Subtype-specific parafollicular localization of the neuropeptide manserin in the rat thyroid gland

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Abstract

The thyroid gland is an endocrine organ which is involved in metabolism, neuroexcitability, body growth and development. The thyroid gland is also involved in the regulation of calcium metabolism, which is not yet fully understood. In this study, we investigated the localization of the granin-derived neuropeptide, manserin, in the adult rat thyroid gland. Manserin immunoreactivity was detected in follicular epithelial cells. Intense manserin signals were also detected in some, but not all, parafollicular cells, indicating that parafollicular manserin is thought to be subtype-specific. These results indicate that thyroid manserin may play pivotal roles in parafollicular cells and follicular epithelial cells such as calcium metabolism and/or thyroid hormone secretion.

Keywords: Manserin, Thyroid gland, Parafollicular cell, Follicular epithelial cell, Neuropeptide, Chromogranin, Secretogranin

Introduction

The thyroid gland plays an important role in the body by promoting metabolism, increasing neuroexcitability, accelerating body growth and development, and increasing cardiac function (Cheng et al., 2010). The thyroid gland is also involved in the regulation of calcium metabolism. A protein called thyroglobulin is secreted by thyroid follicular epithelial cells into thyroid follicles. After iodination, thyroglobulin is again taken up by the follicular epithelial cells (Spitzweg et al., 2000). The hormones thyroxine (T4) and triiodothyronine (T3) are then released (internally secreted) into the circulation (Spitzweg et al., 2000).

Parafollicular cells of the thyroid gland are known to be involved in regulating serum Ca^{2+} levels through calcitonin secretion. Only one type of parafollicular cells is known to date. However, the existence of somatostatin-positive and somatostatin-negative subtypes of parafollicular cells has been proposed (Sawicki et al., 1997), suggesting that parafollicular cells may be heterogeneous. However, the precise morphological and functional distinctions among parafollicular cells have not been elucidated.

We recently isolated manserin, a neuropeptide composed of 40 amino acids, by combining HPLC and affinity purification (Yajima et al., 2004). Manserin is

distributed in the rat pituitary, hypothalamic nuclei, adrenal gland, duodenum epithelial cells, cerebellum, inner ear, and β and δ cells in the pancreatic islets (Yajima et al., 2004, 2008; Kamada et al., 2010; Tano et al., 2010; Ohkawara et al., 2011; Ida-Eto et al., 2012), indicating that manserin plays roles in several endocrine systems. Manserin has not previously been found in the thyroid gland. However, the fact that SgII, a manserin precursor, occurs in thyroid parafollicular cells (Weiler et al., 1989; Schmid et al., 1992) suggests that manserin also might occur in the thyroid gland. Because SgII is reported to have Ca^{2+}-binding ability (Yoo et al., 2007), manserin is also expected to have some roles in Ca^{2+} metabolism. In addition, parafollicular cells secrete numerous regulatory peptides that play important roles in their functions (Sawicki et al., 1995). For these reasons, we sought to identify the localization of manserin in the adult thyroid gland.

Materials and methods

Animals

Four male Wistar rats (8ó12 weeks old) were used. All animal experiments were approved by the Committee of Laboratory Animal Research Center at Mie University.

Tissue preparation

Anesthetized rats were transcardially perfused with 0.9% saline followed by perfusion with 4% paraformaldehyde (PFA) in PBS (Tano et al., 2010). The thyroid and pituitary glands were dissected and immersed in 4.0% PFA in PBS overnight at 4 °C. For cryosectioning, the pituitary gland was cryoprotected in 30% sucrose, embedded in O.C.T compound (Sakura Finetechnical Co. Ltd., Japan), and sectioned at 9 m thickness on a Leica CM1850 cryostat (Leica Microsystems, Wetzlar, Germany). For paraffin sectioning, the thyroid gland was immersed in a graded ethanol series and xylene and embedded in paraffin followed by sectioning at 7 m using a rotary microtome.

