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**Immunohistochemical and immunogold localization of 7B2 (secretogranin V) to large dense-core vesicles
in both neurons and astrocytes of the rat brain: implications for extrasynaptic peptide release**

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Abstract

7B2, also known as secretogranin V, is a granin-family protein that serves as an essential molecular chaperone for the maturation and activation of prohormone convertase 2 (PC2). Although 7B2 is widely distributed in the brain, its relationship to neuronal and glial markers and its subcellular localization have not been systematically characterized. Using a guinea pig antibody raised against the middle region of rat 7B2 (residues 77–90), the specificity of which was verified by immunoblotting, immunogen-peptide preabsorption, and labeling of pituitary melanotrophs and pancreatic islet cells, we examined the adult rat brain by peroxidase-based immunohistochemistry, multiple immunofluorescence labeling, and postembedding immunogold electron microscopy. Light microscopy confirmed widespread 7B2 immunoreactivity in multiple neuronal populations, including olfactory mitral/tufted cells, forebrain interneurons, and cerebellar Purkinje cells, with substantial overlap with PC2. Immunogold labeling was preferentially associated with Golgi cisternae, Golgi-associated vesicular profiles, and individual large dense-core vesicles (LDCVs), whereas small synaptic vesicles were only sparsely labeled. Compartment-based quantification of gold particles indicated a predominance of labeling in neuronal cytoplasm and dendrites rather than at synaptic sites. 7B2-positive LDCVs were also present in the cytoplasm and processes of hippocampal astrocytes and cerebellar Bergmann glia. In both neuronal and glial elements, some labeled LDCVs lay adjacent to the plasma membrane in profiles suggestive of exocytosis. These findings establish 7B2 as a widespread constituent of LDCV-related secretory compartments in both neurons and astrocytes, and provide a morphological basis for extrasynaptic peptide release from glial as well as neuronal elements.

Keywords: secretogranin V; prohormone convertase 2; large dense-core vesicle; postembedding immunogold electron microscopy; astrocyte; Bergmann glia

1 **Introduction**

2 Neuroendocrine protein 7B2, also known as secretogranin V, is the product of the *Scg5* gene and belongs to the
3 granin protein family specifically expressed in neuroendocrine cells (Bartolomucci et al. 2011; Taupenot et al.
4 2003). In neuroendocrine cells, the biosynthesis of bioactive proteins and peptide hormones critically depends on
5 limited proteolytic processing of precursor polypeptides by prohormone convertases (PCs) along the regulated
6 secretory pathway (Nakayama 1997; Seidah and Chrétien 1997; Steiner et al. 1991). During this process, 7B2 acts
7 as a molecular chaperone: in the rough endoplasmic reticulum, it binds to the catalytic domain of PC2 and escorts
8 precursor PC2 (proPC2) as it is transported through the *trans*-Golgi network to immature secretory granules (Braks
9 and Martens 1994). The C-terminal region of 7B2 contains a domain termed the CT-peptide, which functions as
10 an endogenous inhibitor of PC2 activity (Apletalina et al. 2000a, 2000b; Benjannet et al. 1995; Braks and Martens
11 1994; Lindberg et al. 1995; Martens et al. 1994; Muller et al. 1999; Van Horssen et al. 1995, 1998; Zhu et al. 1996,
12 1998). As proPC2 passes from immature to mature granules, exposure to an acidic pH and autocatalytic processing
13 leads to cleavage of the CT-peptide and the generation of fully mature PC2. Thus, the dual role of 7B2 as both a
14 molecular chaperone and an endogenous inhibitor of PC2 has become a widely accepted model of its function
15 (Mbikay et al. 2001; Muller et al. 1997).

16 7B2 was first identified in the pituitary glands of humans and pigs (Hsi et al. 1982; Iguchi et al. 1984;
17 Seidah et al. 1983). Similar to the canonical granins chromogranin A (CgA) and chromogranin B (CgB) and
18 secretogranin II (SgII) and secretogranin III (SgIII), 7B2 has been shown by immunoelectron microscopy to be
19 localized to the secretory granules of peptide hormone-producing cells and to LDCVs of peptidergic neurons
20 (García-Caballero et al. 1997; Kikuchi et al. 2025b; Marcinkiewicz et al. 1986, 1987, 1988; Mbikay et al. 2001;
21 Sato et al. 2010). These observations raise the possibility that 7B2 may function in concert with canonical granins,
22 which are implicated in the sorting and packaging of peptide hormones and neuropeptides into secretory granules
23 and LDCVs (Bartolomucci et al. 2011; Courel et al. 2008; Gerdes et al. 1989; Hosaka et al. 2004, 2005; Kikuchi
24 et al. 2025a; Rosa et al. 1985; Yoo and Albanesi 1990). However, direct evidence for such a cooperative role of
25 7B2 together with granins in cargo sorting and vesicle biogenesis is still lacking.

26 Immunohistochemical studies in the central nervous system (CNS) by Marcinkiewicz and colleagues have
27 provided a detailed analysis of 7B2 distribution, demonstrating its widespread expression in many regions of the
28 brain and spinal cord (Marcinkiewicz et al. 1985, 1986). In contrast, immunoelectron microscopic studies of its
29 subcellular localization have been limited, and 7B2 immunoreactivity has so far been demonstrated only in
30 secretory-like granules of the median eminence and in synaptic vesicles within the interpeduncular and raphe

nuclei (Marcinkiewicz et al. 1986). Biochemical analyses have shown that the content of 7B2 varies among brain regions: 7B2 is relatively abundant in the hypothalamus and basal ganglia, although its concentration per tissue weight is only approximately one-tenth to one-twentieth of that in the pituitary (Iguchi et al. 1985). In hypothalamic subregions with high 7B2 expression, colocalization of 7B2 with peptide hormones and neuropeptides such as vasopressin (Marcinkiewicz et al. 1985), oxytocin (Gebreëls et al. 1997), and neuromedin U (Steel et al. 1988) has been demonstrated. Nevertheless, systematic analyses of 7B2 colocalization with PC2 and other marker proteins in the CNS have not yet been performed, and the precise cellular and ultrastructural distribution of 7B2 in neurons and astrocytes remains largely unresolved.

In previous studies, a rabbit antiserum raised against the amino acids (aa) of human/porcine 7B2 aa23–39 peptide (antiserum #23–39; Iguchi et al. 1984) has been widely used for histological and biochemical analyses of 7B2. We recently generated novel guinea pig antibodies against multiple regions of rat 7B2 and confirmed that one of them, antibody #77–90, produced an immunostaining pattern in endocrine tissues that closely resembled that obtained with antiserum #23–39 (Kikuchi et al. 2025b). By contrast, antibodies recognizing the *N*-terminal region and the *C*-terminal CT-peptide region yielded relatively weak signals; in particular, the CT-peptide recognized by the *C*-terminal antiserum appears to be rapidly degraded and to disappear as PC2 matures (Kikuchi et al. 2025b).

In the present study, we examined the immunohistochemical and ultrastructural localization of 7B2 in the adult rat brain, with particular emphasis on the rhinencephalon, hippocampus, and cerebellum, which have not been adequately characterized in previous studies. Using multiple immunofluorescence labeling and immunoelectron microscopy, we show that 7B2 is distributed in the somata, dendrites, axons, and nerve terminals of various neuronal types, including inhibitory interneurons, as well as in the somata and processes of astrocytes. We were able to identify LDCV-like structures consistent with vesicles undergoing exocytotic release. This widespread intracellular distribution of 7B2 provides morphological basis for the involvement of LDCVs in neuropeptide processing and secretion in both neurons and glial cells. Furthermore, these observations may provide a morphological correlate of volume transmission, in which neuroactive substances are released into extrasynaptic regions of the neuropil. In addition, we performed a compartment-based quantification of immunogold particle distribution to assess the relative association of 7B2 with vesicular compartments.

Materials and Methods

Animals and tissue preparation

Male Wistar rats aged 8–10 weeks ($n = 9$) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). For the immunoblot analysis, the animals were deeply anesthetized with isoflurane inhalation and were perfused intracardially with 50 mL of saline. The brains were surgically removed, dissected into subregions, immediately frozen on dry ice, and stored at -80°C until use. For the light microscopic immunohistochemistry, the brains were meticulously excised and fixed in Bouin's fixative by immersion. The Bouin-fixed tissues underwent a dehydration process and were subsequently embedded in paraffin (Kikuchi et al. 2025b). To prepare immunoelectron microscopy specimens, the rats were perfused intracardially with 50 mL of saline, followed by 250 mL of ice-cold 2% paraformaldehyde-2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C . The brains were removed, coronally dissected into three pieces, and postfixed by immersion in the same fixative for 24 h at 4°C . Fixed brain pieces were embedded in 2% agarose in 0.05 M sodium phosphate buffer (pH 7.4) and sliced coronally on a Microslicer DTK-1000 instrument (Dosaka EM, Kyoto, Japan) at a thickness of $400\text{ }\mu\text{m}$ (Gomi et al. 2021). The target regions were dissected from coronal slices under the observation using an MZ75 stereomicroscope (Leica Microsystems; Wetzlar, Germany). The identification of target regions was referenced using the rat brain atlas (Paxinos and Watson 1986). The dissected small pieces were post-fixed in a 1% osmium tetroxide solution for 1 h and embedded in Epon 812 resin (Nissin EM, Tokyo, Japan). The rat pituitary gland and the mouse pancreatic islet (Yasui et al. 2024) served as positive controls for the immunoelectron microscopy analysis. All animal experiments were performed in accordance with the following guidelines: the Guidelines for the Care and Use of Laboratory Animals of the Institute of Experimental Animal Science, College of Bioresource Sciences, Nihon University (Approval Nos AP24BRS085-1 and AP24BRS089-1 for rat experiments) and the Guidelines for Animal Care, Regulation of Infectious Agents, and Experimental Committee of Gunma University (Experimental Registration No. 16-017 for mouse experiments).

Antibodies

The primary and secondary antibodies utilized in this study are listed in Tables 1 and 2, respectively. The present study utilized guinea pig anti-7B2 synthetic peptide antibodies (#1–14, #77–90, #156–168), which were prepared and characterized as described previously (Kikuchi et al. 2025b). In this study, #77–90 was used for immunoblotting, peroxidase-based immunohistochemistry, immunofluorescence staining, and immunoelectron microscopy. Rabbit monoclonal anti-PC2 antibodies (D1E1S: #14013) were utilized for immunoblotting and

immunofluorescence staining. The following antibodies were utilized for immunofluorescence staining: mouse monoclonal anti-VGluT1 (135 011), anti-VGluT2 (135 421), anti-CgA (C-12: sc-393941), anti-CgB (#32: sc-517541), and anti-GFAP (GF12.24, 61011) antibodies; and rabbit polyclonal anti-calbindin D (AB1778), anti-GAD65 (21760-1-AP), anti-calretinin (82811-1-RR), anti-parvalbumin (ab316220), anti-GS (ab49873), and anti- α -MSH (PHA-AA22-01RBP84). Mouse monoclonal anti- α -tubulin (T5168) antibodies were used for immunoblotting as a loading control.

