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Co-storage and secretion of growth hormone and secretoneurin in retinal ganglion cells

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ABSTRACT

It is well established that growth hormone (GH) and granins are co-stored and co-secreted from pituitary somatotrophs. In this work we demonstrate for the first time that GH- and secretoneurin (SN) immunoreactivity (the secretogranin II (SgII) fragment) are similarly present in retinal ganglion cells (RGCs), which is an extrapituitary site of GH expression, and in quail QNR/D cells, which provide an experimental RGC model. The expression of SgII and chromogranin A in the pituitary gland, neuroretina and QNR/D cells was confirmed by RT-PCR analysis. Western blotting also showed that the SN-immunoreactivity in somatotrophs and QNR/D cells was associated with multiple protein bands (24, 35, 48, 72, 78, 93 and 148 kDa) of which the 72 kDa and 148 kDa bands were most abundant. Secretoneurin was constitutively secreted from QNR/D cells as 35 kDa and 37 kDa proteins and unlike GH, was not increased by exogenous GH-releasing hormone (GHRH). Intracellular analysis by EM showed co-localization of GH and SN in cell bodies and neurites in QNR/D cells. This co-localization was associated with small dark bodies in the neurites. In addition, co-localization of GH and SNAP-25 in the cell surface of QNR/D's plasma membranes suggests GH-release involves specific vesicle-membrane recognition in QNR/D cells. As SN is a marker for secretory granules, GH secretion from RGCs is thus likely to be in secretory granules, as in somatotrophs.

1. Introduction

Chromogranins (CgA, CgB and SgII/CgC) and secretogranins (SgIII–VII) are members of a family of acidic secretory peptides known as granins that are co-stored and processed inside secretory vesicles with other neuropeptides and hormones (Feldman and Eiden, 2003; Helle, 2004; Zhao et al., 2009; Trudeau et al., 2012). They are, for instance, found in somatotrophs of the pituitary gland (Fischer-Colbrie et al., 1995), in which growth hormone (GH) is co-stored and co-released from secretory granules (Hashimoto et al., 1987). Secretogranin (II) mRNA is similarly expressed in GH-secreting GH3B6 (Laverriere et al., 1991) and GH_4C_1 (Hinkle et al., 1992; Scammell and Valentine, 1994) cells. Secretoneurin (SN) has also be found in the retinal ganglion cell (RGC) layer in the human neural retina (Overdick et al., 1996), which is an extrapituitary site of GH expression (Sanders et al., 2010). The possibility that GH and secretoneurin are co-stored and co-secreted from RGC's has therefore been examined in the present study, using QNR/D cells, which provide an experimental model for GH secretion (by GHRH and TRH stimulation) for RGCs as they express conserved RGC molecular cell markers such as Brn3 and Thy1 (Martinez-Moreno et al., 2014a,b).

2. Materials and methods

2.1. Quail neural retina cell line (QNR/D) and cell culture

An embryonic quail neuroretinal cell line (QNR/D, American Type Culture Collection No.: CRL-2532) was obtained from Cedarlane Laboratories (Burlington, Ontario). Cells were defrosted in a 40 °C water bath for 2 min and washed 3 times with DMEM (5 ml each time). Cells were then resuspended with 2 ml of DMEM medium with 10% fetal serum (FBS), 1% antibiotic–antimitotic (containing 10,000 μg/L of penicillin, 10,000 μg/L of streptomycin and 25 μg/L of amphotericin B) and placed in 35 mm diameter tissue culture dishes and incubated at 40 °C in 5% CO_2 in water jacketed incubator for 48 h. The cells were cultured from 1 culture flask into 6 new flasks every 5–6 days, when they reached 80–95% of confluence. The cells used for experiments were used between culture passages 20 and 35, in order to preserve phenotypical characteristics. For immunochemistry analysis, 3×10^5 cells were

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subcultured on glass coverslips treated with poly-L-lysine hydrobromide (Sigma–Aldrich Inc., St. Louis, MO, USA) and laminin (1 μg/cm^2) (Sigma–Aldrich). For Western blot analysis, cells were cultured in 60 mm culture dishes and then incubated with 1 μM GHRH, an effective dose to induce GH release in QNR/D cultures (Martinez-Moreno et al., 2014b) (synthetic peptide 1–29; Bachem Bioscience Inc., No. H-3705.000) for 15 min.

2.2. RT-PCR

Total RNA was extracted from adult quail anterior pituitary glands (from Health Sciences Laboratory Animal Services, University of Alberta, Edmonton), chicken pituitary glands (from heads of slaughterhouse broiler fowl) and QNR/D cells from subcultures. Total RNA isolation was performed using Trizol reagent (Invitrogen, Canada Inc., Burlington, Ontario) and an RNeasy Mini Kit (Qiagen, Ontario, Canada), including a digestion of any contaminating genomic DNA by RNase-Free DNase Set (DNase I treatment; Qiagen, Ontario, Canada). First strand cDNA was synthesized by reverse transcription of 1 μg DNA-free total RNA using Super Script 3 Reverse Transcriptase (Invitrogen, Canada Inc.) according to manufacturer's instructions. One microliter of oligo(dT)$_{20}$ primer was used to reverse transcribe total mRNA. Two microliters of first stand cDNA was amplified in the presence of 10 μM sense and antisense cChA (cChA-F directed to exon-2, 5′-TGAATAAAGGGGACACTAAGG-3′ and cChA-R directed to exon-6, 5′-AGCTCAGCCAGGGATG-3′) and cSG2 primers (cSG2-F, 5′-TCGGTGTTTCAGGAGTTGGG-3′ and cSG2-R 5′-ATTCAGCCA GCTCTTCGTCC-3′). Both primers were designed to amplify avian genes (chromogranin A, XM_421330.4; secretogranin-II, XM_422624.4) and also to cross exon–intron boundaries, as a control for genomic DNA contamination. PCR reactions were performed in the presence of 10× PCR buffer, 50 mM $MgCl_2$, 10 mM dNTP mix, and 0.2 μl Platinum Taq High Fidelity DNA polymerase. The PCR mixtures were first denatured at 94 °C for 5 min, and then subjected to 30 cycles of 94 °C, 30 s denaturation, 55 °C, 30 s annealing, and 72 °C, 30 s elongation, with a final extension at 72 °C using a thermal cycler (Techgene, Fisher Scientific, Canada). The amplified cDNA was identified by the size of the amplicon (337 bp and 943 bp for chromogranin A and secretogranin-II, respectively) compared with a 1 kb plus DNA ladder (Invitrogen, Canada Inc.).