Anti-manserin antibody

Affinity-purified rabbit anti-manserin antibody was prepared as described previously (Kamada et al., 2010). The specificity of the antibody was confirmed by immunoblotting (Yajima et al., 2004) and a preabsorption test.

Immunostaining

The thyroid and pituitary glands were immunostained as described previously (Yajima et al., 2004, 2008). In brief, sections were incubated in 3.0% H_2O_2 in PBS, washed with PBS, and blocked with 10% fetal bovine serum (FBS) in PBS containing 0.1%

Triton X-100 for 1 h. The sections were then labeled with rabbit anti-manserin antibody overnight at 4 °C, incubated with biotin-conjugated anti-rabbit IgG (Chemicon, CA, USA), and washed with PBS. The sections were then stained by the ABC method and visualized with 3,3 -diaminobenzidine (DAB) using the Vectastain Elite ABC Kit (Vector Laboratories, Burlinghame, CA, USA). For counterstaining, sections of the thyroid gland were stained with Mayerøs hematoxylin solution (Wako Pure Chemical Industries Ltd., Japan).

Frozen sections of the pituitary gland were immunofluorescently stained as described previously (Ohkawara et al., 2004; Tano et al., 2010). For single immunostaining, the sections were incubated with anti-manserin antibody (Kamada et al., 2010) at 4 °C overnight. For double immunostaining, the sections were incubated with rabbit anti-manserin antibody (Kamada et al., 2010) and goat anti-thyroid-stimulating hormone (TSH) β chain antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) overnight at 4 °C, followed by incubation with fluorescently labeled secondary antibodies. Concentrations of secondary antibodies used in this study were as follows: Alexa 488-conjugated donkey anti-goat IgG (2 g/ml; Invitrogen, OR, USA) and Alexa 568-conjugated donkey anti-rabbit IgG (2 g/ml; Invitrogen).

Paraffin sections of the thyroid gland were deparaffinized and rehydrated through a graded ethanol series. After rehydration, the sections were treated with citrate buffer (pH 6.0) for target retrieval, followed by blocking with 10% FBS in PBS containing 0.1% Triton X-100 for 1 h. The sections were then incubated with primary antibodies at 4 °C overnight, followed by incubation with fluorescently labeled secondary antibodies. Primary antibodies used for immunostaining of the paraffin sections were as follows: rabbit anti-manserin antibody (Kamada et al., 2010), rabbit anti-SgII antibody (QED Bioscience Inc., CA, USA), and goat anti-calcitonin antibody (Santa Cruz Biotechnology Inc.).

Signals were visualized using an Olympus FV1000 laser scanning microscope (Olympus, Tokyo, Japan) for immunofluorescently stained sections and an Olympus BX50 microscope (Olympus, Japan) for sections stained by the ABC method. These images were processed using Adobe Photoshop CS5.1 (Adobe Systems Inc., CA, USA). Immunoreactive cells were counted using processed images.

Results

Because blood levels of thyroid hormones are controlled by TSH produced in the pituitary gland, we examined whether manserin was present in TSH-expressing cells

in the rat pituitary gland. Manserin was detected in the anterior, intermediate, and posterior lobes of the pituitary gland (Fig. 1A). In our previous study, manserin was not localized in the intermediate and posterior lobes (Yajima et al., 2004). This apparent discrepancy may be explained because of (1) DAB staining, which is less sensitive than immunofluorescent staining, and (2) anti-serum but not affinity-purified anti-manserin antibody used. Double staining with anti-manserin antibody and anti-TSH antibody revealed that manserin was present in TSH-expressing cells (Fig. 1B-D). The TSH-expressing cells were exclusively manserin positive, whereas only some manserin-positive cells were TSH positive (30.5% of 246 manserin-positive cells counted). Since manserin was colocalized with follicle stimulating hormone (FSH)-expressing cells (Yajima, et al, 2004), cells that are manserin-positive and TSH-negative are thought to be FSH-expressing cells. These results indicate that manserin is present in TSH-expressing cells in the rat pituitary gland.