Immunoblotting

Tissue samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting according to standard protocols as described previously (Kikuchi et al. 2025b). Briefly, frozen tissues were homogenized using a lysis buffer that contained 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2.5 mM $MgCl_2$, 1 mM EGTA, 1.0% Triton X-100, and a protease inhibitor cocktail (Complete mini; Roche Diagnostics, Mannheim, Germany). The protein concentration was determined using a Coomassie brilliant blue protein assay reagent (NACALAI TESQUE, Kyoto, Japan). Protein extracts were normalized to the total protein content (30 μ g/lane) and subjected to SDS-PAGE (15% acrylamide gel) and transferred to an Immobilon-PSQ PVDF membrane (Merck Millipore Ltd., Co. Cork, Ireland). The membranes were blocked with 5% skim milk in Tris-buffered saline (pH 7.4) supplemented with 0.05% Tween 20 for 1 h, followed by an overnight incubation with primary antibodies at 4 °C. The primary antibody dilutions employed were 1:1000 for #77–90 and PC2 and 1:3000 for α -tubulin. Peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA, USA), anti-guinea pig IgG (706-035-148), anti-rabbit IgG (111-035-144), and anti-mouse IgG (111-035-146) were diluted (1:5000) and incubated for 1 h at 25 °C. A chemiluminescent HRP substrate (WBKLS0100; Merck/Millipore/Sigma Co. Ltd., Darmstadt, Germany) was used to detect enhanced signals.

Peroxidase-based immunohistochemistry

Peroxidase-based immunohistochemistry was performed according to the protocol as previously described (Kikuchi et al. 2025b). Briefly, paraffin sections mounted on silane-coated glass slides (New Silane II; Muto Pure Chemicals, Tokyo, Japan) were deparaffinized using xylene and subjected to microwave antigen retrieval by boiling in 10 mM sodium citrate buffer (pH 6.0) for 5 min. Following a thorough wash with Milli-Q water, the sections were treated with Dako REAL Peroxidase-Blocking Solution (Dako; Glostrup, Denmark) for 10 min. Subsequently, the sections were blocked with 5% normal goat serum in phosphate-buffered saline (PBS; pH 7.2)

for 30 min at 20 °C. Sections were incubated with primary antibodies for 16 h at 4 °C. Following a thorough washing with PBS, the sections were incubated with biotinylated goat anti-guinea pig IgG (PK-4007, Vector Laboratories, Inc., Newark, CA, USA) for 1 h at 20 °C. Subsequently, the sections were reacted with the avidin biotin complex-horseradish peroxidase (HRP) Reagent (PK-7100, Vector Lab.). The sections were then subjected to a thorough washing process with PBS, followed by visualization with a 3,3'-diaminobenzidine (DAB) Substrate Kit, HRP (SK-4100, Vector Lab.), and counterstained with hematoxylin. Negative control staining was performed by incubating with 7B2 antibodies pretreated with immunogen peptides at a concentration of 5 µg/mL. The images were acquired using a BX51 microscope equipped with a DP72 CCD camera and digitization of images was conducted using DP2-BSW software (Olympus, Tokyo, Japan).

Double and triple fluorescence immunohistochemistry

Deparaffinized sections were processed as above-described antigen retrieval and were incubated with 0.1 M NH₄Cl in PBS for 15 min at 20 °C. These sections were washed with PBS and blocked with 5% normal donkey serum for 30 min at 20 °C. Subsequently, the samples were incubated with primary antibody mixtures for 16 h at 4 °C. Following primary antibody incubation, sections were washed with PBS and then incubated with Alexa Fluor-conjugated secondary antibodies (Invitrogen/Thermo Fisher Scientific Inc., Waltham, MA, USA) for 1 h at 20 °C. The following combinations of secondary antibodies are utilized: (i) Alexa Fluor 488-conjugated donkey anti-guinea pig IgG (Jackson ImmunoResearch, 706-545-148)/Alexa Fluor 555-conjugated donkey anti-rabbit IgG (Invitrogen, A-31572), (ii) Alexa Fluor 488-conjugated donkey anti-guinea pig IgG (706-545-148)/Alexa Fluor 555-conjugated donkey anti-mouse IgG (A-31570), and (iii) a mixture of the antibodies in (ii) plus Alexa Fluor 647-conjugated donkey anti-rabbit IgG (A-31573). Normal animal IgG (Santa Cruz), guinea pig IgG (sc-2711), rabbit IgG (sc-2027), and mouse IgG (sc-2025) were utilized as negative controls. Fluorescence images were acquired using a BX51 microscope equipped with a DP73 CCD camera and digitized using the cellSens Standard 1.8.1 software (Olympus). The acquired images were processed by linear adjustments in brightness and contrast.

Immunoelectron microscopy

Immunogold labeling was performed on 80-nm-thick ultrathin sections as previously described (Yasui et al. 2024; Kikuchi et al. 2025a, 2025b). Sections were mounted on 200-mesh nickel grids and subjected to antigen retrieval at 95 °C for 10 min in 100 µL of 10 mM sodium citrate buffer (pH 6.0) using a GeneAmp PCR System 9700 Fast Thermal Cycler (Applied Biosystems, Waltham, MA, USA). After cooling naturally to 20 °C for approximately

10 min, the grids were washed with Milli-Q water. The sections were incubated in 50 mM glycine in PBS supplemented with 0.01% Tween 20 (PBST) for 15 min to quench residual aldehydes, blocked with 5% normal donkey serum in PBST for 30 min at 20 °C, and then incubated with 7B2 #77–90 primary antibody in PBST containing 5% normal donkey serum for 16 h at 4 °C. After washing, the sections were incubated with 6-nm colloidal gold-conjugated anti-guinea pig IgG secondary antibody (Jackson ImmunoResearch, 706-195-148) for 16 h at 4 °C. For positive controls, rat pituitary gland sections were incubated with 7B2 #77–90 and rabbit anti- α -MSH antibodies, followed by 6-nm colloidal gold-conjugated anti-guinea pig IgG and 15-nm colloidal gold-conjugated anti-rabbit IgG (815.311, AURION Immuno Gold Reagents & Accessories, Wageningen, The Netherlands) secondary antibodies. Mouse pancreatic islet sections were processed in the same manner as an additional positive control. After immunoreaction, sections were washed thoroughly with Milli-Q water, post-fixed with 1% glutaraldehyde in PBST for 5 min, and counterstained with 2% uranyl acetate and 0.4% lead citrate when necessary. Images were obtained using a JEM-1011 transmission electron microscope (JEOL Ltd., Tokyo, Japan) equipped with a MegaView G3 side-mount TEM CCD camera and RAIUS software (EMSIS GmbH, Münster, Germany). Brightness and contrast were adjusted linearly using Adobe Photoshop 2024 (Adobe KK, Tokyo, Japan).

Quantification of immunogold particles in different compartments

To quantify the ultrastructural localization of 7B2, we employed a compartment-based analysis in which each gold particle was assigned to the subcellular structure on which it was located (Burgoyne and Futter 2023). Ultrathin sections were prepared from three rats ($n = 3$), and electron micrographs were acquired from randomly selected fields within the target regions at a final magnification of $\times 60,000$. Fields containing obvious sectioning artifacts, grid bars, or poorly preserved ultrastructure were excluded before particle counting; no other selection criteria were applied. A total of 4,611 gold particles from 230 micrographs were analyzed: 3,588 particles from 155 micrographs of the cerebellar molecular layer and 1,023 particles from 75 micrographs of the hippocampus. Each particle was classified into one of four compartments on the basis of the underlying ultrastructure, and the data are expressed as percentages of the total gold particle count in each region. Compartment A (neuronal axon terminals and synaptic active zones): particles located in regions densely packed with small synaptic vesicles, or within 200 nm of the presynaptic or postsynaptic membrane thickening. Compartment B (neuronal cytoplasm and dendrites): particles located in non-terminal axonal segments, neuronal somata, or dendrites. Compartment C (astrocytes and glial processes): particles located in profiles identified as astrocytic somata or glial processes on the basis of their

1 electron-lucent cytoplasm, irregular outline, and absence of synaptic specializations. Compartment D (others):
2 particles located in myelin sheaths, mitochondria, extracellular space, or other structures not included in the above
3 categories. All compartment assignments were made by a single observer, and the analysis was performed under
4 unblinded conditions. Because the purpose of this analysis was to provide a descriptive overview of the relative
5 distribution of gold particles rather than to test a specific hypothesis, the data are presented as descriptive statistics
6 without inferential statistical testing.

Results

Specificity of 7B2 antibodies

We recently generated three guinea pig antibodies against 7B2 (#1–14, #77–90, and #156–168) that differed in their ability to detect 7B2 in endocrine tissues from several animal species. Among them, #77–90 produced the strongest signal, followed by #1–14, whereas #156–168 yielded the weakest signal (Kikuchi et al. 2025b). To assess the staining characteristics of these antibodies in the brain, we performed immunohistochemical analysis in the rat hypothalamus. In the median eminence and arcuate nucleus, immunoreactivity was strongest with #77–90 (Fig. 1a, c, and e), in agreement with our findings in endocrine tissues (Kikuchi et al. 2025b) and with previous observations in the CNS (Marcinkiewicz et al. 1986). All staining was abolished when the antibodies were preabsorbed with the corresponding antigenic peptides (Fig. 1b, d, and f). Immunoblot analysis of homogenates prepared from brain regions showed that the #77–90 recognized bands of approximately 21 kDa, consistent with those previously identified in endocrine tissues (Kikuchi et al. 2025b). These bands disappeared after preabsorption with the antigenic peptide (Fig. 1g). Under the same conditions, rabbit anti-PC2 detected a band of approximately 70 kDa, as reported previously in endocrine tissues (Fig. 1g'). Together, these findings support the specificity of the #77–90 for 7B2 in the rat brain. Before analyzing brain tissue by immunoelectron microscopy, we assessed the specificity of the #77–90 for postembedding immunogold labeling. Ultrathin sections of the intermediate lobe of the pituitary gland and pancreatic islets were subjected to antigen retrieval and processed for immunolabeling. Double labeling with anti- α -MSH antibodies and #77–90 resulted in clear labeling of secretory granules in melanotrophs of the intermediate lobe (Fig. 1h). In the pancreatic islets, secretory granules in both presumptive α -cells and β -cells were labeled with #77–90 (Fig. 1i). When preabsorbed #77–90 was used, this labeling was almost completely abolished (Fig. 1j and k).