2.3. SDS–PAGE and Western blot analysis

Total proteins in QNR/Ds and chicken pituitary glands were extracted in using homogenization buffer (HCl–Tris 0.05 M, pH 9.0) in the presence of a protease inhibition cocktail (Mini-complete, Roche). Media samples were concentrated 10–1 using Amicon Ultra-15 Centrifugal Filter Devices (Merck Millipore Ltd., Tullagreen, IRL). Equivalent amounts of extracted proteins (50 μg) and 20 μl of concentrated media were separated by 12.5% SDS–PAGE and transferred onto nitrocellulose membranes (Bio-Rad). Nitrocellulose-free binding sites were blocked with 5% non-fat milk (Bio-Rad, Cat. No. 170-6404, Hercules, CA, USA) in PBS (Gibco, NY, USA) for 2 h at room temperature. Then, membranes with protein samples were incubated overnight at 4 °C with a rabbit anti-goldfish SN antibody (1:2000) (Zhao et al., 2006) diluted in TPBS (1× PBS with 0.05% (v/v)). Antibody specificity was determined by antigen preabsorption, in which the SN antiserum (1:2000) was pre-absorbed at 4 °C overnight with 1 μM of goldfish SN. After washing the membranes with TPBS (2 × 5 min) they were then incubated for 2 h with the secondary antibody (biotinylated goat anti-rabbit-IgG (Vector), diluted 1:5000). Membranes were then incubated for 30 min with Vecstatin elite ABC reagent and washed (3 × 10 min) with PBS. Bands were visualized using ECL Blotting Detection Reagent (Amerasham-Pharmacia, Buckinghamshire, UK) on autoradiography film (Fujifilm, Tokyo, Japan). Kaleidoscope molecular weight markers (Bio-Rad) were used as reference for mass determination.

2.4. Immunocytochemistry

QNR/D cells were fixed by immersion in Carnoy's solution (60% methanol, 30% chloroform and 10% acetic acid) for 2 min. They were then washed in PBS and incubated for 1 h in a blocking solu-

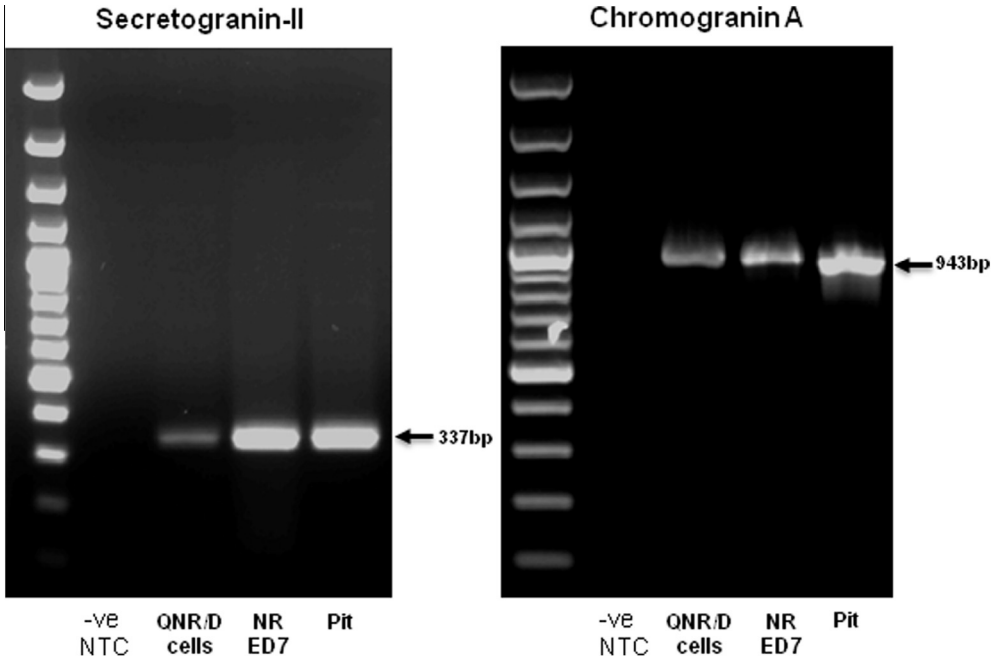

Fig. 1. RT-PCR of secretogranin-II and chromogranin A mRNA in QNR/D cells, chicken embryo neuroretina (NR ED7) and pituitary gland (Pit). Total tissue RNA was extracted and amplified in the presence of oligonucleotide primers (cSG2 and cChA) designed to amplify a 337 bp and 943 bp fragments respectively. These fragments were amplified with reverse transcribed RNA extracted from QNR/D's, neuroretina and pituitary. cDNA was then amplified for 30 cycles and stained by ethidium bromide. This fragment was not evident when PCR was performed in the absence of template (−ve NTC). The data shown is representative of three samples.

