Secretogranin II and its derivative peptide, manserin, are differentially localized in Purkinje cells and unipolar brush cells in the rat cerebellum

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Abstract

The cerebellum has long been recognized as the primary center of motor coordination in the central nervous system. Cerebellar neuropeptides have been postulated to be involved in such motor coordination, though this role is not fully understood. We herein investigated the localization of novel neuropeptide, “manserin” in the adult rat cerebellum. Punctate signals of manserin immunoreactivity were observed in the granular layer of the rat cerebellum. Manserin signals were also observed in the fibers and fiber terminals in the granular layer as well as the molecular layer. Manserin did not localize in Purkinje cells. Interestingly, cerebellar manserin was preferentially colocalized with unipolar brush cells, a class of excitatory granular layer interneuron, which are known to be involved in vestibullocerebellar functions. These results indicate that manserin plays pivotal roles in the cerebellar functions.

Keywords: cerebellum, chromogranin, manserin, neuropeptide, secretogranin, unipolar brush cell

Introduction

The cerebellum has long been recognized as the primary center of motor coordination in the central nervous system (Baillieux et al., 2008). In addition, the cerebellum is also known to be involved in the regulation of sensorimotor, autonomic and cognitive processes (Manzoni et al., 2007). Morphologically, the cerebellar cortex has a tri-layered structure, and its neural circuits are composed of characteristic afferent and efferent fibers (Ito, 2006). Precise cellular and molecular mechanisms regarding regulation of such motor coordination including head, body and eye movement are not fully known to date.

Several types of interneurons are known to exist in cerebellar neural circuits (Ito, 2006). These include granule cells, Golgi cells and Lugaro cells. Recently, a new type of interneuron called unipolar brush cells (UBCs), was reported (Braak and Braak, 1993; Diño et al., 1999; Mugnaini and Floris, 1994). UBCs are excitatory granular layer interneurons that are enriched in vermal and vestibullocerebellar lobules and partake in fast and slow mechanisms for the regulation of head and body posture and eye movement (Diño and Mugnaini, 2008; Rossi et al., 1995; Sekerková et al., 2005), but precise functions of UBCs have not been elucidated. Since UBCs are known to contain the highest density of granins including neurotransmitters such as

neuropeptide (Miyazaki et al., 2011), UBCs may play important roles in cerebellar functions through neurotransmission.

We have recently isolated manserin, a neuropeptide consisting of 40 amino acids by combining the techniques of HPLC and affinity purification (Yajima et al., 2004) and found that manserin is distributed in the rat pituitary, hypothalamic nuclei, adrenal gland, duodenum epitherial cells and pancreas islet β, δ cells (Kamada et al., 2010; Tano et al., 2010; Yajima et al., 2004, 2008). Manserin was discovered by the analysis of a suspected processing sequence for secretogranin II (SgII), which is one of the granin proteins that occurs in the secretory vesicles (Fischer-Colbrie et al., 1995; Zhao et al., 2009, 2010). There already were reports of other processed peptides from SgII such as secretoneurin (SN) (Kirchmair et al., 1993) and EM66 (Anouar et al., 1998). Recently, expression of SgII in the cerebellum was reported in terminals of parallel fibers, climbing fibers, mossy fibers, Purkinje cells and granular layer interneurons, which contain UBCs (Miyazaki et al., 2011; Nunzi M. G. and Mugnaini E., 2009). Interestingly, chromogranin A and chromogranin B, other granin family proteins, are expressed in stellate cells, Purkinje cells, granule cells and UBCs (Nunzi M. G. and Mugnaini E., 2009). Not only these, neuropeptides and amines in the cerebellum have been suggested to take part in

such cerebellar mechanisms (Ito, 2009), suggesting they have roles in cerebellar function. In the present study, we investigated localization of manserin in adult rat cerebellum.

Materials and methods

Animals

Twelve-week old male Wistar rats were used in the present study. All animal experiments were approved by the Committee of Laboratory Animal Research Center at Mie University.