Regional distribution of 7B2 in rat brain

The regional distribution of 7B2 was examined in detail in brain sections. In the forebrain, strong immunoreactivity was observed in the cerebral cortex (Fig. 2a), caudate-putamen (Fig. 2b), habenular nucleus (Fig. 2c), hippocampus (Fig. 2d–f and Supplementary Fig. 1a), and suprachiasmatic nucleus (Supplementary Fig. 1c). In the hippocampus, moderate labeling was present in pyramidal cells and putative interneurons in the cornu ammonis 1 (CA1) region (Fig. 2d), whereas stronger labeling was observed in the stratum lucidum of the cornu ammonis 3 (CA3) region (Fig. 2e) and in the hilus of the dentate gyrus (DG) (Fig. 2f). In the brainstem, strong labeling was detected in the pontine nucleus (Fig. 2g). In the cerebellum, moderate labeling was present throughout the cortex (Supplementary

Fig. 1k). The intensity of 7B2 immunoreactivity in Purkinje cell somata and dendrites varied among cells and across lobules (Fig. 2h and Supplementary Fig. 1m). In the molecular layer, a distinctive row of coarse punctate profiles was present in lobule X (Fig. 2i). In addition, moderate labeling was observed in neurons considered likely to be basket cells or stellate cells on morphological grounds (Fig. 2j and k). In the granular layer, relatively strong labeling was present in large irregular profiles corresponding to synaptic glomeruli and low-level labeling was also observed in neurons resembling Golgi cells (Fig. 2j). Unipolar brush cells (UBCs) are known to be distributed at high density in the vestibulocerebellum and flocculus (Mugnaini et al. 1997). Prominent 7B2 labeling was observed in cells morphologically consistent with UBCs in lobules IX (Fig. 2l). Moderate immunoreactivity was also observed in large neurons of the deep cerebellar nuclei (Fig. 2m). Because information on the distribution of 7B2 in the olfactory system is limited, we examined major relay sites in both the main olfactory system, which processes general odor information (olfactory bulb, anterior olfactory nucleus, olfactory tubercle, anterior piriform cortex [APC], and cortical amygdaloid nucleus), and the accessory olfactory system, which processes pheromonal cues (accessory olfactory bulb, bed nucleus of the stria terminalis, and medial amygdaloid nucleus) (Fig. 2n). In the olfactory bulb, strong immunoreactivity was observed in the glomeruli, which contain olfactory nerve terminals and the dendrites of mitral/tufted cells, as well as in the perikarya of periglomerular cells and mitral/tufted cells. Moderate labeling was present in the external plexiform layer, internal plexiform layer, and granule cell layer (Fig. 2o). In the anterior olfactory nucleus and the dorsal part of the APC, robust labeling was observed in neuronal perikarya (Fig. 2p and Supplementary Fig. 2a). In contrast, in the ventral part of the APC, strong immunoreactivity was present mainly in the neuropil, and fiber bundles in the olfactory tubercle were also intensely labeled (Supplementary Fig. 2b). In the cortical amygdaloid nucleus, moderate immunoreactivity was present in the perikarya and neuropil of a subset of neurons (Supplementary Fig. 2c). In the accessory olfactory bulb, the strongest signal was detected in the mitral/tufted cell layer (Supplementary Fig. 2g). Moderate labeling was also present in the bed nucleus of the stria terminalis and medial amygdaloid nucleus (Supplementary Fig. 2h and i). All of these signals were abolished by preabsorption of the antibodies with the immunogen peptide (Supplementary Fig. 1b, d–j, l, n, 2d–f, and j–o).

Double immunofluorescence labeling of 7B2 with PC2, VGluT1, and VGluT2 in the forebrain

We examined the colocalization of 7B2 with PC2, VGluT1, and VGluT2 by double immunofluorescence labeling. In the median eminence, the distribution of PC2 and VGluT2 closely resembled that of 7B2, particularly in the external zone, where the processes of neuroendocrine neurons converge (Fig. 3a and c), whereas no overlap

between VGluT1 and 7B2 was detected (Fig. 3b). In the internal zone and in the arcuate nucleus immediately dorsal to it, PC2 and VGluT2 showed a punctate distribution similar to that of 7B2 (Fig. 3a and c). In the habenular nucleus, PC2, VGluT1, and VGluT2 all overlapped with 7B2, and the overlap was more extensive in the medial than in the lateral habenular nucleus (Fig. 3d–f). In the hippocampus, PC2 overlapped weakly with 7B2 in the perikarya of CA1 pyramidal cells (Fig. 3g), in the stratum lucidum of CA3 (Fig. 3j), and in the polymorphic layer of the DG (Fig. 3m). The two VGluTs showed complementary patterns: VGluT2 overlapped with 7B2 in CA1 region (Fig. 3i) but not in CA3 region or the DG (Fig. 3l and o), whereas VGluT1 overlapped with 7B2 in CA3 region (Fig. 3k) and the DG (Fig. 3n) but not in CA1 region (Fig. 3h).

Multiple immunofluorescence labeling of 7B2 with PC2 and cerebellar marker proteins

Detailed cellular-level information on the distribution of 7B2 in the cerebellum has been limited. To identify cerebellar neurons and terminals containing 7B2, we performed multiple immunofluorescence labeling with antibodies against PC2, GAD65, parvalbumin, calretinin, VGluT1, VGluT2, and calbindin. GAD65 was used as a marker for Purkinje cells, molecular layer interneurons, and Golgi cells; parvalbumin for Purkinje cells and molecular layer interneurons, including basket and stellate cells; calretinin for a subset of unipolar brush cells (UBCs) in the vestibulocerebellar granular layer; VGluT1 for parallel fiber terminals in the molecular layer and for mossy fiber-related components of cerebellar glomeruli; VGluT2 for climbing fiber terminals in the molecular layer and a subset of mossy fiber terminals in the granular layer; and calbindin for Purkinje cells. 7B2 immunoreactivity was present in the perikarya of Purkinje cells, molecular layer interneurons, and Golgi cells, identified by overlap with GAD65 or parvalbumin (Fig. 4a, b, e, and f). 7B2 was also present in the pinceau, the basket cell axonal plexus surrounding the initial segment of the Purkinje cell axon, visualized with parvalbumin in lobule III and with GAD65 in lobule X (Fig. 4b and e). Calretinin-positive UBCs, which were more densely distributed in lobule X of the vestibulocerebellum than in lobule III, were also labeled for 7B2 (Fig. 4c and g). In the molecular layer, fine punctate 7B2 labeling overlapped with VGluT1-positive parallel fiber terminals (Fig. 4i and k). At VGluT2-positive climbing fiber terminals apposed to calbindin-positive Purkinje cell dendrites, overlap with 7B2 was observed in lobule IX but not in lobule V (Fig. 4j and l). In the granular layer, strong 7B2 labeling was present in mossy fiber terminals labeled for VGluT1 or VGluT2 (Fig. 4i–l). Double labeling for 7B2 and PC2 revealed a difference between lobules III and X in the large, irregular profiles of the granular layer, which corresponded to synaptic glomeruli in lobule III and to UBC somata in lobule X (Fig. 4d and h). Weak but detectable overlap with PC2 was also present in aligned punctate profiles resembling climbing fibers in the

1 molecular layer of lobule X (Fig. 4h). In the deep cerebellar nuclei, 7B2 in large excitatory neurons overlapped
2 more extensively with VGluT2 than with VGluT1 (Fig. 4m and n), whereas these neurons showed little PC2
3 immunoreactivity (Fig. 4o). Smaller calretinin-positive inhibitory neurons were only weakly labeled for 7B2 (Fig.
4 4p). Thus, 7B2 was distributed in the somata, dendrites, and axon terminals of several cerebellar neuronal types.

5 6 **Double immunofluorescence labeling of 7B2 with calcium-binding proteins and chromogranins in the** 7 **olfactory bulb**

8 In the olfactory bulb, calbindin and calretinin are expressed in distinct neuronal populations (Crespo et al. 1997),
9 and calbindin-positive and calretinin-positive periglomerular cells are recognized as different interneuron
10 subpopulations (Kosaka et al. 1995). 7B2 overlapped with both calbindin and calretinin in the perikarya of
11 periglomerular cells and in dendrites extending toward the center of the glomeruli (Fig. 5a, b, d, and e). Some
12 glomeruli showed particularly intense calretinin immunoreactivity (Fig. 5d and e). In mitral/tufted cells, calbindin
13 immunoreactivity was moderate and overlapped with 7B2, whereas calretinin immunoreactivity was minimal (Fig.
14 5c and f). From the internal plexiform layer to the granule cell layer, 7B2 overlapped weakly with calbindin-
15 positive process-like profiles and with calretinin-positive cell bodies (Fig. 5c and f). We next examined the
16 relationship of 7B2 to CgA and CgB, both of which are implicated in the condensation and stabilization of granule
17 contents through pH- and Ca^{2+} -dependent aggregation (Winkler et al. 1992; Yoo, 1995). Within the glomeruli,
18 CgA and CgB each overlapped weakly with 7B2 in olfactory nerve terminals and in the dendrites of periglomerular
19 cells, and CgB immunoreactivity was weaker than that of CgA (Fig. 5g, h, j, and k). The distribution of CgA
20 resembled that of calretinin, including the glomeruli with intense labeling (Fig. 5g and h). In mitral/tufted cells,
21 by contrast, the overlap with 7B2 was more evident for CgB than for CgA (Fig. 5i and l). In the external plexiform,
22 internal plexiform, and granule cell layers, fine punctate signals of both CgA and CgB overlapped with 7B2 (Fig.
23 5i and l).

24 25 **Double immunofluorescence labeling of 7B2 with parvalbumin and calretinin in the cerebral cortex and** 26 **hippocampus**

27 We next examined the relationship of 7B2 to parvalbumin and calretinin. In the parietal cortex, 7B2 labeling was
28 present in neurons labeled for either marker (Fig. 6a and b). In the hippocampus, 7B2 labeling was present in a
29 small number of scattered neurons in the pyramidal cell layer of CA1 (Fig. 6c and d) and CA3 (Fig. 6e and f)
30 regions. In the DG, 7B2 labeling was present in parvalbumin-positive neurons within the granule cell layer and in

calretinin-positive neurons at the border between the granule cell layer and the polymorphic layer (hilus) (Fig. 6g and h). Although these neurons most likely correspond to GABAergic interneurons in the rat, they cannot be assigned to a single defined category on the basis of morphology and marker expression alone. Because parvalbumin and calretinin are not coexpressed in the rat hippocampus (Miettinen et al. 1992), these findings indicate that 7B2 is present in more than one neuronal subtype.

Localization of 7B2 in astrocytes

Because 7B2 has been regarded as a neuronal and neuroendocrine protein, we next asked whether it is also present in astrocytes. Double immunofluorescence labeling for 7B2 together with the astrocyte markers GS and GFAP was performed in the median eminence, arcuate nucleus, habenular nucleus, hippocampus, and cerebellum. Overlap was restricted to defined subsets of astrocytic profiles rather than being widespread (Fig. 7). Overlap with GS appeared as linear or punctate labeling in glial processes of the median eminence and arcuate nucleus (Fig. 7a), in processes within the habenular nucleus (Fig. 7b), in relatively thick processes near the cell bodies in the stratum lucidum of hippocampal CA3 (Fig. 7c), and in the cytoplasm and processes of astrocytes adjacent to Purkinje cells in the cerebellum (Fig. 7d). Overlap with GFAP was present in Bergmann glial processes extending into the cerebellar molecular layer, particularly in processes showing relatively low GFAP immunoreactivity (Fig. 7e). By contrast, many strongly GFAP-positive processes showed little or no detectable 7B2 immunoreactivity (Fig. 7f), and astrocytes in the cerebellar granular layer, white matter, and deep cerebellar nuclei showed minimal or no overlap (Fig. 7g and h).

Ultrastructural localization of 7B2 in the olfactory bulb and habenular nucleus

In the olfactory bulb, immunogold-labeled LDCVs were observed in dendrites of mitral cells and periglomerular cells within glomeruli (Fig. 8a), in mitral cell somata (Fig. 8b), and in dendritic profiles within the external plexiform layer (Fig. 8c). Similar labeling was also observed in dendrites of mitral/tufted cells in the accessory olfactory bulb (Fig. 8d). In the medial habenular nucleus, immunogold labeling was present in neuronal somata on Golgi membranes and on a subset of coated vesicles, whereas other coated vesicles in the same fields were unlabeled (Fig. 8e). These coated vesicles were positioned near the Golgi membranes (Fig. 8e and f). Additionally, we observed coated vesicles in close proximity to microtubules within axons that are presumably afferent axons within the medial habenular nucleus (Fig. 8g).

Ultrastructural localization of 7B2 in the hippocampus

In the hippocampus, immunogold particles clustered prominently on LDCVs within the cytoplasm of pyramidal cells in CA1 and CA3 regions (Fig. 9a and c). LDCVs in astrocytes were similarly labeled, and lower densities of gold particles were also present on vesicular membranes near the Golgi apparatus (Fig. 9b). In the DG, labeling was observed in granule cell dendrites extending into the molecular layer (Fig. 9d), as well as on LDCVs located distally from the active zone in synaptic terminals of interneuron-like profiles (Fig. 9e). In the giant mossy fiber terminals of CA3, immunogold-labeled LDCVs were present among abundant small synaptic vesicles (Fig. 9f). In granule cells, immunogold labeling was also associated with the lumen of Golgi cisternae and with Golgi vesicular profiles (Fig. 9g).