Because manserin is colocalized with the TSH-expressing cells in the pituitary gland as mentioned above, the peptide is assumed to be localized in thyroid gland. Intense but scattered manserin signals were observed in cells around follicles (Fig. 2A and C). At higher magnification, intense manserin-positive cells were exclusively localized in the space between follicles, i.e., the interfollicular space, suggestive of parafollicular

cells (Fig. 2E, arrows). Under careful observation, manserin immunoreactivity was also observed in the follicular epithelial cells, although the immunoreactivity was faint less than parafollicular cells (Fig. 2E and G). These results indicate that manserin is present in follicular epithelial as well as parafollicular cells. No signal was detected when the manserin antibody was preabsorbed with a recombinant peptide (10^{6} M; Fig. 2B) and when the primary antibody was omitted (data not shown). SgII, a precursor of manserin that has been reported to be present in the thyroid gland (Schmid et al., 1992; Weiler et al., 1989), showed a staining profile quite similar to that of manserin (Fig. 2D, F).

To confirm the presence of manserin in parafollicular cells, we tested the colocalization of manserin with calcitonin, a parafollicular hormone that regulates blood Ca^{2+} levels. As reported (Foster, 1968), anti-calcitonin antibody stains almost all parafollicular cells (Fig. 3B, E). However, only 35.8% of 447 calcitonin-expressing cells counted were manserin positive (Fig. 3A, C), suggesting the existence of distinct forms of parafollicular cells. On the other hand, almost all (96.4%) of the calcitonin-expressing cells counted were SgII positive (Fig. 3D, F). These results suggest that manserin may localize only in a specific subtype of the parafollicular cells.

Discussion

This is the first study to demonstrate that manserin is present in thyroid follicular epithelial cells and in parafollicular cells. This result suggests that thyroid manserin may play some roles in the thyroid functions such as Ca^{2+} metabolism and hormone secretion.

We detected manserin signals as fine puncta in the cytoplasm of thyroid follicular epithelial cells. Because thyroid hormones are produced by the proteolytic cleavage of iodinated thyroglobulin in lysosomes and released by follicular epithelial cells into the blood, the punctate signals of manserin in the follicular epithelial cells indicate that manserin may reside in the lysosome where thyroid hormones were produced. Granin family proteins play a role in the formation of secretory vesicles (Iacangelo and Eiden, 1995; Kim et al., 2001), and there is evidence that the SgII-derived peptide SN is involved in stimulating the production and release of LH (Zhao et al., 2011). Thus, granin family proteins and their derived peptides play important roles in hormone secretion. Accordingly, we propose that manserin regulates the body metabolic rate by modulating thyroid hormone secretion, although further experiments are necessary to resolve this issue.

The presence of manserin in only approximately one-third of parafollicular cells

indicates that manserin may be a marker for one subtype of the parafollicular cells. Sawicki (1995) reported that somatostatin was present in only a subset of these cells. The characterization of functionally different subtypes of the parafollicular cells awaits further studies of thyroid manserin.

The manserin precursor, SgII, was present in almost all parafollicular cells, whereas manserin was found in only one subset. Most parafollicular cells contain prohormone convertase (PC) 1 and PC2, which cleave precursor proteins including SgII at paired basic sites to yield bioactive peptides that include SN, EM66, and manserin (Kurabuchi and Tanaka, 2002). The same authors reported that PC2 was equally distributed in all of the parafollicular cells, but that the content of PC1 was different among the parafollicular cells. One possibility is that manserin is produced by the cleavage of SgII by PC1 and is accordingly found only in the parafollicular cells expressing relatively higher levels of PC1.

Manserin localizes in TSH-expressing cells and follicular epithelial cells in the pituitary and thyroid glands, respectively. TSH activates follicular epithelial cells and stimulates synthesis and secretion of thyroid hormones, T3 and T4 (Szkudlinski et al., 2002). The production and secretion of TSH is suppressed by blood levels of thyroid hormones. Thus, blood TSH levels are tightly controlled by a negative-feedback loop.