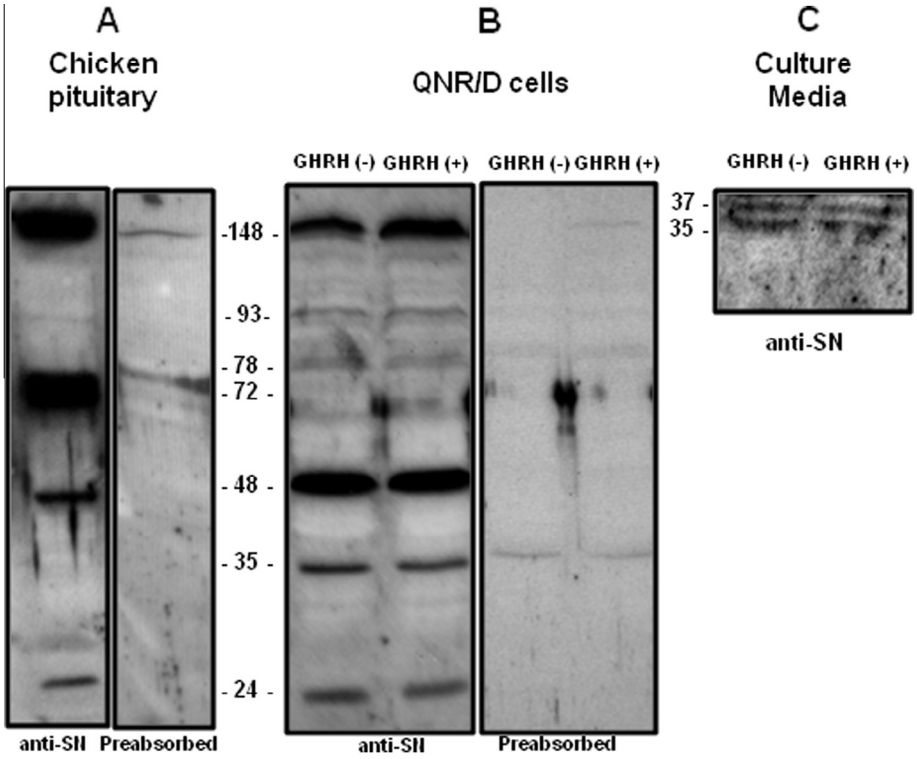

Fig. 2. Representative Western blot of SN-immunoreactivity in extracts of pituitary gland (A), QNR/D's (B) and culture media (C). QNR/D cells and incubation media treated with GHRH (1 μM) were analyzed after a short incubation (15 min). The extracts were separated by SDS–PAGE under reducing conditions (RC) and after transfer to nitrocellulose membranes, the proteins cross-reacting with a rabbit anti-SN antiserum (1:2000) were visualized using biotin conjugated anti-rabbit IgG (1:5000), Vecstatin ABC peroxidase system and enhanced chemiluminescence. X-ray films were exposed for 30 s and overexposed for 30 min. These results are representative of 4 experiments. SN antiserum (1:2000) was pre-absorbed at 4 °C overnight with 1 μM of SN synthetic peptide as negative control. The data shown is representative of 5 samples.

tion containing PBS with 3% non-fat dry milk (Bio-Rad). The fixed QNR/D's and 7 μm slides of chicken pituitary glands or embryonic day (ED7) chick retina sections were incubated overnight at 4 °C with a sheep anti-chicken GH antibody (used previously by Sanders et al., 2010; Cheung and Hall, 1990 diluted 1:1000 with TPBS), or rabbit anti-SN (at 1:2000) and/or with a mouse monoclonal anti-SNAP25 (Clone S-7B8, Exalpha Biologicals Inc., Shirley, MA, USA) Cells/slides were washed with PBS (3 × 10 min), then incubated for 2 h in a dark cold room with donkey anti-sheep-IgG (FITC) (ab6896-1, Abcam), Alexa Fluor 594 anti-rabbit-IgG (Cat. No. A11012, Invitrogen, Eugene, OR, USA) and Alexa Fluor 488 goat anti-rabbit (Cat. No. A11078, Invitrogen) (at 1:1000 in TPBS and 1% milk) respectively. The samples were then washed in PBS (3 × 10 min) and incubated for 5 min in NucBlue Fixed Cell ReadyProbe (DAPI; Molecular Probes, Eugene, OR, USA) as per manufacturer's instructions. Finally, the coverslips were washed with PBS 2 × 10 min each prior to mounting on microscope slides (Superfrost Plus, Fisher Scientific, USA) using Prolong Gold mounting medium (Invitrogen No. P36930). Slides were visualized using a Leica TCS SP5 confocal microscope. Subsequent image processing was performed using the ImageJ (free software developed by National Institutes of Health; http://imagej.nih.gov/).

2.5. Immunoelectron microscopy

QNR/D cells were cultured in 35 mm glass bottom culture dishes and fixed in a mixture of 0.5% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 20 min at 40 °C. Samples were dehydrated with a graded alcohol series (30%, 50%, 70%, and 80% ethanol), and then infiltrated with LR White (London Resin Co., Berkshire, UK). Infiltrated samples were embedded in gelatin capsules and polymerized under UV light for 24 h at 4 °C. Following polymerization, ultrathin sections (60 nm) were cut and loaded onto a 300-mesh nickel grid without coating. Immunogold labeling was carried out as described previously by Ilkow et al. (2010) with slight modifications. Prior to incubation with antibodies, the dried ultrathin sections were blocked overnight at 4 °C with 8% bovine serum albumin in Tris-buffered saline (pH 7.4). Double labeling with primary antibodies, rabbit anti-SN (1:5000) and guinea pig anti-chicken-GH (1:10,000) was performed at 4 °C overnight. Incubations with the 12-nm colloidal gold-conjugated goat anti-rabbit IgG (1:20) and 18 nm-colloidal gold-conjugated donkey anti-guinea pig IgG (1:40) secondary antibodies were performed for 40 min each at room temperature. All antibodies were diluted with Tris-buffered saline containing 1% bovine serum albumin. After incubations with the primary and secondary antibodies, the sections were contrasted with 2% aqueous uranyl acetate for 15 min before viewing with a Philips 410 transmission electron microscope at 80 kV equipped with a charge-coupled device camera (MegaView III Soft Imaging System; Olympus).

3. Results

3.1. Secretogranin II (SgII) and chromogranin A (CgA) expression in the chicken neuroretina and QNR/D cells

The expression of secretogranin-II (the precursor of SN) and chromogranin A (CgA) were determined by RT-PCR (Fig. 1), which showed the presence of 943 bp (CgA) and 373 bp (SgII) cDNA fragments in the total RNA extracted from QNR/D cells, from the chicken embryo (ED) 7 neural retina and from slaughterhouse chicken pituitary glands. In each extract the amplicon length was consistent with their predicted size and no other bands were evident in the gel, confirming the absence of genomic DNA or tran-