Ultrastructural localization of 7B2 in the cerebellum

In the cerebellar molecular layer, 7B2-labeled LDCVs were observed in parallel fiber profiles, at the apposition between Purkinje cell dendritic spines and adjacent Bergmann glial processes, in climbing fiber terminals, and in presumptive inhibitory interneuron terminals (Fig. 10a, b, and g). Occasional gold particles were also associated with small synaptic vesicles, but labeling was far more prominent on LDCVs (Fig. 10b). In Purkinje cells, 7B2-labeled LDCVs were present in the cytoplasm, and some were closely associated with microtubules (Fig. 10c and d). In addition, immunogold labeling was detected in Golgi cisternae and along Golgi membranes in Purkinje cells (Fig. 10e). 7B2-labeled LDCVs were also present in Bergmann glial processes (Fig. 10f).

In the granular layer, a few gold particles were associated with small synaptic vesicles in mossy fiber terminals, whereas LDCVs in the same terminals were unlabeled (Fig. 11a). By contrast, in lobule X, immunogold-labeled LDCVs were readily identified in cerebellar glomeruli (Fig. 11b). Profiles morphologically suggestive of exocytotic release were also observed in the granular layer and are presumed to be associated with astrocytic processes (Fig. 11c). In addition, immunogold-labeled LDCVs were observed in presumptive Golgi cell dendrites and terminals (Fig. 11d and e), and in basket cell terminals forming the pinceau around Purkinje cell somata (Fig. 11f).

Distribution of immunogold particles among subcellular compartments

The distribution of gold particles among Compartments A–D in the hippocampus and the cerebellar molecular layer is summarized in Fig. 12. A similar distribution pattern was observed in both regions. The largest proportion of particles was assigned to Compartment B (the cytoplasm and dendrites of neurons), accounting for 63.0% in

1 the hippocampus and 59.9% in the cerebellum. Compartment A (synaptic region) represented 14.5% in the
2 hippocampus and 15.8% in the cerebellum, while Compartment C (processes of astrocytes and glial cells)
3 constituted 20.6% and 19.8%, respectively. Compartment D, which likely contains a significant background
4 component, accounted for 2.0% in the hippocampus and 4.5% in the cerebellum. Thus, in both regions, 7B2
5 labeling was more frequently associated with non-synaptic neuronal compartments than with synaptic profiles,
6 and a consistent minority of particles was associated with profiles identified as astrocytic. These proportions are
7 expressed as fractions of the total particle count rather than as area-normalized densities, and are therefore intended
8 as a descriptive overview of the relative distribution of labeling.
9

Discussion

In the present study, we examined the cellular and ultrastructural localization of 7B2 in the adult rat brain using newly generated anti-7B2 antibodies (Kikuchi et al. 2025b), multiple immunofluorescence labeling, and immunogold electron microscopy. The main findings can be summarized as follows. First, the guinea pig antibodies directed against the middle region of 7B2 (#77–90) provided robust and specific labeling in the rat brain. Second, 7B2 was widely distributed in multiple brain regions and in morphologically and neurochemically distinct neuronal populations. Third, 7B2 showed extensive overlap with PC2 in several regions. Fourth, at the subcellular level, 7B2 was associated predominantly with Golgi-related compartments and LDCVs, with only limited labeling of small synaptic vesicles. Finally, 7B2-positive LDCVs were identified not only in neurons but also in astrocytes, including Bergmann glia, and some exhibited profiles suggestive of exocytosis. Together, these observations extend the pioneering observations regarding the distribution of 7B2 in the CNS (Marcinkiewicz et al. 1985, 1986) and provide an anatomical framework for understanding its association with regulated secretory compartments.

Regional and cellular distribution of 7B2

Immunoreactivity was observed in the cell bodies, dendrites, axons, and nerve terminals of diverse neuronal populations in the olfactory system, hippocampus, cerebellum, and other forebrain and brainstem regions. Particularly prominent labeling was observed in mitral/tufted cells of the olfactory bulb, in Purkinje cells and several classes of cerebellar interneurons, and in hippocampal regions associated with mossy fiber circuitry. These findings reaffirm that 7B2 is incorporated into diverse neural systems and is not restricted to classical hypothalamic neuroendocrine pathways.

Relationship between 7B2 and PC2

The close spatial relationship between 7B2 and PC2 observed in several regions is consistent with the established role of 7B2 as a molecular chaperone and endogenous regulator of PC2 maturation (Braks and Martens 1994; Lindberg et al. 1995; Mbikay et al. 2001; Muller et al. 1997). In the median eminence, arcuate nucleus, habenular nucleus, and hippocampal CA3 and dentate hilar regions, the distributions of the two proteins were highly similar. These observations support the interpretation that 7B2 is localized to compartments in which PC2-dependent processing is likely to occur. On the other hand, information on the cellular distribution of PC2 in the cerebellum is limited (Schäfer et al. 1993; Winsky-Sommerer et al. 2000), and its colocalization with 7B2 had not been analyzed. In the present study, it was shown that the two signals were not completely superimposable in all

1 structures, particularly in the cerebellar cortex and deep cerebellar nuclei. It has been reported that PC2 mRNA
2 expression is more regionally restricted and is absent from some regions where 7B2 is expressed at high levels
3 (Seidel et al. 1998). The present protein-level analysis is consistent with this observation. Such regional differences
4 may reflect cell-type-specific variation in the intracellular trafficking, maturation, or turnover of the two proteins
5 along the regulated secretory pathway, rather than a simple dissociation of their functions. Alternatively, they may
6 suggest that 7B2 has an additional function in cells that contain very little PC2.

7 8 **Subcellular localization to LDCVs and association with the secretory pathway**

9 A notable finding of the present study is the preferential localization of 7B2 to LDCVs. This distribution agrees
10 with previous work in endocrine and peptidergic systems showing localization of 7B2 to secretory granules and
11 dense-core vesicles (García-Caballero et al. 1997; Kikuchi et al. 2025b; Marcinkiewicz et al. 1986, 1987, 1988;
12 Sato et al. 2010). In the present material, 7B2 labeling was also observed in Golgi cisternae and in vesicular profiles
13 in the vicinity of the Golgi apparatus. These observations are consistent with the current model in which 7B2
14 associates with proPC2 early in the secretory pathway and is transported through Golgi-related compartments to
15 immature and mature dense-core vesicles (Braks and Martens 1994; Mbikay et al. 2001; Muller et al. 1997).
16 Immunogold particles were concentrated on individual LDCVs in neuronal somata, dendrites, axons, and terminals,
17 whereas only occasional particles were associated with small synaptic vesicles. Quantitative analysis of
18 immunogold particle distribution across four compartment categories in the hippocampus and cerebellar molecular
19 layer supported this observation. Because LDCVs are predominantly located in neuronal somata, dendrites, and
20 non-terminal axonal segments, whereas synaptic active zones are dominated by small synaptic vesicles, we
21 compared particle distribution between these compartments. Particles located in neuronal cytoplasm and dendrites
22 accounted for approximately 60% of the total, whereas those associated with synaptic active zones accounted for
23 approximately 15%, consistent with the preferential association of 7B2 with LDCVs rather than small synaptic
24 vesicles. Thus, the present ultrastructural findings provide morphological support in the brain for a trafficking
25 pathway that has been defined mainly in endocrine cells.

26 The labeling of coated vesicular profiles, particularly in the medial habenular nucleus, is also of interest.
27 Although the precise identity of these vesicles remains uncertain, the presence of electron-dense coats at the
28 cytoplasmic face of the vesicle membrane suggests that they may correspond to clathrin- or coatamer-coated
29 vesicles (Rabouille and Klumperman 2005). Their distribution near Golgi membranes and within axonal profiles
30 suggests that 7B2 is present not only in mature LDCVs but also in earlier transport or sorting intermediates. This

observation is compatible with the possibility that 7B2 participates more broadly in the organization of secretory compartments. In this context, it is notable that 7B2 showed partial overlap with CgA and CgB in the olfactory bulb. The presence of CgA (Lahr et al. 1990, 1992), CgB (Forss-Petter et al. 1989), SgII (Leitner et al. 1997), and SgIII (Gomi et al. 2021) has been reported in the olfactory bulb. These canonical granins are thought to contribute to the condensation, sorting, and stabilization of secretory cargoes within the regulated secretory pathway (Bartolomucci et al. 2011; Hosaka and Watanabe 2010; Hotta et al. 2009; Maeda et al. 2018; Winkler et al. 1992; Wollam et al. 2017; Yoo 1995). Although the present study does not directly address molecular interactions among granin family proteins, the observed anatomical overlap is consistent with previous findings that 7B2 is incorporated into granin-rich secretory granules in the pituitary (Kikuchi et al. 2025b; Sakai et al. 2003; Westphal et al. 1999).

Association of 7B2 with specific neural circuits

The regional patterns observed here further suggest that 7B2 is associated with distinct neuronal systems rather than being distributed uniformly throughout the brain. In the hippocampus, strong labeling in the stratum lucidum of CA3 and in the dentate hilus, together with extensive overlap with VGluT1, points to a prominent association with mossy fiber-related circuitry. The presence of 7B2-positive LDCVs in giant mossy fiber terminals is consistent with the well-known structural specialization of this pathway. Notably, the distribution pattern of 7B2 closely resembled that of SgII in the mouse hippocampus (Miyazaki et al. 2011). In the cerebellum, 7B2 was present in Purkinje cells, basket and stellate cells, Golgi cells, presumptive UBCs, and mossy and climbing fiber-related elements, indicating that it is incorporated into both inhibitory and excitatory components of cerebellar circuitry. The selective overlap with calretinin in the vestibulocerebellum reflects this region's known anatomical specialization, where UBCs are particularly abundant (Mugnaini et al. 1997). This overlap may also be related to UBC subtypes and their granin distribution patterns (Nunzi and Mugnaini 2009; Sekerková et al. 2014). Furthermore, strong labeling in mitral/tufted cells, periglomerular cells, and dendritic profiles in both the main and accessory olfactory bulbs, as well as in downstream structures of the main and vomeronasal olfactory pathways, indicates that 7B2 is broadly associated with olfactory processing circuitry. Biochemical analyses have shown that 7B2 content in the brain varies significantly among regions (Iguchi et al. 1985). Taken together, these findings point to marked regional and cellular heterogeneity in the distribution of 7B2.

Potential role of 7B2 in astrocytic secretion

Another noteworthy observation is the demonstration of 7B2 in astrocytic elements. At the light microscopic level, overlap with GS and GFAP was limited and heterogeneous. However, immunoelectron microscopy identified 7B2-positive LDCVs in the cytoplasm and processes of hippocampal astrocytes and cerebellar Bergmann glia. A compartment-based analysis indicated that approximately 20% of gold particles were located in profiles identified as astrocytic on morphological grounds. These findings suggest that at least a subset of astrocytes contains dense-core vesicular compartments bearing 7B2. Direct ultrastructural descriptions of peptide-related secretory compartments in astrocytes have been scarce, and the present observations therefore fill a specific gap in the anatomical description of these cells. The weak association of 7B2 with strongly GFAP-positive processes further suggests that its distribution within astrocytes may vary among cellular compartments or astrocyte subtypes.