Tissue preparation

Rats were anesthetized and transcardially perfused with 4.0 % paraformaldehyde (PFA) in PBS (Tano et al., 2010). The cerebellum was dissected out and was immersed in 4.0 % PFA in PBS overnight at 4 ℃. For cryosectioning, the cerebellum was cryoprotected in 30 % sucrose, and embedded in O.C.T compound (Sakura Finetechnical Co. Ltd., Japan) and sectioned at 8 μm in the sagittal plane with Leica CM1850 cryostat (Leica Microsystems, Germany). In some experiments, the cerebellum was embedded in paraffin and sectioned at 7 μm in the sagittal plane.

Anti-manserin antibody

Affinity-purified rabbit anti-manserin antibody was prepared as described previously (Kamada

et al., 2010). The specificity of anti-manserin antibody has been confirmed by immunoblotting (Yajima et al., 2004).

Immunostaining

Immunostaining was performed as described previously (Yajima et al., 2004, 2008). Briefly, for single immunostaining, frozen sections were incubated in 3.0 % H_2O_2 in PBS and washed with PBS followed by blocking with 10 % fetal bovine serum (FBS) in PBS containing 0.1 % Triton X-100 for 1 hr. Sections were labeled with rabbit anti-manserin antibody (Kamada et al., 2010) overnight at 4 ℃, followed by incubation with biotin-conjugated anti-rabbit IgG (Chemicon, CA, USA). After washing with PBS, sections were stained by the ABC method and visualized with 3,3'-diaminobenzidine using VECTASTAIN Elite ABC Kit (Vector Laboratories, CA, USA).

Immunofluoresent staining using frozen sections were performed as described previously (Ohkawara et al., 2004; Tano et al., 2010). For single immnostaining, 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 either of following mouse monoclonal antibodies: Tuj1 (Covance, CA, USA); anti-calretinin (CR, Millipore, CA, USA); anti-metabotropic glutamate receptor 1 alpha (mGluR1α, Becton, Dickinson and Company, NJ, USA); anti-vesicular glutamate transporter 2 (VGluT2, Abcam, UK); anti-calbindin-D-28K (Sigma, MO, USA) overnight at 4 ℃, followed by incubation with fluorescently labeled secondary antibodies. Concentrations of secondary antibodies used in this study were as follows: Alexa 488-conjugated goat anti-mouse IgG (2 μg/ml; Invitrogen, OR, USA); Alexa 568-conjugated goat anti-rabbit IgG (2 μg/ml; Invitrogen, OR, USA). In some experiments, paraffin sections were deparaffinized and rehydrated through a graded series of ethanol. After rehydration, 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 hr. Then sections were incubated with primary antibodies at 4 ℃ overnight, followed by incubation with fluorescently labeled secondary antibodies. Primary antibodies used in the immunostaining for paraffin sections were as follows: rabbit anti-SgII antibody (QED Bioscience Inc., CA, USA); mouse anti-calbindin-D-28K antibody (Sigma, MO, USA); mouse anti-CR antibody (Millipore, CA, USA).

Signals were visualized using Olympus FV1000 laser scanning microscope (Olympus, Japan).

Results

Localization of manserin in the rat cerebellum was investigated using immunohistochemical staining of frozen section. Intense manserin immunoreactivity was widely detected in the granular layer of the rat cerebellum (Fig. 1A, B). At higher magnification, manserin immunoreactivity was also found at the fibers with beaded branches (Fig. 1C) in the molecular layer. Higher magnification of the granular layer also revealed manserin signals in fiber terminals (Fig. 1D arrows) and cytoplasm (Fig. 1D arrowhead). Staining was not observed when the primary antibody was omitted (Fig. 1E). Furthermore, staining was not observed when anti-manserin antibody was preabsorbed with 10^{-6} M recombinant manserin peptide (Fig1F). These results indicate that manserin exists in the rat cerebellum mainly in the granular layer, as well as, to some extent, in the molecular layer.

