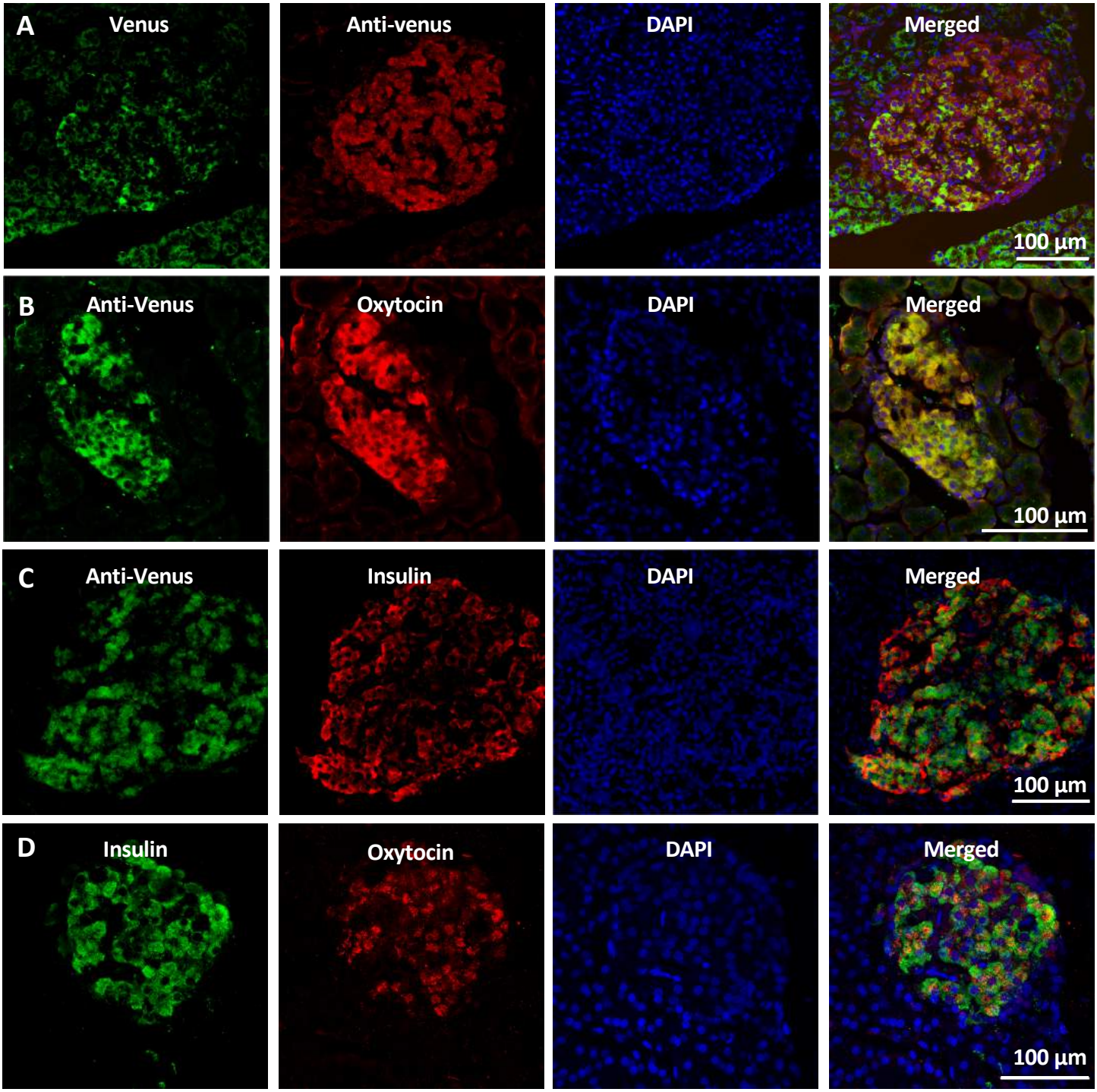

Figure 4: Immunohistochemistry of Venus, oxytocin and insulin expression in the adult rat pancreas. A) Examples of islet of Langerhans expressing Venus (green) and after signal amplification with antibody against Venus (red), yellow in merged images. **B)** Example of a viral-transfected islet (green) which is also stained by fluorescence histochemistry for oxytocin (red). **C)** Example of a viral-transfected islet (green) co-stained for insulin. **D)** Example for double fluorescence histochemistry for insulin (green) and oxytocin (red). Nuclear marker (DAPI) in blue.