The presence of manserin in TSH-expressing cells and follicular epithelial cells suggests that it plays a role in the maintenance of homeostasis using this negative-feedback mechanism. Further investigations of manserin are required to resolve this question. Since manserin was shown to be expressed depending on the stress (Kamada et al., 2010), homeostasis of calcium regulation depending on the stress is also thought to be maintained through manserin. Further studies using stress animals should be necessary to resolve this issue.

As a result of this study, we conclude that the novel peptide manserin is localized in TSH-expressing cells in the adult rat pituitary gland and in follicular epithelial cells in the thyroid gland. Although the manserin precursor protein SgII was found in almost all parafollicular cells, manserin was found in only approximately one-third of these cells. These results suggest that manserin performs subtype-specific functions in the parafollicular cells.

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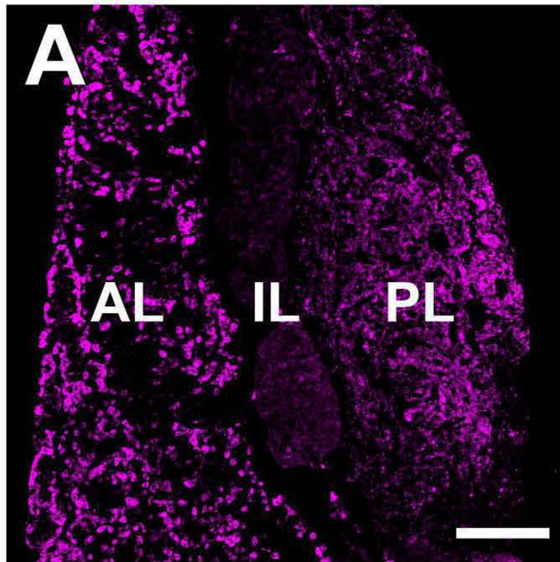
Figure legends

Fig. 1. Localization of manserin in TSH-expressing cells in the rat pituitary gland. Antigenóantibody complexes were detected by immunofluorescent staining (AóD). Manserin signals were detected in the anterior, intermediate, and posterior lobes of the pituitary gland (A). Double immunostaining with anti-manserin antibody (B) and anti-TSH antibody (C) was also shown. D is a merged image. AL, anterior lobe; IL, intermediate lobe; PL, posterior lobe. Scale bars = 200 m (A) and 10 m (B-D).

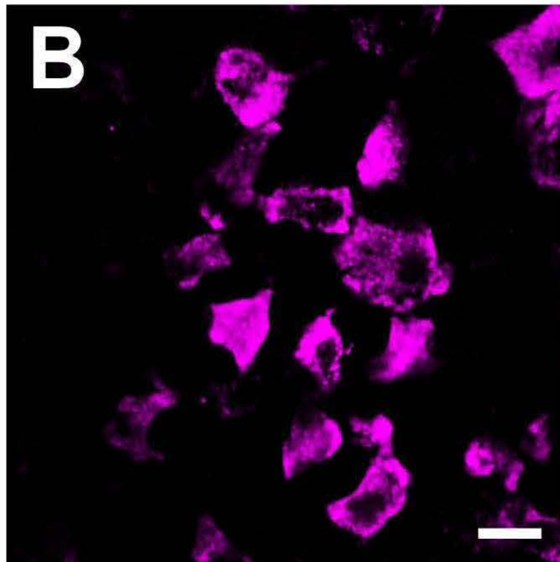
Fig. 2. Distribution of manserin in the thyroid gland. Antigenóantibody complexes were detected by DAB staining (*brown,* AóG). The specificity of anti-manserin antibody was confirmed by the preabsorption test. In the preabsorption test, manserin signals were detected when anti-manserin antibody was incubated with PBS (A), but no signal was detected when anti-manserin antibody was preabsorbed with a recombinant peptide (10 ^{6} M) (B). In the thyroid gland, manserin signals (C, E, G) and SgII signals (D, F) were detected. *Arrows* in E indicate localization of manserin in the cells located in the interfollicular space. *Arrowheads* in E indicate localization of manserin in the follicular epithelial cells. G shows higher magnification view of the boxed area in E. In CóG, nuclei were counterstained with Mayerøs hematoxylin solution. f, follicle. Scale bars = 50 m (A-D), 10 m (E, F) and 2 m (G).