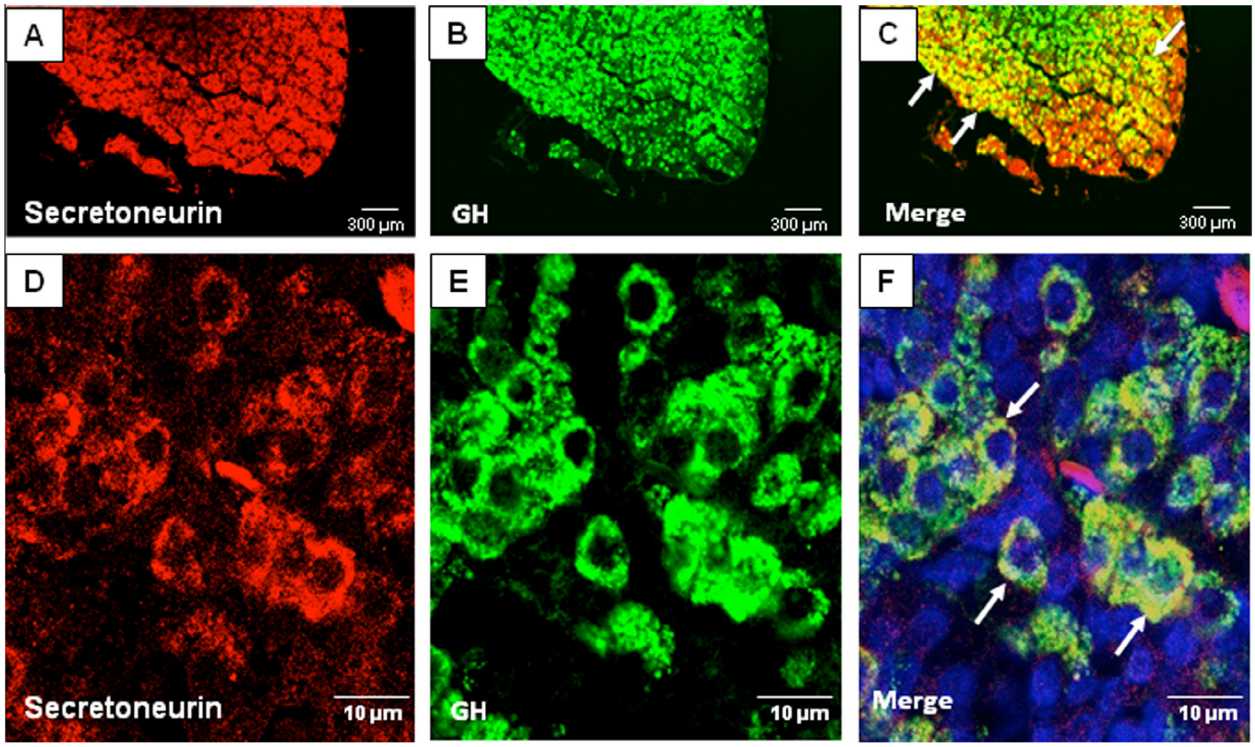

Fig. 3. (A–F) Co-localization of growth hormone (GH) and secretoneurin (SN) immunoreactivity in the chicken pituitary gland. Confocal images of double stained sections of pituitary show: SN-ir cells (red, 1st Ab: polyclonal rabbit anti-goldfish SN at 1:2000; 2nd Ab: donkey anti-sheep IgG-Alexa Fluor 594 at 1:1000) and GH (green, 1st Ab: a polyclonal sheep anti-chicken GH at 1:1000; 2nd Ab: donkey anti-sheep IgG-FITC at 1:1000). Co-localization of GH and SN in the pituitary gland is shown in yellow (C and F). Arrows show examples of co-localization. The lower panels (D–F) show somatotrophs at higher magnification. Nuclear staining was developed with DAPI (F; blue). Scale bars, 300 µm (A–C), 10 µm (D–F). The data shown is representative of 4 slides. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

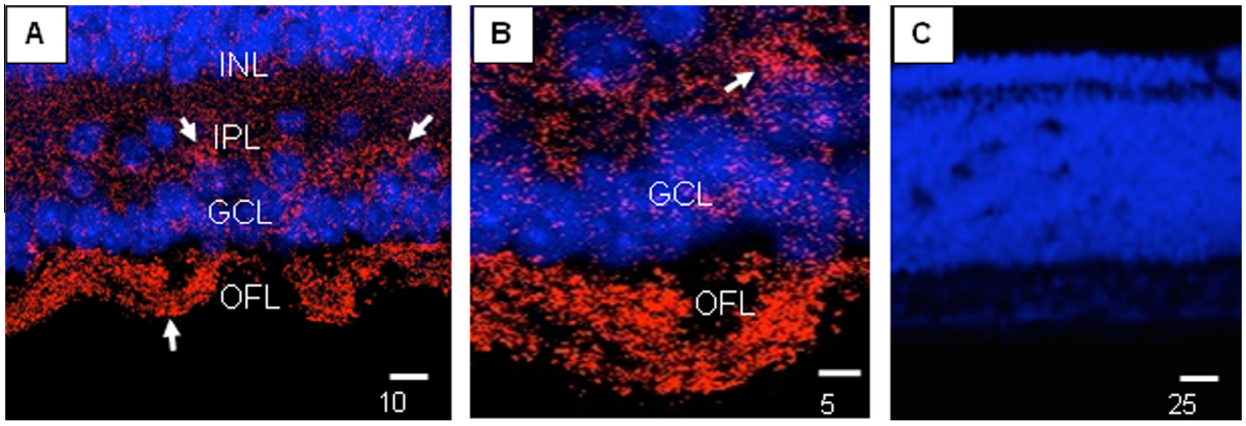

Fig. 4. Immunocytochemical staining in the neural retina of ED7 chick embryo eyes for secretoneurin (red). Arrows indicate the SN positive staining in RGC's using SN antiserum at 1:200 (A and B). Interior nuclear layer (INL), interior plexiform layer (IPL), ganglion cell layer (GCL), optic fiber layer (OFL). (C) Negative control. The data shown is representative of 4 slides. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

scriptional variants. Negative controls in the absence of template, showed no cDNA amplification after 30 PCR cycles.

3.2. SN-in the chicken pituitary gland and in QNR/D cells

Western blotting with the SN antibody demonstrated a number of SN immunoreactive bands on the chicken pituitary gland and in QNR/D cells and in their incubation media (Fig. 2). In the chicken pituitary, immunoreactive protein bands of 24, 48, 72 and 148 kDa were clearly evident, of which the 72 and 148 kDa bands were the most abundant. The specificity of staining was shown by its loss (for the 28 and 48 bands) and diminution (by >95% for the 72 and 148 kDa) following the preabsorption of the antibody with excess SN peptide. QNR/D cells had similar 24, 48, 72 and 148 kDa SN-IR bands, although the 72 kDa band was only minimally present. Faint bands of 35, 78 and 93 kDa were also visible in the QNR/D cell extracts. The specificity of this staining was shown by the preabsorption of the SN antibody with the SN peptide, which resulted in an almost complete loss of SN staining.