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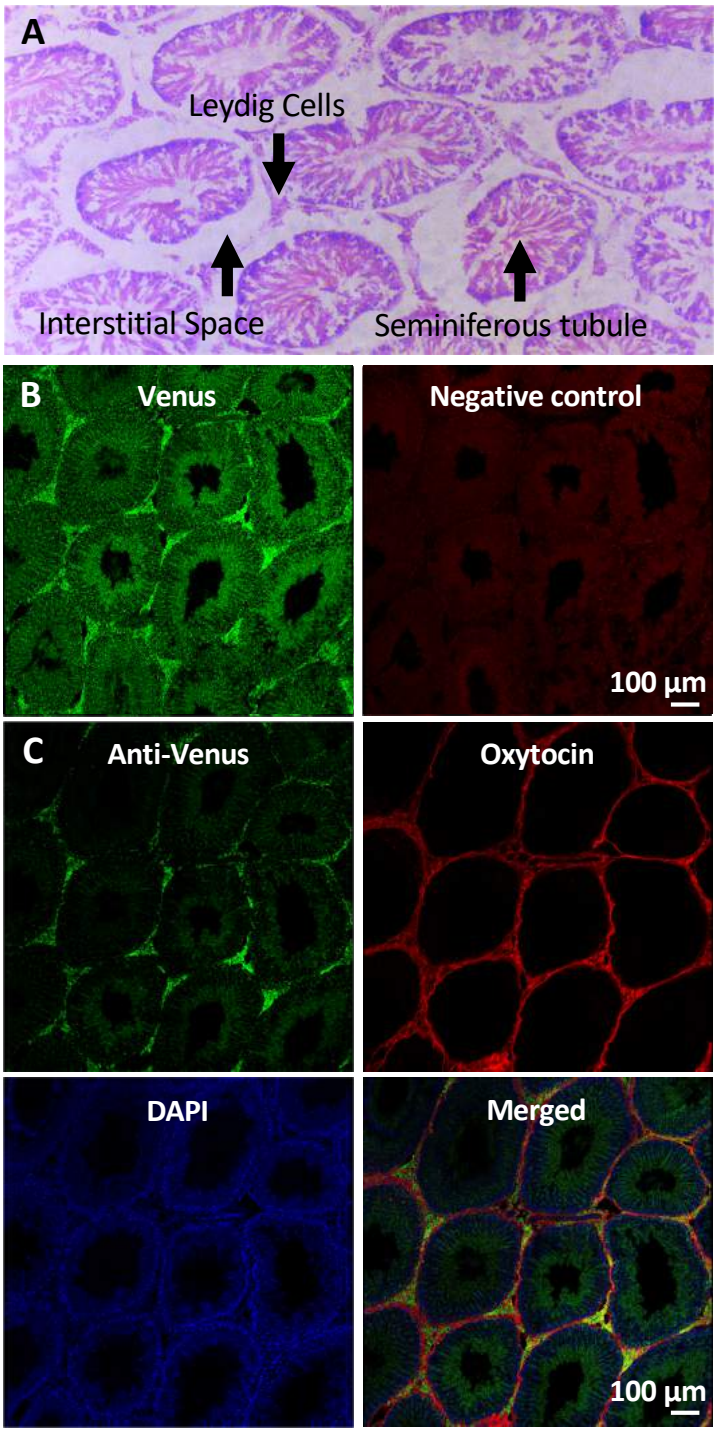

Figure 5: Immunohistochemistry of Venus and oxytocin expression in the adult rat testes. A) Haematoxylin and eosin staining of a section from the testes **B)** Examples of testes tissue expressing Venus (green) and negative control without primary antibody treatment. **C)** Example of a viral-transfected tissue (green) which is also stained by fluorescence histochemistry for oxytocin-neurophysin (red). The overlay shows oxytocin expression in the Leydig cells. Nuclear marker (DAPI) in blue.

Paiva et al., Fig. 5