In order to identify the manserin immunoreactive cell type, sections were double-immunofluorescently stained with anti-manserin antibody and Tuj1 antibody, a marker for neuron. As shown in Fig 2A-C, manserin immunoreactive cells were, by visual inspection, most if not all Tuj1 positive.

Since SgII, a precursor of manserin, and its related peptide, SN, occurs in UBCs, a class of cerebellar interneuron (Miyazaki et al., 2011; Nunzi and Mugnaini, 2009), we tested whether manserin also occurs in UBCs using markers for UBCs such as CR or mGluR1α (Nunzi et al., 2002). UBCs are recently identified excitatory granular layer interneurons that are distinguished at least two subtypes by differential expressions of specific proteins, including CR and mGluR1α (Kalinichenko and Okhotin, 2005; Nunzi et al., 2002). As shown in Fig 2 D-F, CR positive cells were preferentially manserin positive (98.2 % of 109 CR positive cells counted). Another marker of UBCs, mGluR1α, was also tested (Fig.2 G-I). Only 6.0 % of mGluR1α-positive UBCs were manserin-positive (of 215 mGluR1α positive cells counted). These results indicate that vast majority of CR-expressing UBCs and to some extent mGluR1α -positive UBCs, contain manserin peptides. However, small proportion of manserin-positive cells may also occur in neurons of granular layer other than UBCs, indicative of granule cells, Golgi cells and Lugaro cells.

As shown in Fig. 1C and D, apparent positive labeling was found at the fibers in the molecular layer as well as fiber terminals in the granular layer. In order to identify the origin of these fibers, colocalization of manserin with VGluT2, a marker of climbing fibers, mossy fibers

and axon terminals of CR-positive UBCs (Fremeau et al., 2001; Hioki et al., 2003; Nunzi et al., 2003), was tested. As shown in Fig. 2J-O, VGluT2 was colocalized with manserin at the fibers in the molecular layer (Fig. 2J-L) as well as fiber terminals in the granular layer (Fig. 2M-O arrows). These results indicate that, in addition to neuronal localization, manserin distributes at climbing fibers in the molecular layer, mossy fibers and/or axon terminals of CR-positive UBCs in the granular layer.

Previous reports demonstrate that Purkinje cells express SgII, a manserin's precursor (Cozzi et al., 1989; Miyazaki et al., 2011; Nunzi and Mugnaini, 2009). Therefore, we examined whether manserin also occurs in Purkinje cells. As consistent with previous papers, SgII localized to Purkinje cells as confirmed by double staining with anti-calbindin antibody (Fig. 3A-D). On the other hand, no manserin immunoreactive Purkinje cells were observed (Fig 3E-G).

Discussion

In the present study, we demonstrated, for the first time, that manserin exists in the cerebellum. Cerebellar manserin was preferentially colocalized with specific subtypes of UBCs, a class of excitatory interneuron. Interestingly, unlike SgII, manserin does not exist in Purkinje cells. These results indicate that the cerebellum exerts its physiological functions through granins and/or its related peptides.

Manserin did not exist in Purkinje cells. This was quite unexpected because manserin's precursor, SgII, has been repeatedly reported to localize in Purkinje cells (Miyazaki et al., 2011; Nunzi and Mugnaini, 2009). However, this apparent discrepancy may be explained as follows; the antibodies against SgII used in their experiments are thought to recognize not only full-length SgII but also fragments or peptides which are cleaved from SgII (Miyazaki et al., 2011; Nunzi and Mugnaini, 2009). Since proteolytic processing of SgII to fragments is thought to occur at high efficiency (Fischer-Colbrie et al., 1995; Kirchmair et al., 1993; Vaudry and Conlon, 1991), the fact that manserin does not exist in Purkinje cell while its precursor exist might be due to simply recognition of SgII antibodies to its fragments rather than full-length SgII. Because our manserin antibodies used in the present study has been repeatedly confirmed

its high affinity to manserin, we can conclude that Purkinje cells contain only fragments of SgII not including manserin.