Fig. 3. Distribution of manserin or SgII in the parafollicular cells. Double immunostaining was performed with anti-calcitonin antibody (B, E) and anti-manserin (A) or anti-SgII antibody (D). Merged images are also shown (C, F). Scale bar = 20 m (A-F).

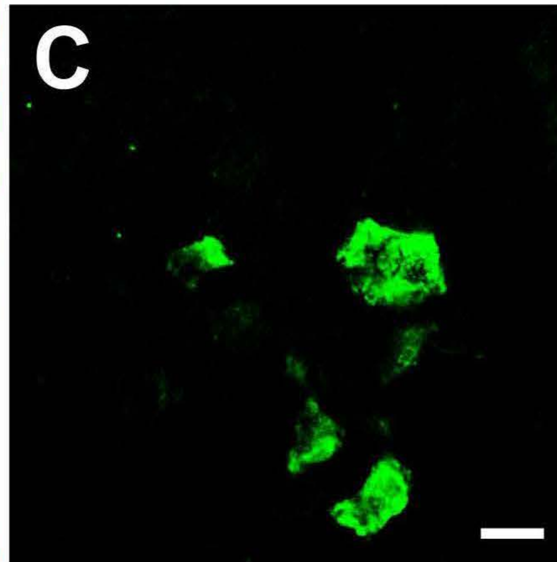
anti-manserin Ab



anti-manserin Ab



anti-TSH Ab



Merged

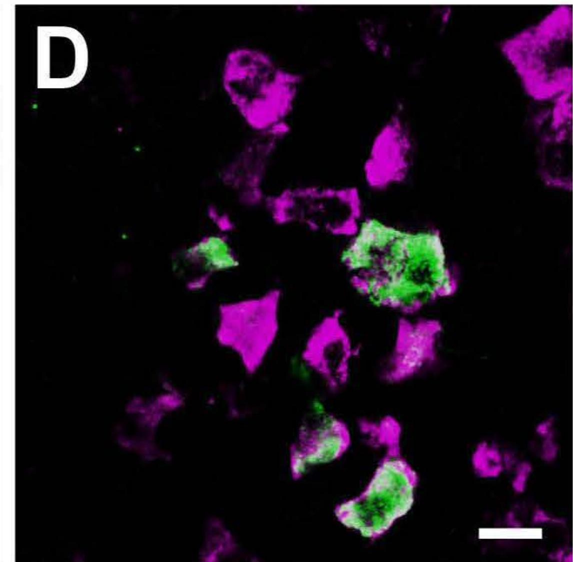


Fig. 1_Ohkawara et al.

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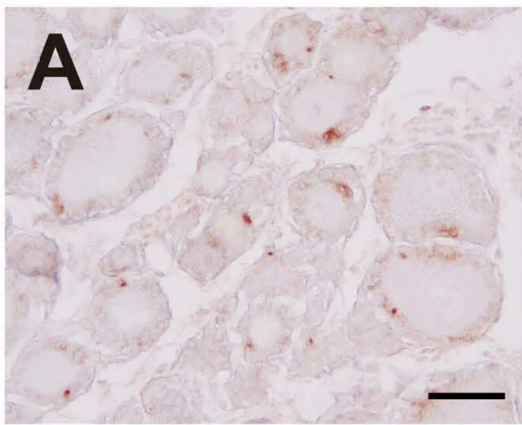
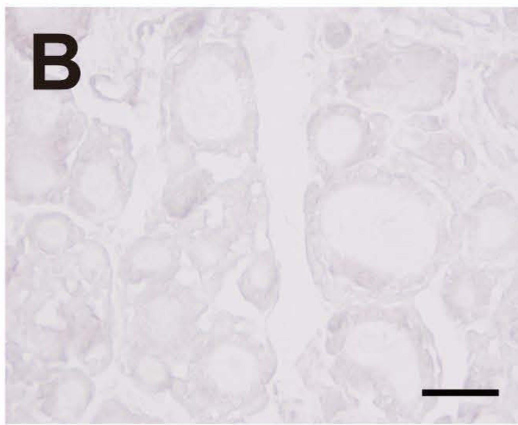
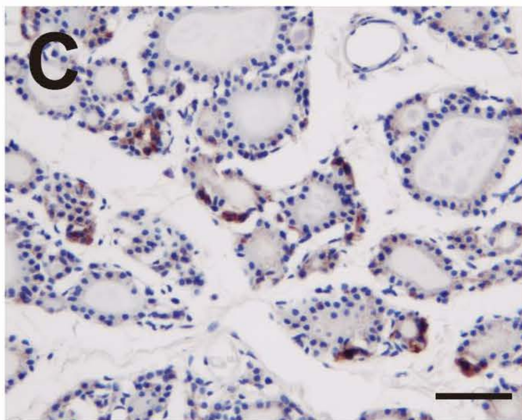