SN was secreted from these cells, since immunoreactive bands of 35 kDa and 37 kDa were present in the concentrated incubation media, in the absence of other stained protein bands. GHRH treatment of the cells increased the cell and media GH content (Martinez-Moreno et al., 2014b) but GHRH incubation did not alter

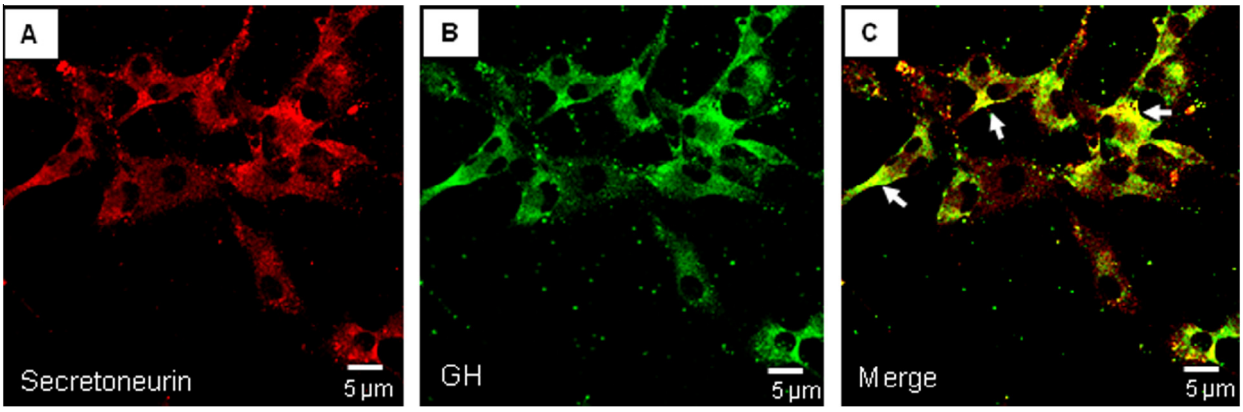

Fig. 5. Co-localization of GH- and SN-immunoreactivity in quail QNR/D cells. GH-IR (green, B) was detected using a specific sheep anti-chicken antibody (at 1:1000) and SN-ir (A) was developed with anti-SN antiserum (red). Examples of co-localization are indicated with arrows (yellow; C). Scale bars, 10 µm (A), 5 µm (B), 25 µm (C). The data shown is representative of 5 cell cultures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

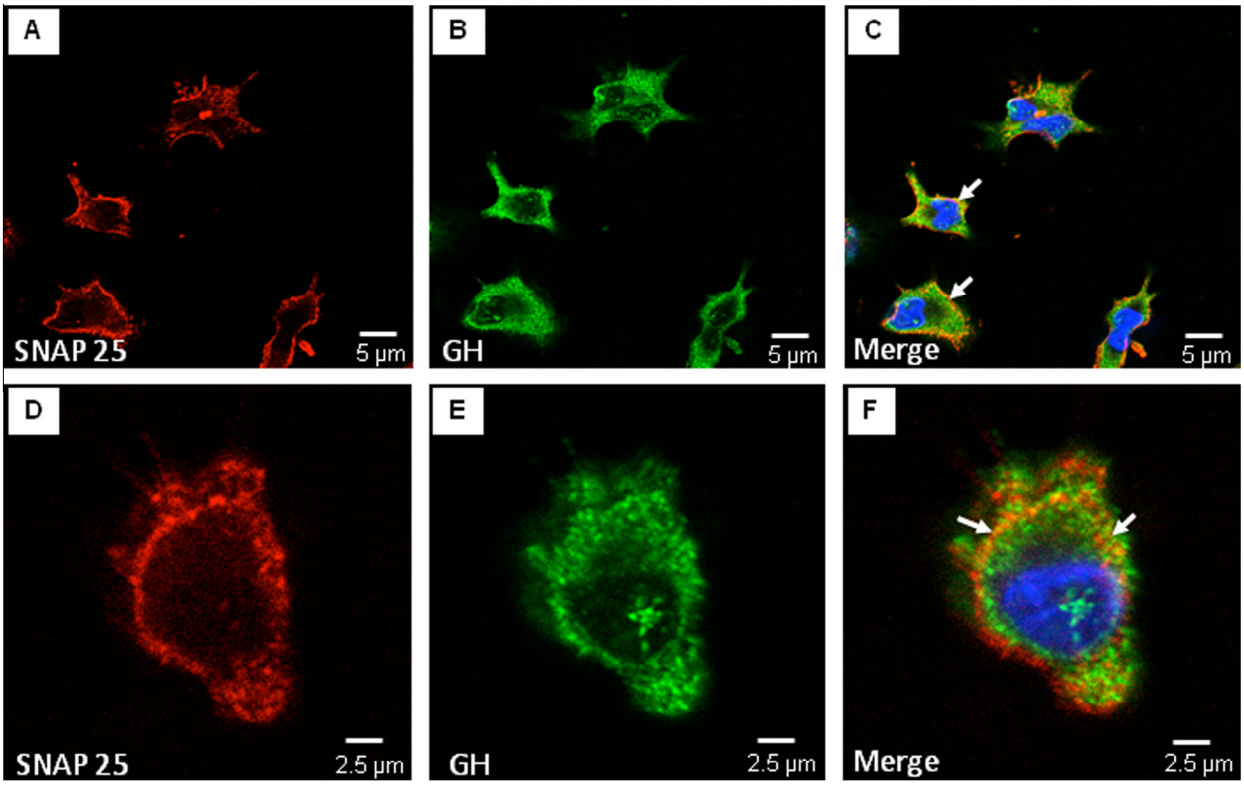

Fig. 6. Co-localization of growth hormone (GH) and SNAP-25 immunoreactivity (ir) in QNR/D cells. GH-IR (green) was detected using a specific rabbit anti-chicken GH (Harvey et al., 2012) (at 1:1000), whereas SN-ir (red) was detected using rabbit anti-SN antiserum (at 1:200). Arrows show intense co-localization (yellow) Nuclei (blue) is visualized with DAPI. Scale bars, 5 µm (A–C) and 2.5 µm (D–F). The data shown is representative of 4 cell cultures. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the abundance or relative proportion of any SN-immunoreactive bands.

3.3. Co-localization of SN and GH in somatotrophs

As expected, GH immunoreactivity in the chicken pituitary gland was restricted to somatotrophs in the caudal lobe (Fig. 3B and E). SN-immunoreactivity was also colocalized (Fig. 3E and F) in somatotrophs (Fig. 3A and D) and in other caudal lobe cells located in the periphery of the gland that were presumably gonadotrophs (Fig. 3A and C).