Manserin-positive granular layer cells were Tuj1- and CR-positive, indicating that manserin immunoreactive cells were CR-positive UBCs. The granular layer is known to contain several types of interneurons such as granule cells, Golgi cells, Lugaro cells and UBCs (Ambrosi et al., 2007). Although precise roles of cerebellar UBCs have not been elucidated to date, UBCs are thought to receive excitatory inputs from the primary and secondary vestibulocerebellar mossy fibers (extrinsic mossy fibers) and give rise to axons (intrinsic mossy fibers) which innervate granule cells and other UBCs (Diño et al., 2000; Nunzi et al., 2001). The fact that manserin occurs in UBCs might be helpful for the elucidation of the function of UBCs. Alternatively, as suggested by Nunzi and Mugnaini (2009), the occurrence of SgII or manserin in UBCs may confer a specific neurosecretory phenotype to this neuronal subtype, endowing parcellation of the granular layer and further differentiating the two microcircuits. Further experiments are necessary to resolve manserin functions in UBC.

Two antithetic findings regarding the colocalization between SgII and the subtypes of UBCs were revealed; one is SgII's existence in the CR-expressing UBCs (Nunzi and Mugnaini, 2009),

while in mGluR1α -expressing UBCs (Miyazaki et al., 2011). Our present results that manserin exists in almost all CR-positive UBCs may lead to speculation that SgII also exists in CR-positive UBCs. Further experiments are necessary to resolve this issue.

In the granular layer, nerve fibers and fiber terminals were manserin-immnoreactive. Manserin's existence in the fibers were initially suspected by their characteristic varicose fibers and rosette-like ending, and further confirmed by double-staining with anti-VGluT2 antibody. Manserin's existence in the fibers is not surprising because neuropeptides such as calcitonin-gene related peptide and corticotrophin-releasing factor are reported to exist in large dense core vesicle (LDCV) of neuronal fiber compartments (Morara et al., 1989; Palkovits et al., 1987; Sakanaka et al., 1987). In addition, SgII, a precursor of manserin, is also localized in the cerebellar fibers (Miyazaki et al., 2011; Nunzi and Mugnaini, 2009). Our results are, however, still striking because the localization of manserin in the fibers and fiber terminals as well as cytoplasm suggests its active synthesis and storage process in the cerebellum. Further experiments are necessary to resolve this issue.

Conclusion

The present study suggests localization of manserin in most CR- expressing UBCs and some mGluR1α-expressing UBCs in adult rat cerebellum. Furthermore manserin signals were also localized at climbing fibers, mossy fibers and/or axon terminals of CR-positive UBCs. These results suggest that manserin, a novel neuropeptide, plays important roles in the cerebellar functions.

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Figure legends

Fig. 1 Manserin immunoreactivity in the rat cerebellum. Frozen sections were incubated with anti-manserin antibody (A-D), and antigen-antibody complexes were detected using diaminobenzidine staining (*brown*, A) or immunofluorescent staining (B-D). Staining was not observed when anti-manserin antibody was omitted (E), or preabsorbed with 10^{-6} M recombinant manserin peptide (F). At higher magnification, manserin immunoreactivity was found at the fibers (C) with beaded branches in the molecular layer and at fiber terminals (D; arrows) and cytoplasm (D; arrowhead) in the granular layer. Scale bars represent 100 μ m (A, B, E, and F) and 10 μ m (C, D).

Fig. 2 Double immunostaining for colocalization of manserin (*magenta*, A, D, G, J, and M) and different marker proteins (*green*, B; Tuj1, E; CR, H; mGluR1α, K and N; VGluT2). C, F, I, L, and O are merged images of A and B, D and E, G and H, J and K, M and N, respectively. Arrows in O indicate colocalization of manserin with VGluT2 at fiber terminals in the granular layer. Scale bars represent 10 μ m (F, I, L, and O) and 30 μ m (C).

Fig. 3 Cerebellar immunofluorescent staining for colocalization in Purkinje cells. Paraffin

sections were incubated with anti-SgII antibody (A). Double immunostaining was performed with anti-calbindin antibody (C, F) and anti-SgII antibody (B) or anti-manserin antibody (E). Merged images were also shown (D, G). Scale bars represent 100 μ m (A) and 10 μ m (D, G).