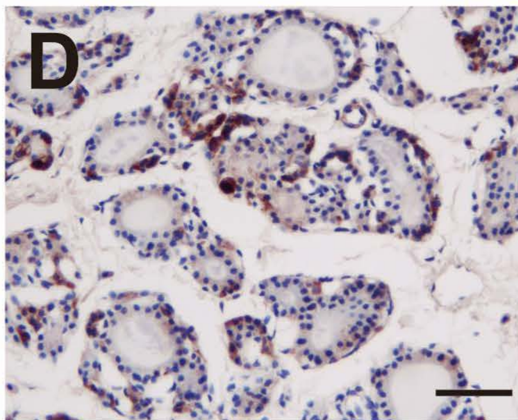
1 μM peptide

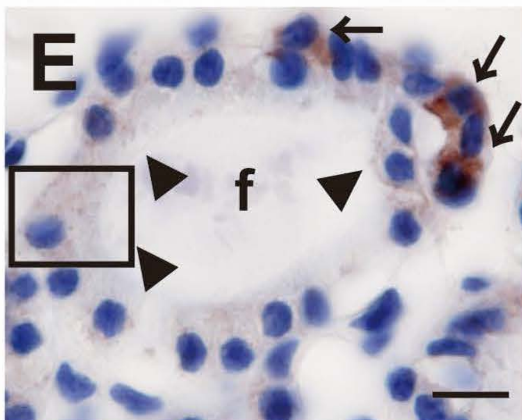


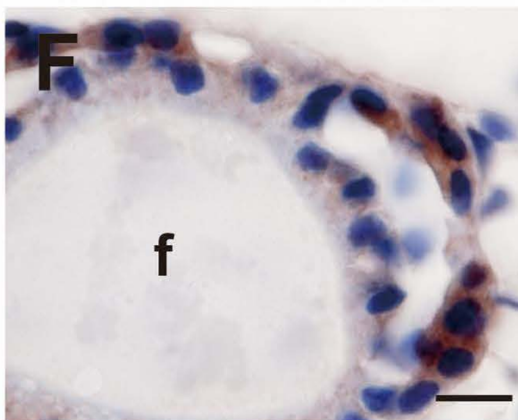
anti-manserin Ab