3.4. SN-immunoreactivity in the chicken embryo neural retina

Secretoneurin-immunofluorescence (red) was widespread in the neural retina of ED7 embryos and present in the inner plexiform layer and the ganglion cell layer (Fig. 4) but it was most intense in the optic fiber layer (OFL) formed by axons of the RGC's (Fig. 4A and B), which are known to have intense GH immunoreactivity (Baudet et al., 2003). In contrast, SN immunoreactivity was not present in the inner nuclear layer (Fig. 4A), nor in the retinal pigmented epithelium, nor in photoreceptor cells (data not shown). Negative controls incubated with non-immune serum showed no immunoreactivity (Fig. 4C).

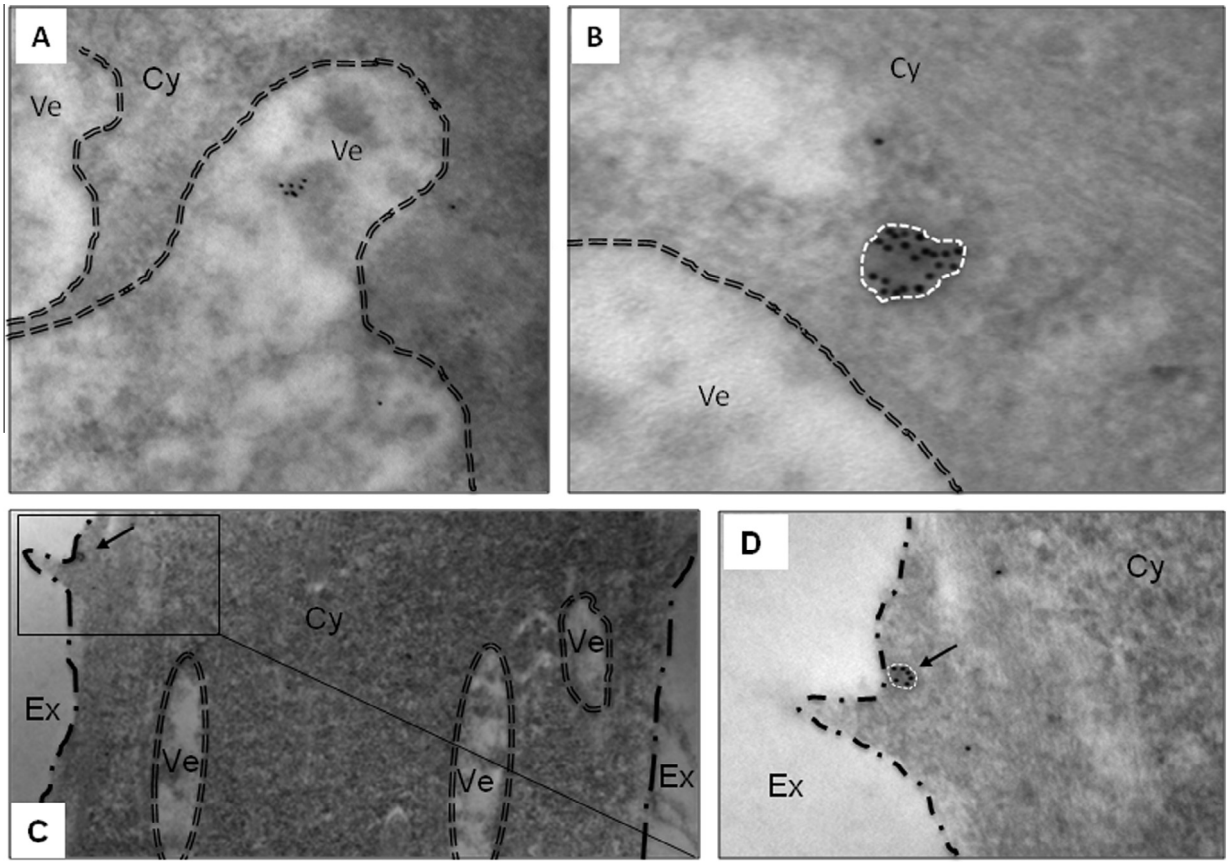

Fig. 7. Electron micrographs of QNR/D cells showing the presence of secretoneurin. SN-ir in QNR/D's was determined using 1st Ab: a polyclonal rabbit anti-SN at 1:5000 and 2nd antibody a goat anti-rabbit coupled to 12-nm colloidal gold particle at 1:20. In (A) and (B) gold particles (arrows) show SN-ir in vesicles (Ve). In (C) and (D) clear GH-immunostaining is observed in the cytoplasm close to the cell surface. Cy, cytoplasm; Nu, nucleus; Ex, exterior; Cell membrane, Me. Inserted square in (C) is shown in (D) at higher magnification. Black dotted line indicates cell membrane; white dotted line delimitates secretory granule electrodensity; double black dotted line delimitates vesicles. The data shown is representative of 3 cell cultures.

3.5. Co-localization of SN and GH in QNR/D cells

GH- and SN-immunoreactivities were intense in the cytoplasm of QNR/D cells in which colocalization was evident in both cell bodies and neurites (Fig. 5A–C). Negative controls incubated with preabsorbed antibodies (using an excess of the corresponding peptide) showed no immunoreactivity (data not shown).

3.6. Co-localization of SNAP-25 and growth hormone (GH) in QNR/D cells

SNAP-25 is a protein involved in specific interactions between synaptic vesicles and plasma membranes (Rizo and Sudhof, 2002). In QNR/D cells, SNAP-25 immunoreactivity was restricted to the outer membranes of the cells and was not present in the inner cytoplasm (Fig. 6A and D). This contrasts with the distribution of GH, which is mostly cytoplasmic and, to a lesser extent, nuclear (Fig. 6B and E). Colocalization of SNAP-25 and GH was observed, but only on the periphery of the cells (Fig. 6C and F), which may reflect immunoreactivity on the cell surface or plasma membrane.

3.7. Intracellular distribution of SN and GH in QNR/D cells

Using electron microscopy, immunolabeling with a gold-conjugated (12 nm particles) antibody showed the presence of SN immunoreactivity in large vesicles with low electron density close to the nucleus (Fig. 7A and B). SN-immunoreactivity was also present in small darker bodies inside the neurites (Fig. 7C and D). GH-immunoreactivity (labeled with 18 nm gold particles), in comparison, was more ubiquitously distributed in the cytoplasm (Fig. 8). Double labeling of the QNR/D cells shows that SN (arrows) and GH (arrowheads) were colocalized (in electron dense organelles) close to the plasma membrane of the neurite outgrowths (Fig. 9B) and cell bodies (Fig. 9A).