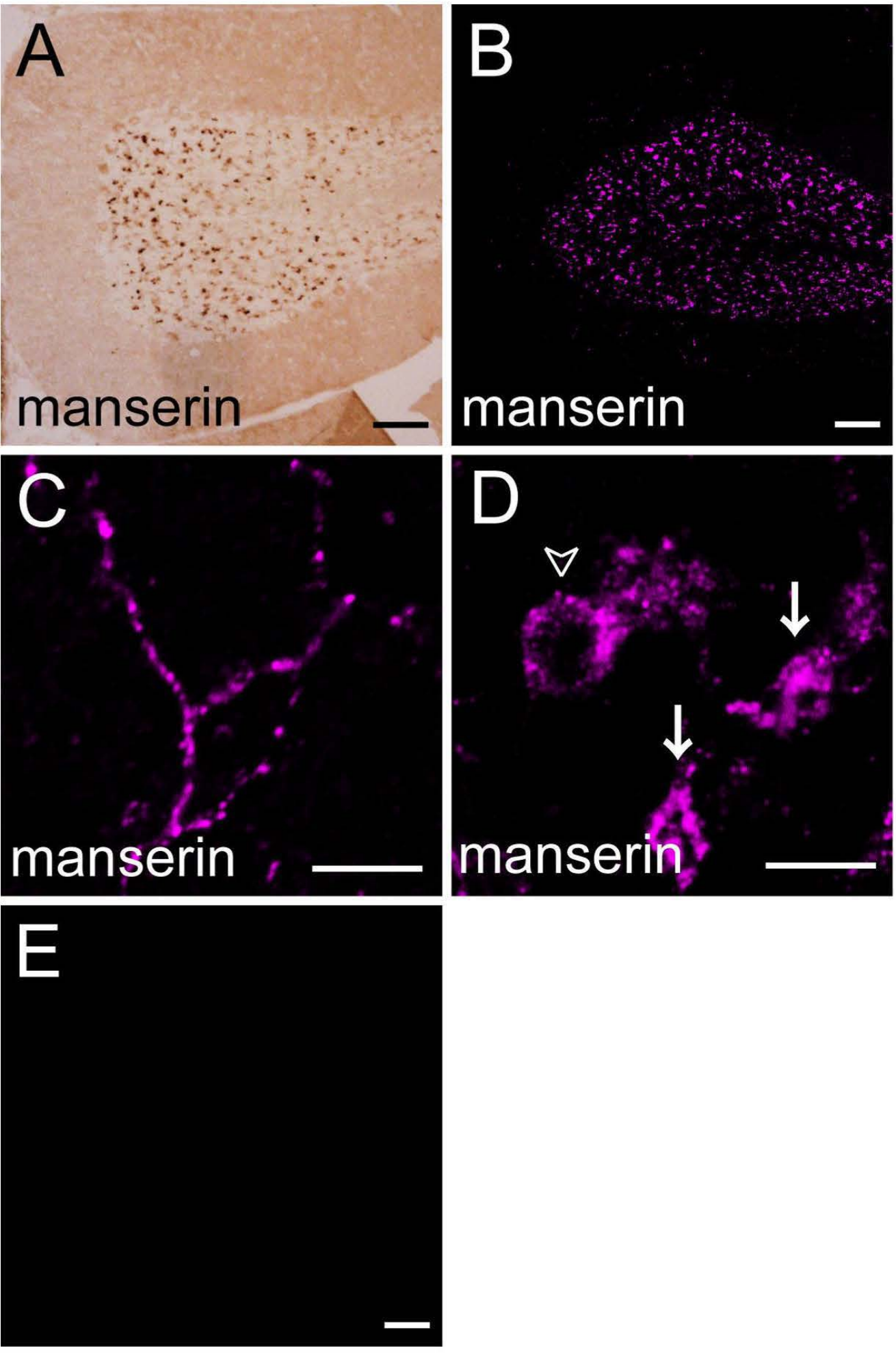

Fig. 1 Ohkawara et al