To date, accumulated evidence indicates that astrocytes are active participants in tripartite synapses (Araque et al. 1999; Perea et al. 2009). These cells are thought to release neurotransmitters, such as glutamate, GABA, ATP, and D-serine, via regulated exocytosis from synaptic-like microvesicles (Agulhon et al. 2008; Cali 2024; Le Meur et al. 2012; Mielnicka and Michaluk 2021; Prada et al. 2011; Sahlender et al. 2014; Tanaka et al. 2013). On the other hand, it has been hypothesized that specific peptides derived from granin proteins and brain-derived neurotrophic factor are sorted into LDCVs and processed by PCs (Bathina and Das 2015; Fulmer et al. 2014; Hur et al. 2010; Majdoubi et al. 1996; Miyazaki et al. 2011; Paco et al. 2010; Woulfe et al. 1999; Zhan et al. 2018a, 2018b). However, electron microscopic evidence for the presence of secretory granules or LDCVs in astrocytes has remained limited (Calegari et al. 1999; Hur et al. 2010). The precise localization of 7B2 to LDCVs in astrocytes, as demonstrated in this study, provides morphological support for the interpretation that at least a subset of astrocytes possesses secretory compartments potentially associated with PC2-dependent peptide processing.

Morphological correlates of 7B2 with exocytotic release

Profiles morphologically suggestive of exocytotic release were observed in both neuronal and glial elements. In these profiles, 7B2-positive LDCVs were situated adjacent to plasma membranes showing local indistinctness or apparent interruption, and vesicular contents appeared to be present in the extracellular space. Although static ultrastructural images cannot establish membrane fusion directly, these observations are consistent with the interpretation that at least some 7B2-positive LDCVs are positioned for exocytotic release. From a morphological standpoint, such findings may represent a structural correlate of extrasynaptic release from dense-core vesicles. The presence of 7B2-labeled LDCVs in neuronal dendrites, distant from synaptic active zones, may be related to dendritic release (Kennedy and Ehlers 2011; Ludwig and Leng 2006; Ludwig et al. 2016). Similarly, the release

of LDCVs from astrocytes may represent morphological correlates of volume transmission (Gianni and Pasqualetti 2023; Özçete et al. 2024). Nevertheless, further research is needed to determine the functional significance of these morphological features.

Open questions and future directions

Two aspects of the present findings warrant particular attention in future work. The first concerns the identity of the cargo. Although the localization of 7B2 to LDCVs is consistent with a role in PC2-dependent processing, the peptides stored in these vesicles remain to be identified, and it is not yet known whether they differ among the neuronal and glial populations described here. Combining 7B2 immunogold labeling with markers for candidate peptide cargoes would help resolve this question and clarify whether the regional heterogeneity observed here reflects distinct peptidergic repertoires. The second concerns the relationship between 7B2 and the canonical granins. The partial overlap with CgA and CgB in the olfactory bulb, and the resemblance of the hippocampal 7B2 pattern to that of SgII (Miyazaki et al. 2011), raise the question of whether these proteins occupy the same vesicle population or define partially distinct dense-core compartments. Double-labeling immunogold analysis at the level of individual vesicles would address this directly and would bear on whether 7B2 participates in cargo condensation and granule biogenesis alongside the canonical granins. Finally, the profiles suggestive of exocytosis described here define a structural question rather than answer a functional one. Whether these vesicles undergo regulated release, and under what conditions, will require ultrastructural analysis of stimulated preparations or complementary live-imaging approaches. The present observations provide the anatomical reference against which such studies can be designed.

Conclusion

The present study shows that 7B2 is widely distributed in neurons and astrocytes of the adult rat brain and is associated predominantly with Golgi-related compartments and LDCVs rather than with conventional small synaptic vesicles. Its substantial overlap with PC2 supports the view that 7B2 is a widespread component of peptide-related secretory compartments in the CNS. The identification of 7B2-positive LDCVs in both neuronal and glial elements further extends the known anatomical distribution of this protein and provides a morphological basis for future analyses of its role in regulated secretion. More broadly, the presence of 7B2-positive LDCVs in non-synaptic neuronal compartments and in astrocytic profiles indicates that the 7B2–PC2 system operates well beyond the classical neuroendocrine pathway in which it was first described.

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Competing interests

The authors declare no competing interests.

Ethics approval

All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Institute of Experimental Animal Science, College of Bioresource Sciences, Nihon University (Approval Nos AP24BRS085-1 and AP24BRS089-1 for rat experiments) and the Guidelines for Animal Care, Regulation of Infectious Agents, and Experimental Committee of Gunma University (Experimental Registration No. 16-017 for mouse experiments).

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Data availability

The data supporting the findings of this study are available within the article and its supplementary information files. Additional data, including the original electron micrographs, are available from the corresponding author upon reasonable request.

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Author contributions

HG designed the study, performed experiments, analyzed the data, and wrote the original manuscript draft, with contributions from ST, MH, and TY. All authors discussed the results, edited the manuscript, contributed important intellectual content, and approved the final manuscript.

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Table 1. Primary Antibodies Used in the Present Study

Target [†]	Host sp. [‡]	Dilution (Usage) [§]	Supplier [¶] (Catalog No.)	Immunogen [†]	RRID
7B2 (SgV)	Gp	1:1000–1:2000 (IH)	H. Gomi, Nihon Univ. (#1-14)	Rt 7B2 aa 1–14	AB_3751456
7B2 (SgV)	Gp	1:1000–1:2000 (IB, IH, IF, IE)	H. Gomi, Nihon Univ. (#77-90)	Rt 7B2 aa 77–90	AB_3751453
7B2 (SgV)	Gp	1:500 (IH)	H. Gomi, Nihon Univ. (#156-168)	Rt 7B2 aa 156–168	AB_3751454
PC2	Rb	1:1000 (IB, IF)	Cell Signaling (D1E1S; #14013)	Hu PC2 aa 194 surrounding	AB_2631285
VGluT1	Mo	1:1000 (IF)	Synaptic Systems (135 011)	Rt VGluT1 aa 542–560	AB_2617087
VGluT2	Mo	1:500 (IF)	Synaptic Systems (135 421)	Rt VGluT2 aa 510–582	AB_2619823
Calbindin	Rb	1:500 (IF)	Chemicon/Millipore (AB1778)	Mo Calbindin D	AB_2068336
GAD65	Rb	1:200 (IF)	Proteintech (21760-1-AP)	Hu GAD65 aa 1–180	AB_2878916
Calretinin	Rb	1:500 (IF)	Proteintech (82811-1-RR)	Hu Calretinin aa 1–271	AB_3670560
Parvalbumin	Rb	1:1000 (IF)	Abcam (ab316220)	Proprietary information	AB_3751472
CgA	Mo	1:50 (IF)	Santa Cruz (C-12, sc-393941)	Hu CgA aa 442–457	AB_2801371
CgB	Mo	1:50 (IF)	Santa Cruz (#32, sc-517541)	Rt CgB aa 200–389	AB_3764835
GS	Rb	1:20,000 (IF)	Abcam (ab49873)	Mo GS aa350–373	AB_880241
GFAP	Mo	1:100 (IF)	PROGEN (GF12.24, 61011)	Hu Intermediate filament	AB_2920743
α -MSH	Rb	1:25,000 (IE)	Gunma Univ. (PHA-AA22-01RBP84)	α -MSH aa1–13	AB_3751473
α -tubulin	Mo	1:3000 (IB)	Merck/Sigma-Aldrich (T5168)	Su sperm axoneme	AB_477579

Abbreviations:

[†]SgV, secretogranin V; PC2, prohormone convertase 2; VGluT1, vesicular glutamate transporter 1; VGluT2, vesicular glutamate transporter 2; GAD65, glutamic acid decarboxylase 65; CgA, chromogranin A; CgB, chromogranin B; GS, glutamine synthetase; GFAP, Glial fibrillary acidic protein; α -MSH, α -melanocyte-stimulating hormone.

[‡]Host species, Gp, guinea pig; Rb, rabbit; Mo, mouse

[§]IB, immunoblotting; IH, peroxidase-based immunohistochemistry; IF, immunofluorescence staining; IE, immunoelectron microscopy.

[¶]Cell Signaling Technology (Danvers, MA, USA), Synaptic Systems GmbH. (Göttingen, Germany), Chemicon/Millipore/Merck/Sigma-Aldrich, Co. LLC (St. Louis, MO, USA), Proteintech Group, Inc. (Rosemont, IL, USA), Abcam Ltd. (Cambridge, UK), Santa Cruz Biotechnology Inc. (Dallas, TX, USA), PROGEN Biotechnik GmbH. (Heidelberg, Germany), Gunma University, Antibody donation project of Institute for Molecular and Cellular Regulation, prepared by Dr. K. Wakabayashi (Maebashi, Japan).

[†]Rt, rat; Hu, human; Mo, mouse; Su, Sea urchin.

Table 2. Secondary Antibodies Used in the Present Study

Name of antibodies	Species [†]	Dilution (Usage) [‡]	Supplier [§] (Catalog No.)	RRID
Peroxidase-conjugated anti-guinea pig IgG	Dk	1:5000 (IB)	Jackson (706-035-148)	AB_2340447
Peroxidase-conjugated anti-rabbit IgG	Gt	1:5000 (IB)	Jackson (111-035-144)	AB_2307391
Peroxidase-conjugated anti-mouse IgG	Gt	1:5000 (IB)	Jackson (111-035-146)	AB_2307392
Biotinylated anti-guinea pig IgG	Gt	1:200 (IH)	Vector (PK-4007)	AB_2336816
Alexa Fluor 488-conjugated anti-guinea pig IgG	Dk	1:2000 (IF)	Jackson (706-545-148)	AB_2340472
Alexa Fluor 555-conjugated anti-rabbit IgG	Dk	1:2000 (IF)	Invitrogen (A-31572)	AB_162543
Alexa Fluor 555- conjugated anti-mouse IgG	Dk	1:2000 (IF)	Invitrogen (A-31570)	AB_2536180
Alexa Fluor 647-conjugated anti-rabbit IgG	Dk	1:2000 (IF)	Invitrogen (A-31573)	AB_2536183
6 nm colloidal gold-conjugated anti-guinea pig IgG	Dk	1:50 (IE)	Jackson (706-195-148)	AB_2340464
15 nm colloidal gold-conjugated anti-rabbit IgG	Dk	1:50 (IE)	Aurion (815.311)	not_applicable

Abbreviations:

[†]Dk, donkey; Gt, goat.

[‡]IB, immunoblotting; IH, peroxidase-based immunohistochemistry; IF, immunofluorescence staining; IE, immunoelectron microscopy.

[§]Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA), Vector Laboratories (Burlingame, CA, USA), Invitrogen/Thermo Fisher Scientific Inc. (Waltham, MA, USA), Aurion Immuno Gold Reagents & Accessories (Wageningen, The Netherlands).