4. Discussion

The results of this study clearly show the colocalization of SN and GH in chicken somatotrophs, as also occurs in mammalian (Fischer-Colbrie et al., 1995; Hashimoto et al., 1987) pituitary glands. It is therefore likely that SN and GH are co-secreted from the chicken pituitary, as SN is thought to be co-stored and co-secreted with the neuropeptides and proteins present within the same secretory vesicles (Feldman and Eiden, 2003; Helle, 2004; Trudeau et al., 2012). It is therefore not surprising that the pituitary gland is thought to be the source of most of the SN and pituitary hormones present in peripheral plasma (Trudeau et al., 2012). SN-immunoreactivity was also observed in some other pituitary cells, that were probably gonadotrophs, since this is the only other cell type in the caudal lobe (Liu et al., 2005) and SN staining in gonadotrophs would be consistent with previous studies in goldfish (Blazquez et al., 1998).

In the chicken pituitary, at least 3 SN-immunoreactive protein bands were discerned. This is consistent with the finding of 5 immunoreactive secretoneurin moieties in the range of 40–90 kDa in extracts of the chicken brain (Leitner et al., 1998). Numerous SN-immunoreactive proteins derived from the ~600

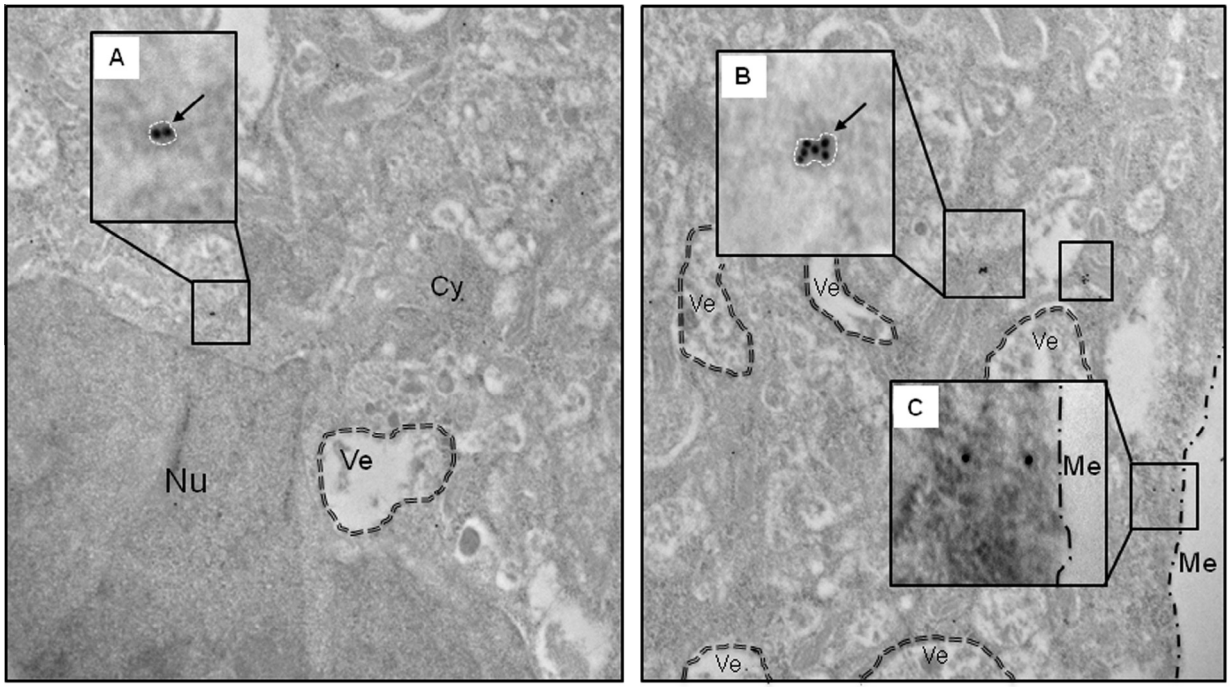

Fig. 8. Electron micrographs demonstrating the presence of GH in QNR/D cells. Immunocytochemistry for GH of chicken using specific polyclonal antibody (guinea pig anti-chicken GH at 1:10,000) and second antibody (donkey anti-guinea pig coupled to 18-nm colloidal gold particle, 1:40). Positive GH immunoreactivity (IR) in cytoplasm of QNR/D's. Inserted squares show picture magnifications. Arrow shows examples of positive immunoreactivity. Cy, cytoplasm; Nu, nucleus; Ex, exterior; Vesicle, Ve. Inserted squares (A–C) show immunolabeling at higher magnification. Black dotted line indicates cell membrane (Me); white dotted line delimitates secretory granule electrodensity; double black dotted line delimitates vesicles. The data shown is representative of 3 cell cultures.