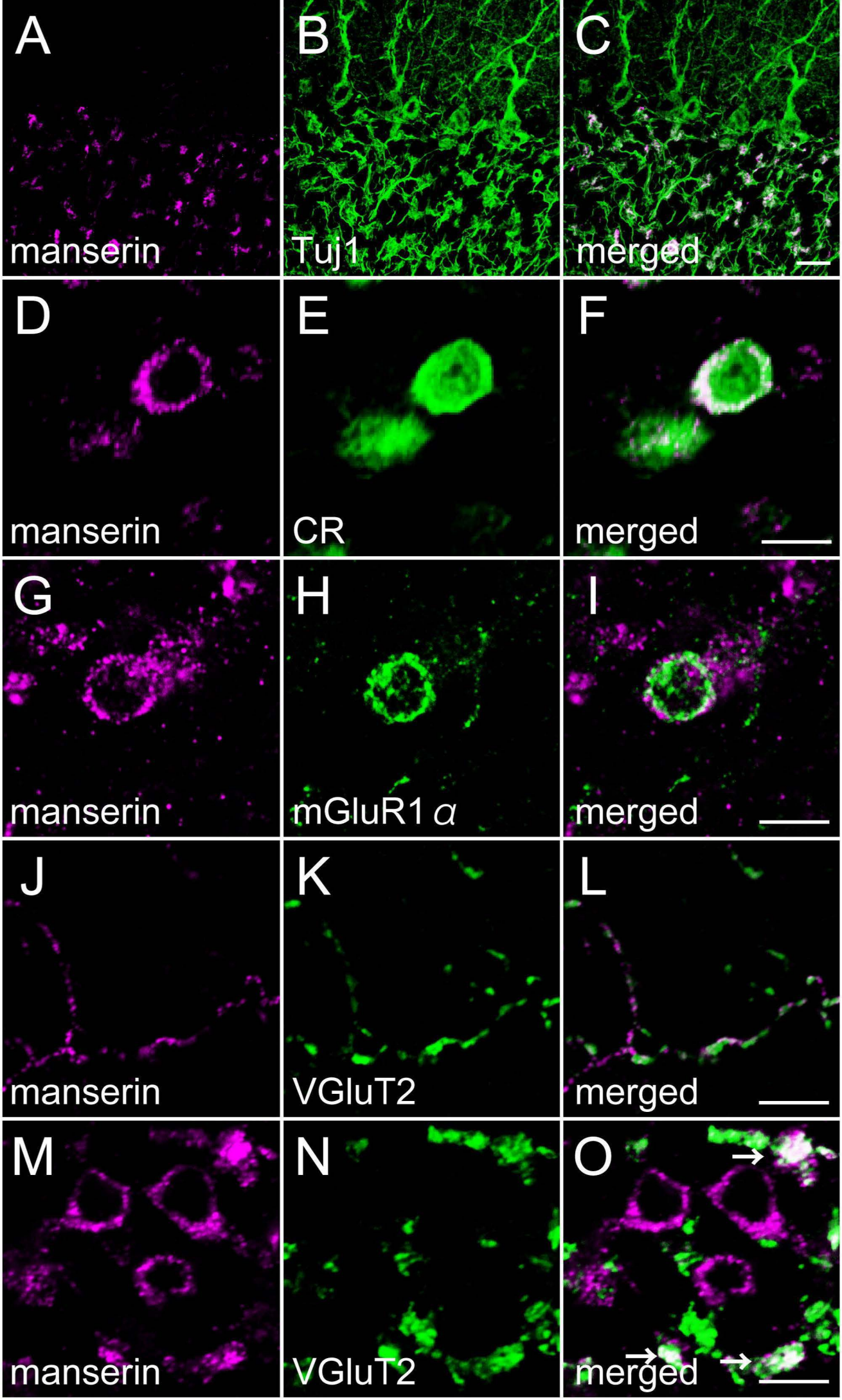

Fig. 2 Ohkawara et al