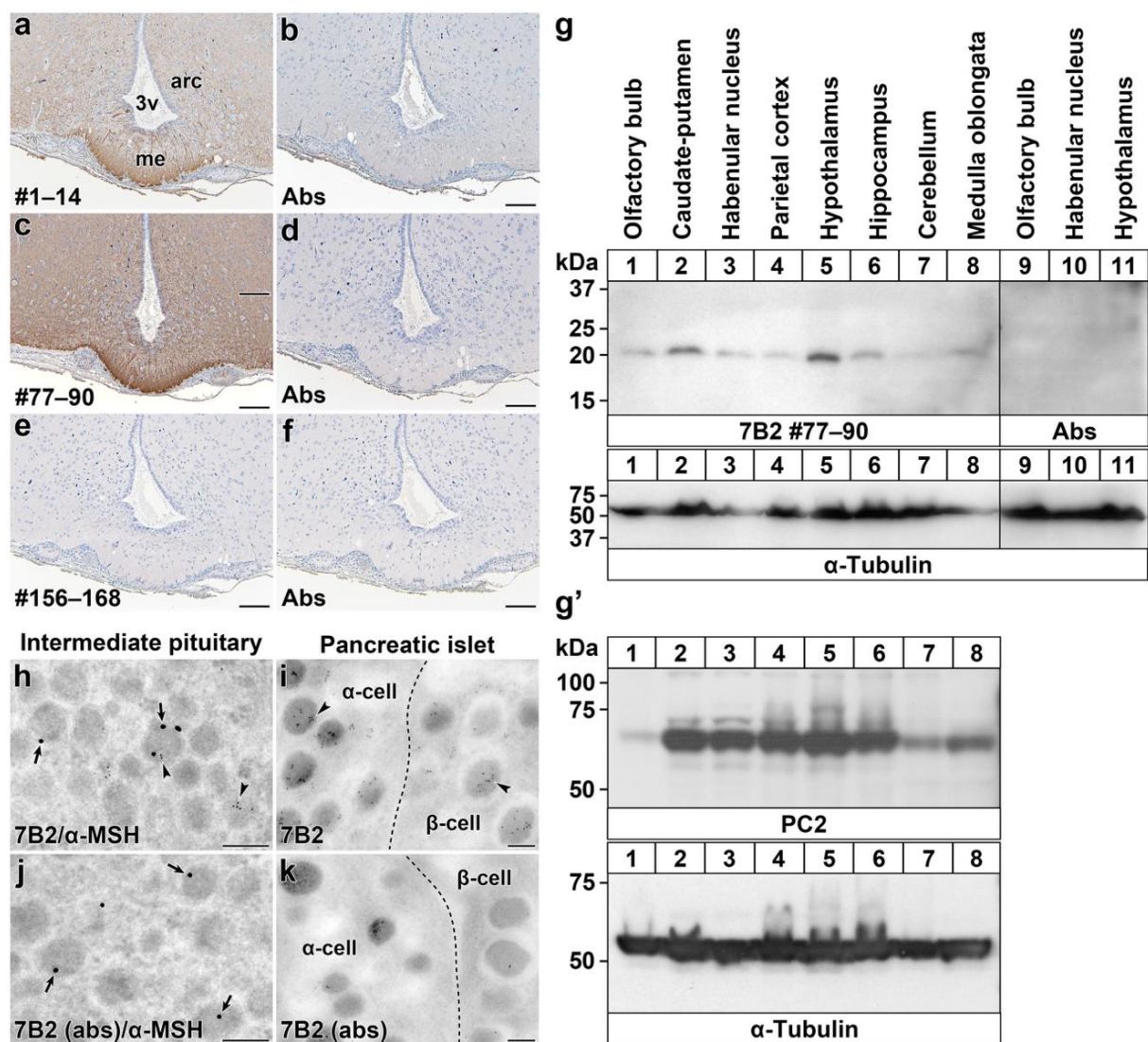


Fig. 1

Fig. 1 Specificity of 7B2 antibodies. Immunohistochemistry in coronal hypothalamic sections reacted using three guinea pig #1-14, #77-90, and #156-168 antibodies (a, c, and e) or preabsorbed antibodies with immunogen peptides (b, d, and f). (g) Immunoblot analysis of protein extracts (30 μ g) from brain regions using #77-90 antibodies (lanes 1-8); negative control blots were incubated with preabsorbed antibodies (lanes 9-11, corresponding to three representative regions). (g') The same lanes probed for PC2 and α -tubulin as loading controls. The positions of protein size markers are indicated on the left. Immunogold labeling in ultrathin sections reacted using #77-90 antibodies. (h) Intermediate lobe of the pituitary gland. Secretory granules in rat melanotrophs were labeled for 7B2 (6 nm gold particles, arrowheads) and α -MSH (15 nm gold particles, arrows). (i) Pancreatic islets. Secretory granules in presumptive α - and β -cells were labeled for 7B2 (6 nm gold particles, arrowheads). Negative control sections of the pituitary intermediate lobe (j) and pancreatic islet (k) were incubated

- 1 with preabsorbed antibodies. 3v, third ventricle; arc, arcuate nucleus; kDa, kilodalton; me, median eminence. Scale
- 2 bars: 100 μm (**a–f**) and 200 nm (**h–k**)
- 3

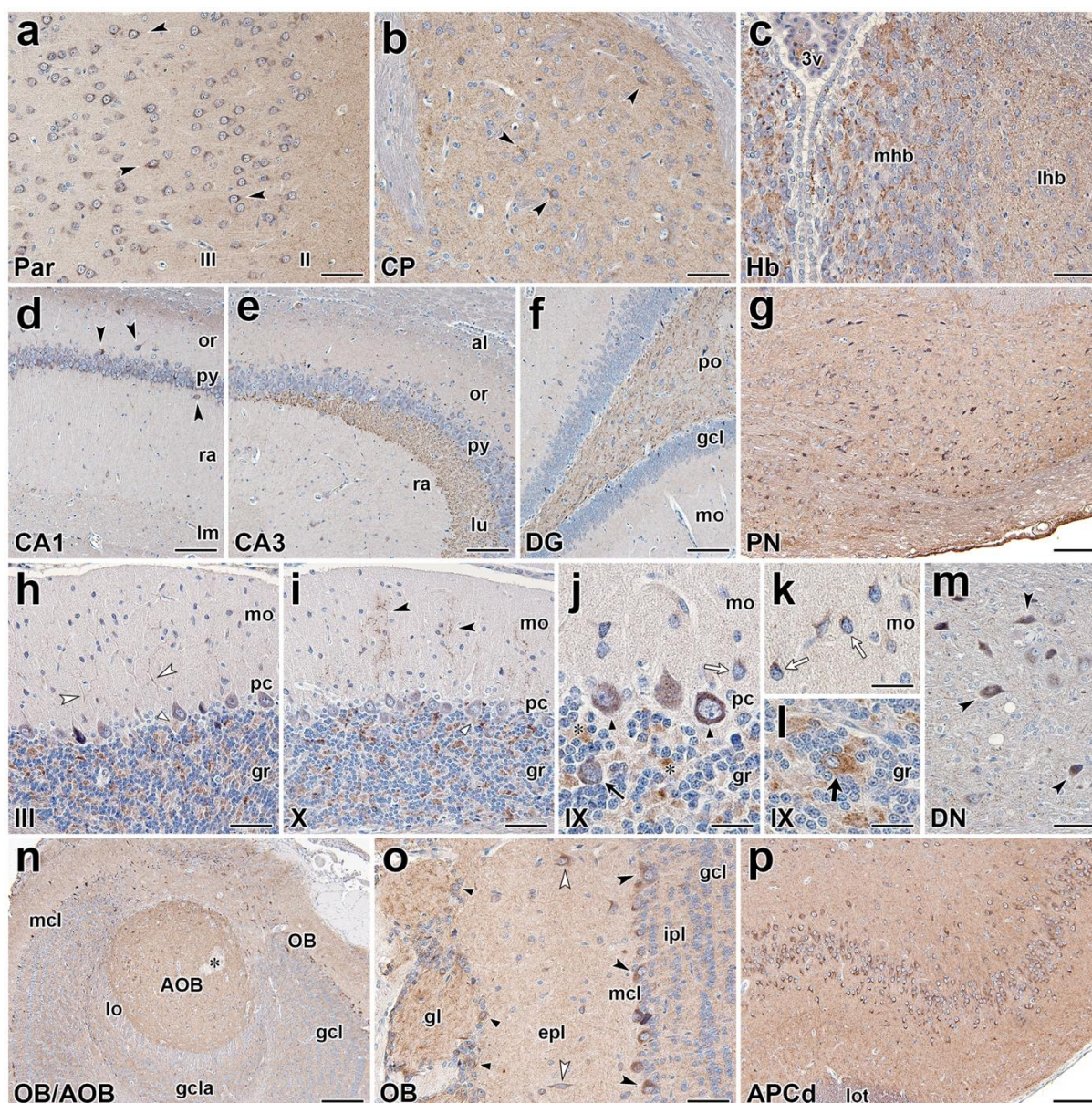


Fig. 2

Fig. 2 Immunohistochemistry of 7B2 in rat brain. (a and b) In layers II and III of the parietal cerebral cortex (Par) and caudate-putamen (CP), perikarya (arrowheads) and neuropil were labeled. (c) In the habenular nucleus (Hb), fiber structures were strongly labeled. (d) In the hippocampal CA1 region, pyramidal cells and putative inhibitory interneurons (arrowheads) were labeled. (e and f) Strong labeling in the stratum lucidum of the CA3 region and the hilus of the dentate gyrus (DG). (g) Fibers in the pontine nucleus (PN) were moderately labeled, and perikarya of PN neurons were strongly labeled. (h–l) In cerebellar sagittal sections, various elements were labeled in lobules III (h), IX (j–l), and X (i). Large white arrowheads in h and i indicate Purkinje cell dendrites and the small white arrowhead indicates the pinceau formation. Large black arrowheads in i indicate profiles resembling climbing fibers. Small black arrowheads in j indicate strongly labeled Purkinje cell perikarya and the thin black arrow

indicates a putative Golgi cell. Asterisks in **j** indicate synaptic glomeruli. White arrows in **j** and **k** indicate putative basket cells and stellate cells in the molecular layer, respectively. The thick black arrow in **l** indicates the cell body of a putative UBC. (**m**) In deep cerebellar nucleus (medial nucleus) (DN), small black arrowheads indicate large glutamatergic projection neurons. (**n**) In the olfactory system, accessory olfactory bulb (AOB) and surrounding olfactory bulb (OB) were strongly labeled. The asterisk indicates the vomeronasal nerve layer. (**o**) In OB, large black or white arrowheads indicate mitral cells and tufted cells, respectively, and small black arrowheads indicate periglomerular cells. (**p**) In anterior piriform cortex, pyramidal neurons located in the dorsal part (APCd) were strongly labeled. 3v, third ventricle; al, alveus hippocampi; epl, external plexiform layer; gcl, granule cell layer; gela, granule cell layer of accessory olfactory bulb; gl, olfactory glomerulus; ipl, internal plexiform layer; lhb, lateral habenular nucleus; lm, stratum lacunosum-moleculare; lot, lateral olfactory tract; lu, stratum lucidum; mcl, mitral cell layer; mhb, medial habenular nucleus; mo, molecular layer; or, stratum oriens; po, polymorphic layer; py, pyramidal cell layer; ra, stratum radiatum. Scale bars: 20 μm (**j–l**), 50 μm (**a–c**, **h**, **i**, **m**, and **o**), 100 μm (**d–g** and **p**), and 200 μm (**n**)