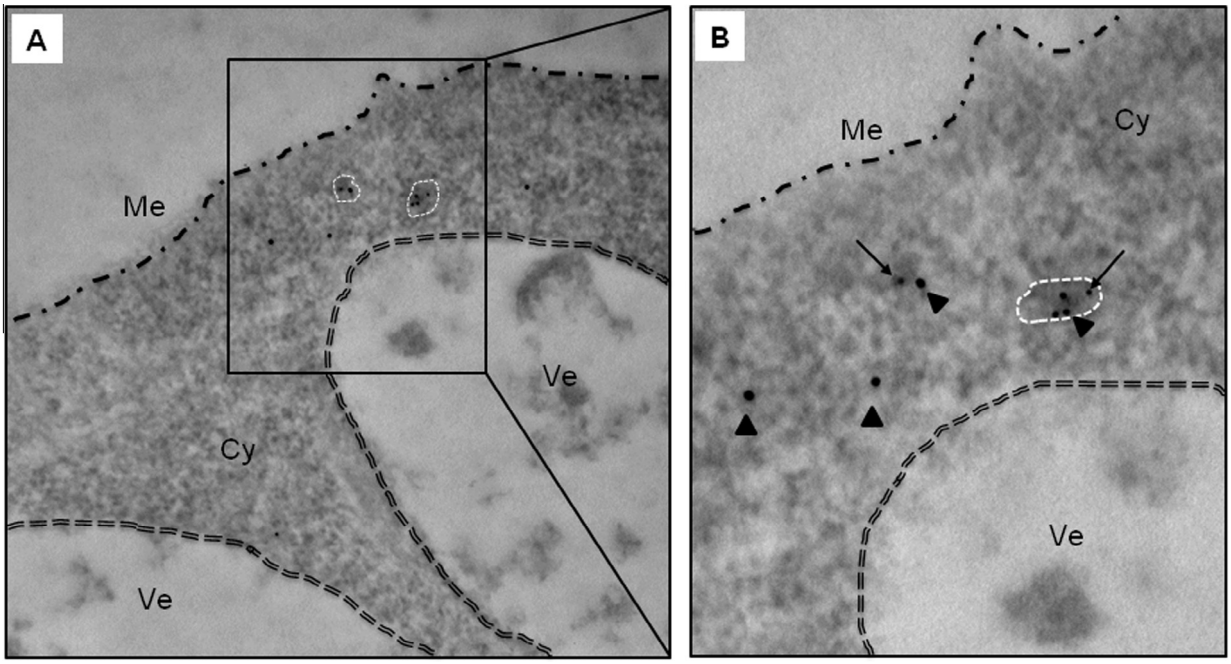

Fig. 9. Double immunostaining for GH and SN in QNR/D cells. GH and SN were detected with specific primary antibodies (at 1:5000 and 1:1000 respectively). The 12-nm immunogold particles (arrow) show positive labeling for SN and the 18-nm gold particles for GH-ir (arrow heads). Inserted square in (A) shows magnified area in (B). Black dotted line indicates cell membrane (Me); white dotted line delimitates secretory granule electrodensity; double black dotted line delimitates vesicles (Ve). The data shown is representative of 3 cell cultures.

amino acid SgII precursor are characteristically present in all vertebrates (Leitner et al., 1998; Van Horssen and Martens, 1999; Zhao et al., 2006) and are generated through pro-protein convertase activity (Zhao et al., 2010). In this work, we find a 72 kDa SN-immunoreactive band which corresponds with the predicted size for the chicken SgII (72.8 kDa). The SN-immunoreactive bands with molecular weights lower than 72 kDa present in QNR/D cells and pituitary gland extracts (24, 35 and 48 kDa) is likely to derive from the SgII precursor by proteolytic cleavage. The absence of the SN precursor (72 kDa) and the intense SN-ir band of 48 kDa suggest

a fast and efficient proteolytic mechanism. The large molecular size (148 kDa) of one SN-immunoreactive band is likely to result from the aggregation of smaller SN-immunoreactive moieties or from interactions with other proteins. For example, intracellularly, SgII binds to secretogranin III (SgIII) and participates in the formation of secretory vesicles containing orexin, neuropeptide Y and proopiomelanocortin in hypothalamic neurons (Hotta et al., 2009).

In addition to somatotrophs, SN immunoreactivity was found in the RGC layer and OFL of the chicken embryo neural retina, in which we have previously found intense GH immunoreactivity (Baudet et al., 2003). It is thus possible that SN-immunoreactive peptides are similarly colocalized with GH in this extrapituitary site of GH production. The finding of SN-immunoreactivity in the neural retina is consistent with its presence in neurons that innervate the iris/ciliary body complex on the rat eye (Troger et al., 2005, 2007) and its presence in amacrine cells and RGCs in the human eye (Overdick et al., 1996). In view of its location, the RGC layer may thus be the source of the SN found in the vitreous fluid of the eye (Lorenz et al., 2008), as it is for vitreal GH (Harvey et al., 2009). It is thus of interest that the vitreal levels of both SN and GH are reduced in patients with diabetic retinopathy (Lorenz et al., 2008; Ziaei et al., 2009), possibly as result of RGC cell death.

In addition to RGCs, SN was also found in QNR/D cells, in which it was again colocalized with GH. This is consistent with the use of these cells as an experimental model for determining roles of the retinal GH in RGC function (Martinez-Moreno et al., 2014a). SN-immunoreactive bands of 35 and 37 kDa size were also found in the incubation media of these cells, although at lower molecular weights than in the cells, possibly reflecting secretory forms.

The release of SN from cultured bovine adrenochromaffin cells is stimulated by PACAP (Turquier et al., 2001). Our finding that GHRH, another member of the glucagon superfamily, had no effect on SN release from QNR/D cells was therefore unexpected. This is also surprising since under the same experimental conditions, GHRH was found to increase GH synthesis and release from QNR/D cells (Harvey et al., 2012; Martinez-Moreno et al., 2014b). Secretagogue-induced GH release from these cells may therefore be from secretory granules lacking SN-immunoreactive peptides. In contrast, constitutive GH release from these cells (Martinez-Moreno et al., 2014b) may involve granules containing SN-immunoreactive peptides. However, the latter is an unlikely possibility given the extensive evidence that SN-immunoreactivity is a marker of regulated secretory pathways (Fischer-Colbrie et al., 1995; Helle, 2004). It is also possible that other secretagogues of GH, such as TRH and ghrelin, could be involved in the regulation of the SN synthesis and release, especially as they are also found in RGC's and QNR/D cells (Harvey et al., 2012).

Within the QNR/D cells, the colocalization of SN and GH was also demonstrated by immunogold electron microscopy. Both proteins were localized in compact granules with electron-dense material that are likely to be secretory vesicles (Helle, 2004; Hendy et al., 2006), as also seen in cultured hypothalamic neurons (Kagotani et al., 1991). Secretoneurin was, however, also found inside large vesicles with low electron density, which may reflect SN endocytosis, since similar endocytotic vesicles containing chromogranin A has been reported in human kidneys and in the kidneys of rats and cattle (Weiler et al., 1991) and SgII containing neurons in the rat hypothalamus (Kagotani et al., 1991).

In this study we also found that GH in QNR/D cells was co-localized with SNAP-25 (synaptosomal-associated protein 25), suggesting GH release occurs following the specific fusion of vesicles with the plasma membrane of the cell soma and neurite outgrowths. It is therefore relevant that GH release from human pituitary adenomas (Rotondo et al., 2008) and rat anterior pituitary glands (Matsuno et al., 2003, 2011) involves a specific fusion complex, SNARE, which includes SNAP 25 (Hodel, 1998).

In summary, this study shows that GH is colocalized with SN in the somatotrophs of the chicken pituitary gland and in GH-producing RGCs and quail-derived QNR/D cells. SN- and GH-immunoreactivity was observed in the same intracellular structures in the QNR/D cells, which demonstrates, for the first time, ultrastructural co-storage and possibly constitutive co-secretion. As SN is a marker for secretory granules, GH release from retinal cells is thus likely to be in secretory granules, as in somatotrophs of the pituitary. The presence of SN and GH in the axons and neurites of RGCs and QNR/D cells may suggest physiological roles in synaptogenesis and axogénesis in the chicken neuroretina.

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