anti-SgII Ab







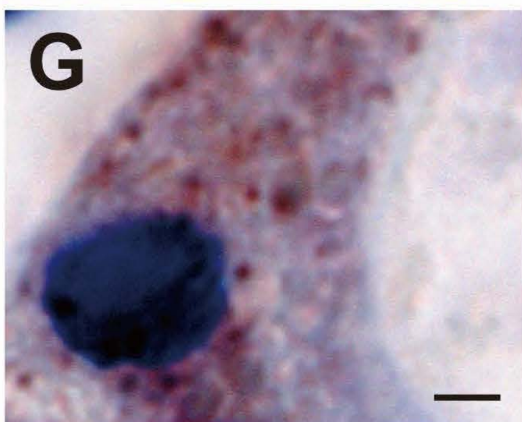


Fig. 2_Ohkawara et al

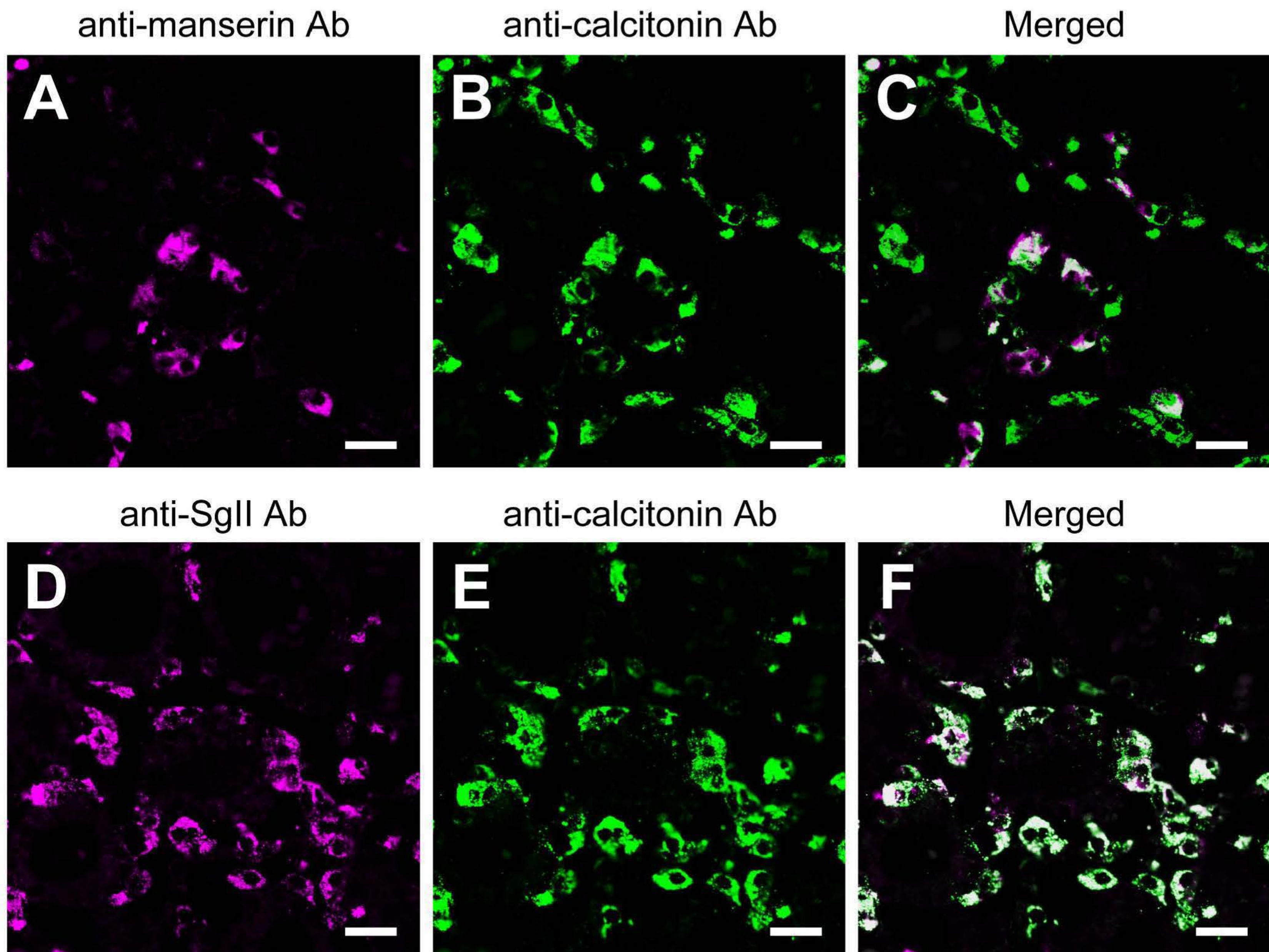


Fig. 3_Ohkawara et al.