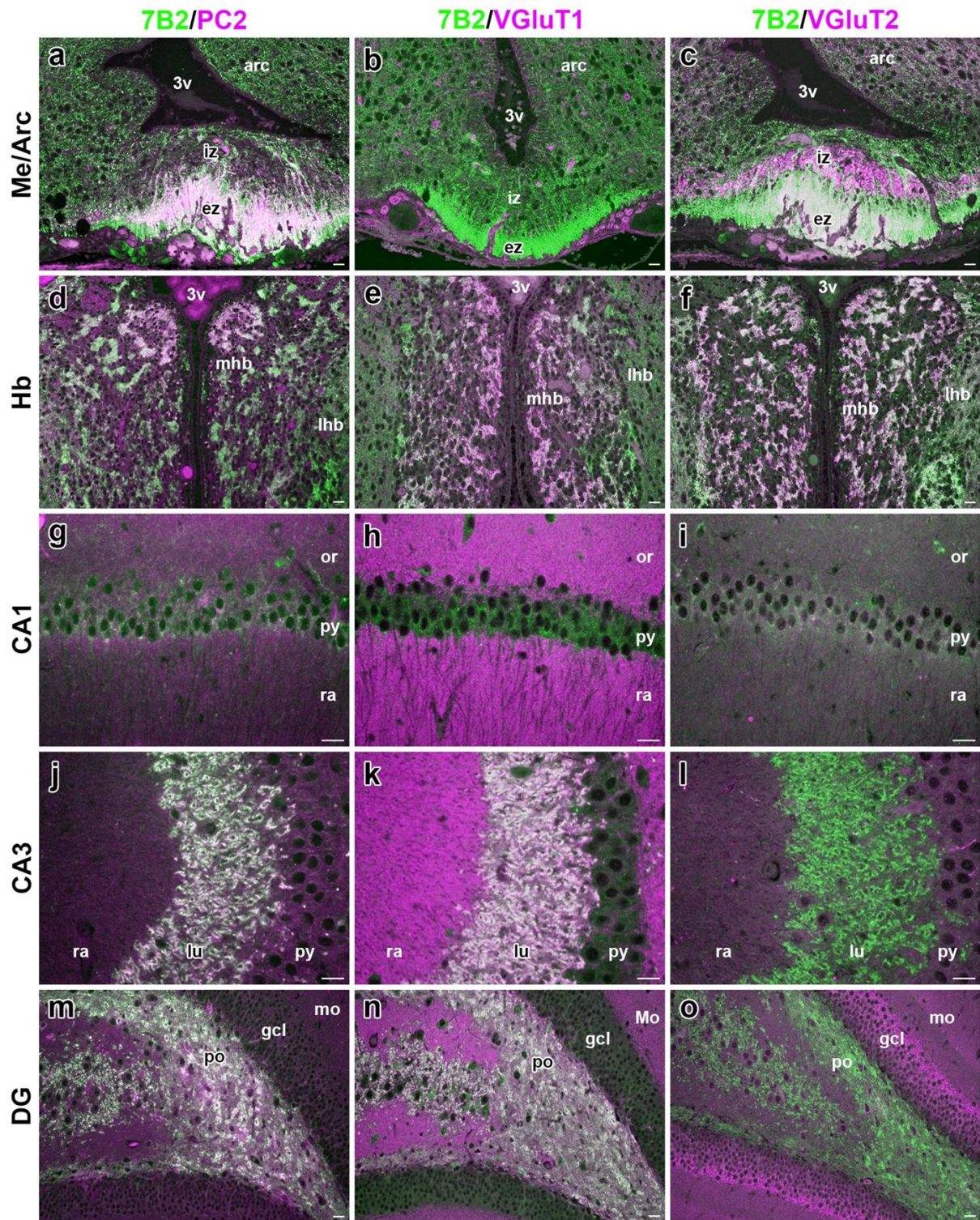


Fig. 3

Fig. 3 Double immunofluorescence labeling of 7B2 with PC2, VGlut1, and VGlut2 in the forebrain. 7B2 is shown in green and the markers in magenta: PC2 (a, d, g, j, and m), VGlut1 (b, e, h, k, and n), and VGlut2 (c, f, i, l, and o). (a–c) Median eminence and arcuate nucleus (Me/Arc). (d–f) Habenular nucleus (Hb). (g–i) Hippocampal CA1 region. (j–l) CA3 region. (m–o) Dentate gyrus (DG). 3v, third ventricle; ez, external zone; gcl, granule cell layer; iz, internal zone; lhb, lateral habenular nucleus; lu, stratum lucidum; mhb, medial habenular

- 1 nucleus; mo, molecular layer; or, stratum oriens; po, polymorphic layer; py, pyramidal cell layer; ra, stratum
2 radiatum. Scale bars: 20 μm
3

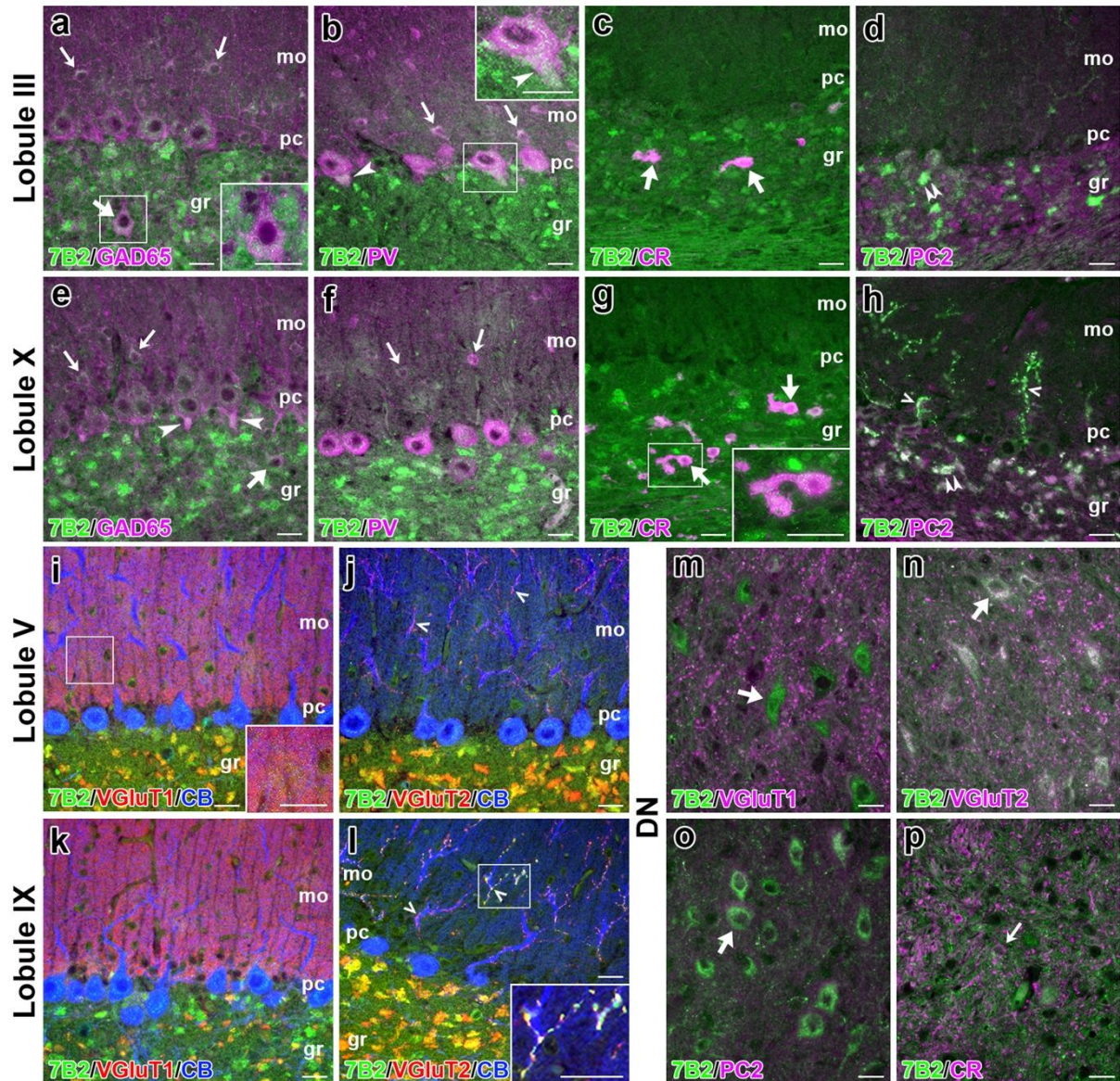


Fig. 4

Fig. 4 Multiple immunofluorescence labeling of 7B2 with PC2 and cerebellar marker proteins. (**a–d**) Lobule III. (**e–h**) Lobule X. (**i and j**) Lobule V. (**k and l**) Lobule IX. (**m–p**) Deep cerebellar nucleus (medial nucleus) (DN). 7B2 is shown in green, and the markers are shown in the following colors: GAD65 (**a and e**), parvalbumin (PV) (**b and f**), calretinin (CR) (**c, g, and p**), PC2 (**d, h, and o**), VGluT1 (**m**), and VGluT2 (**n**), magenta; VGluT1 (**i and k**) and VGluT2 (**j and l**), red; and calbindin (CB) (**i–l**), blue. Thin arrows in **a, b, e, and f** indicate the inhibitory interneurons (stellate cells and basket cells) in the molecular layer. Thick arrows in **a and e** indicate Golgi cells, and those in **c and g** indicate the calretinin-expressing UBCs. Arrowheads in **b and e** indicate the pinneau formation, visualized with parvalbumin in lobule III and with GAD65 in lobule X. Double arrowheads in **d and h** indicate large, irregular profiles in the granular layer, corresponding to synaptic glomeruli in lobule III and to UBC somata in lobule X. Open arrowheads in **h, j, and l** indicate profiles resembling climbing fiber terminals. Thick arrows in

1 **m–o** and thin arrow in **p** indicate large excitatory neurons strongly labeled for 7B2 and calretinin-positive smaller
2 inhibitory neuron weakly labeled for 7B2, respectively. Insets show magnified views of the following elements:
3 **a**, Golgi cell; **b**, Purkinje cell perikaryon and pinceau; **g**, UBCs; **i**, parallel fiber terminals; **l**, climbing fiber
4 terminals. gr, granular layer; mo, molecular layer; pc, Purkinje cell layer. Scale bars: 20 μ m