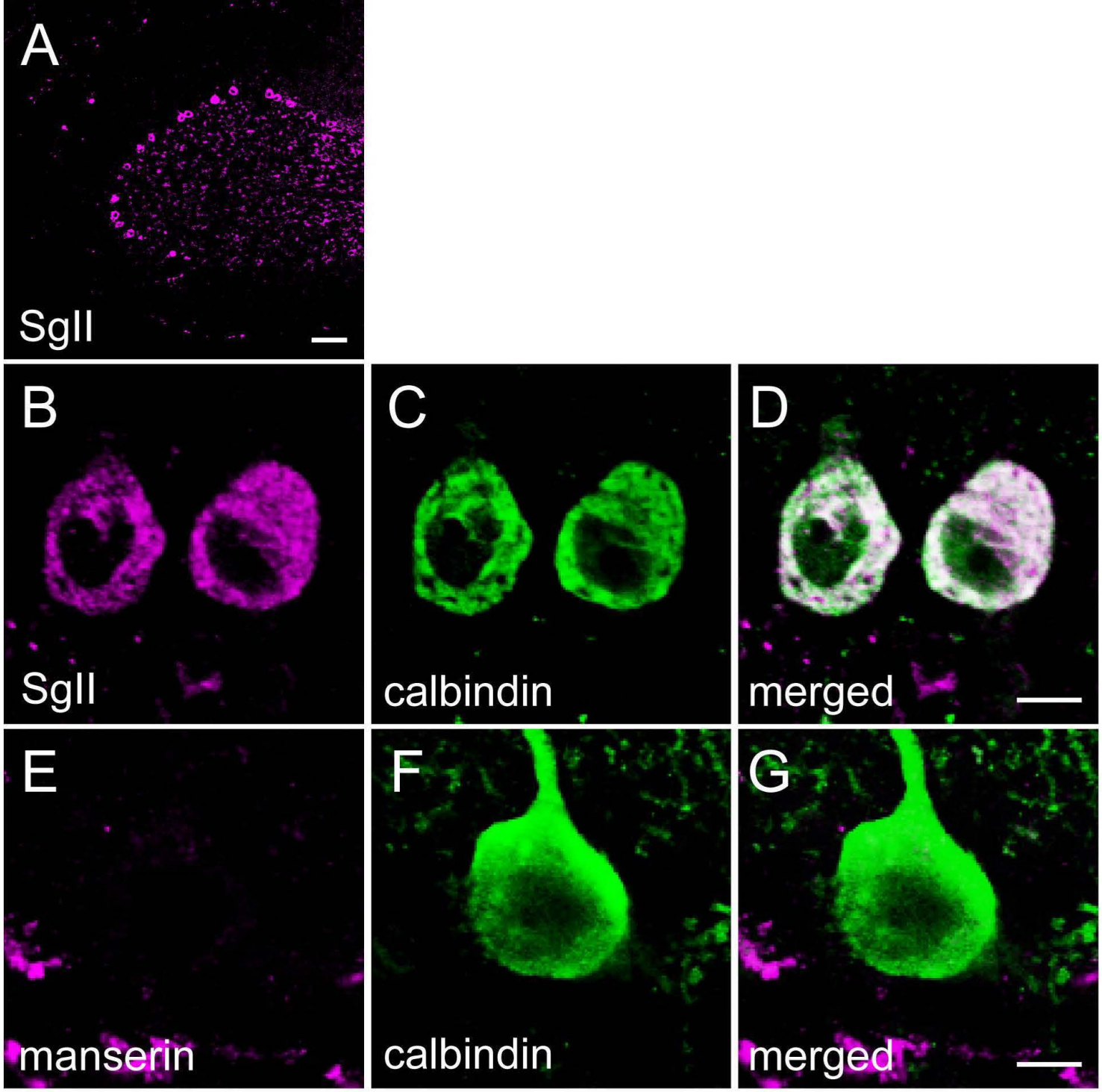

Fig. 3 Ohkawara et al