5

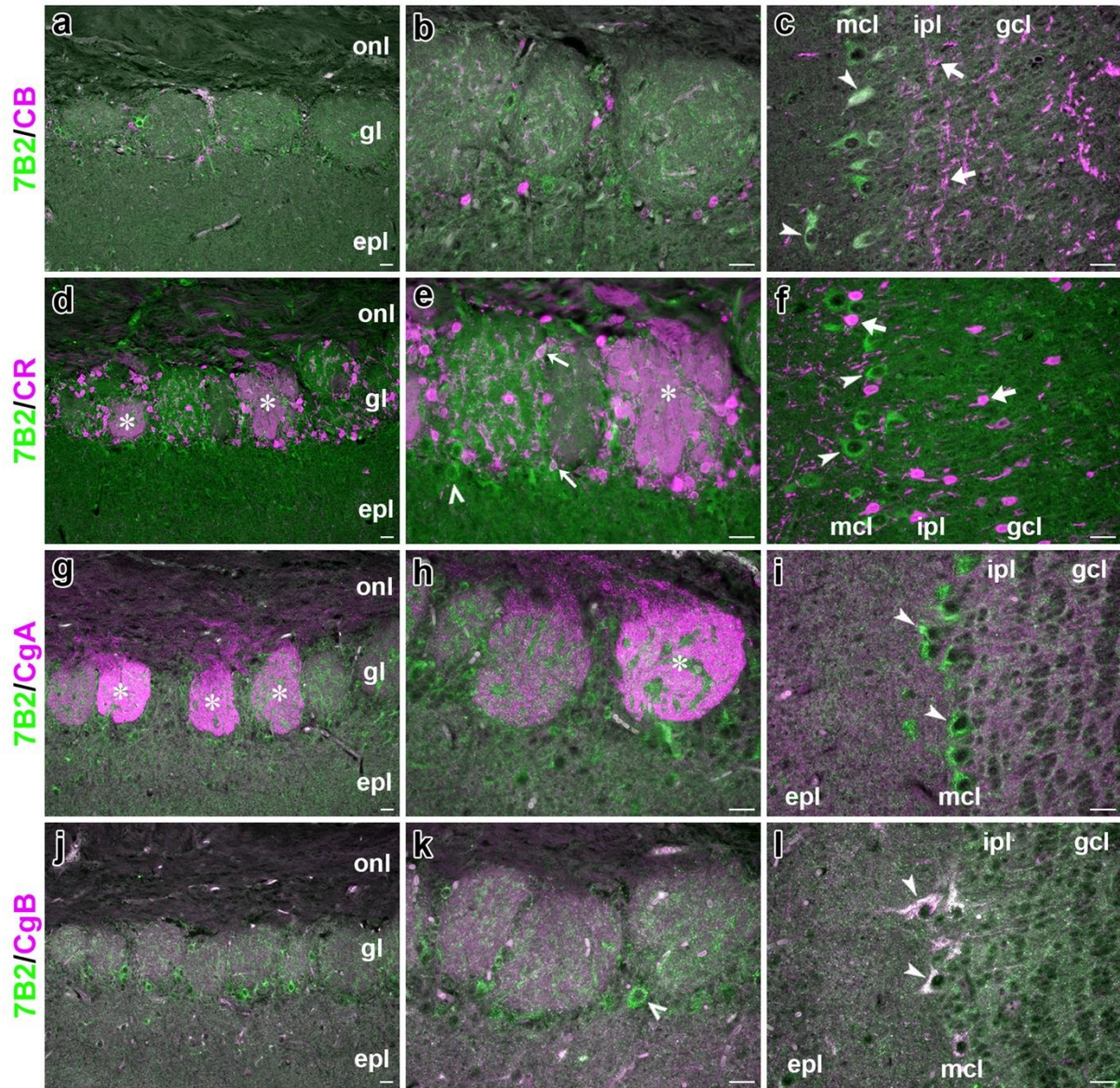


Fig. 5

Fig. 5 Double immunofluorescence labeling of 7B2 with calcium-binding proteins and chromogranins in the olfactory bulb. 7B2 is shown in green and the markers in magenta: calbindin (CB) (a–c), calretinin (CR) (d–f), Chromogranin A (CgA) (g–i), and Chromogranin B (CgB) (j–l). Low-magnification (a, d, g, and j) and high-magnification (b, e, h, and k) images of the olfactory glomerulus, and images of the mitral cell layer and deeper layers (c, f, i, and l). Asterisks indicate glomeruli strongly labeled for CR (d and e) or for CgA (g and h). Thin arrows in e indicate periglomerular cells with intense overlap of 7B2 and CR, and open arrowheads in e and k indicate periglomerular cells without overlap. Arrowheads indicate mitral/tufted cells with moderate or no overlap of CB or CR signals (c and f, respectively) and with no or strong overlap of CgA or CgB signals (i and l, respectively). Thick arrows in c and f indicate weak overlap of 7B2 with CB in processes and with CR in cell

- 1 bodies of interneurons, respectively. epl, external plexiform layer; gcl, granule cell layer; gl, olfactory glomerulus;
- 2 ipl, internal plexiform layer; mcl, mitral cell layer. Scale bars: 20 μm
- 3

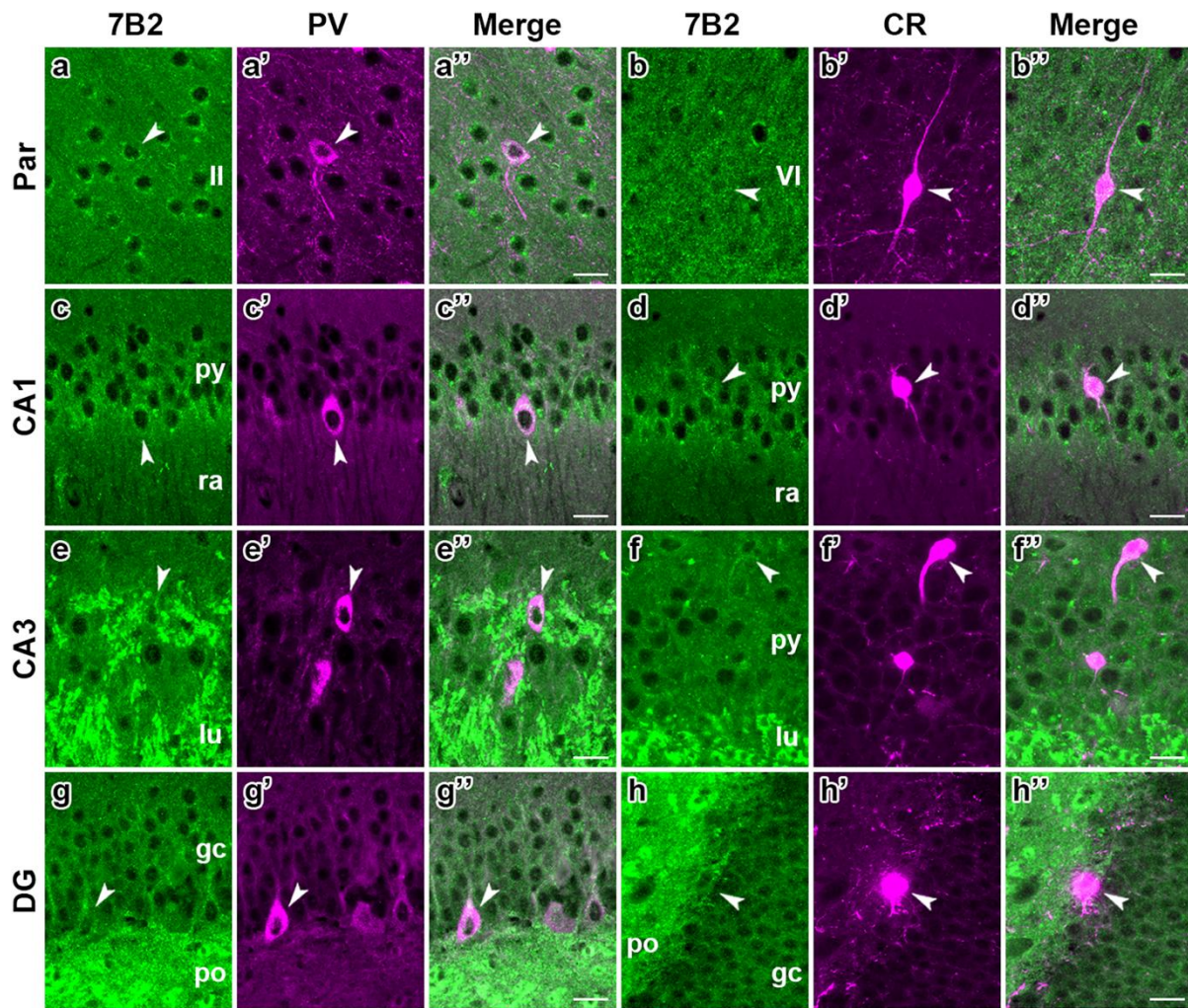


Fig. 6

Fig. 6 Double immunofluorescence labeling of 7B2 with parvalbumin and calretinin in the cerebral cortex and hippocampus. 7B2 is shown in green and the markers in magenta. (**a** and **b**) Parietal cerebral cortex (Par). (**c** and **d**) Hippocampal CA1 region. (**e** and **f**) CA3 region. (**g** and **h**) Dentate gyrus (DG). Each set shows 7B2 (**a–h**), the marker (**a'–h'**), and the merged image (**a''–h''**); the markers are parvalbumin (PV) (**a', c', e', and g'**) and calretinin (CR) (**b', d', f', and h'**). Arrows indicate neurons immunoreactive for both 7B2 and the respective marker. gc, granule cell layer; lu, stratum lucidum; po, polymorphic layer; py, pyramidal cell layer; ra, stratum radiatum; II, cortical layer II; VI, cortical layer VI. Scale bars: 20 μ m

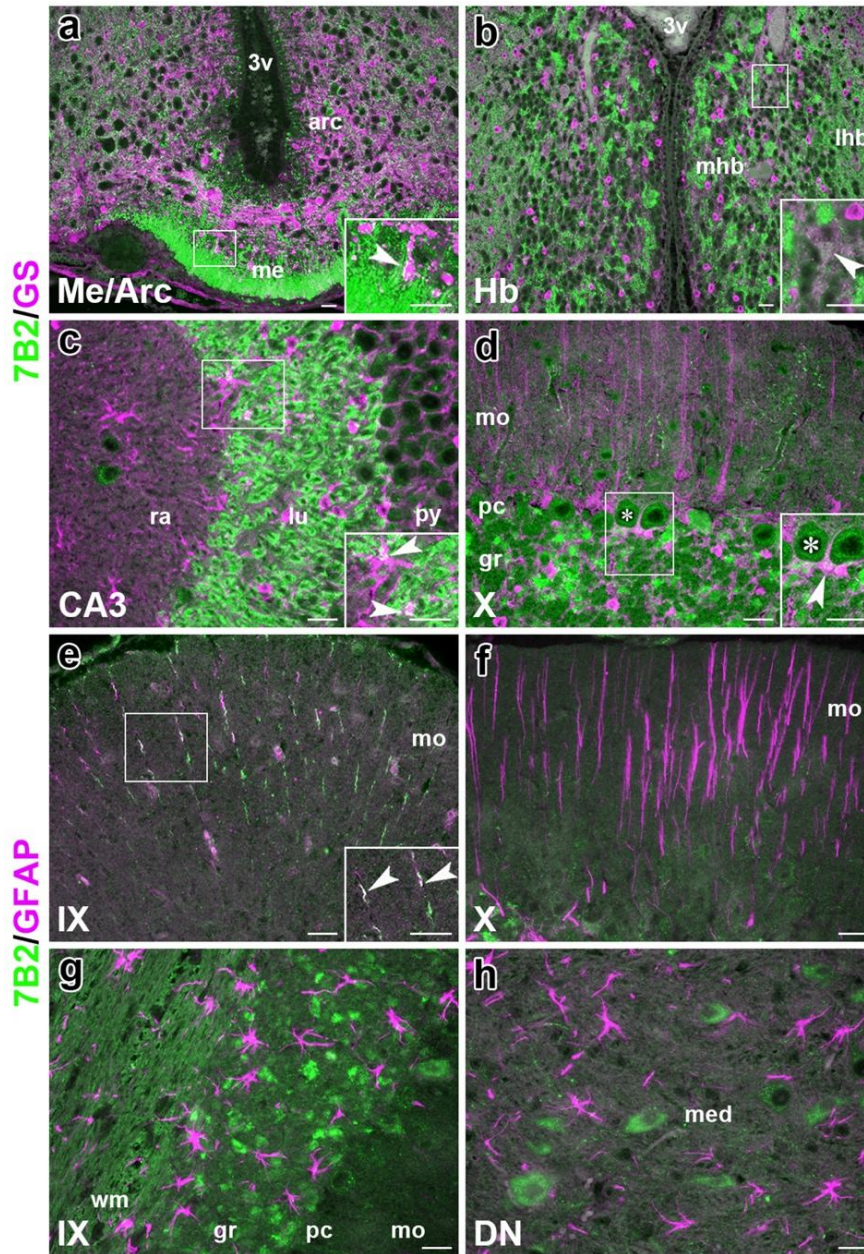


Fig. 7

Fig. 7 Double immunofluorescence labeling of 7B2 with astrocyte markers. 7B2 is shown in green and the astrocyte markers in magenta: glutamine synthetase (GS) (a–d) and glial fibrillary acidic protein (GFAP) (e–h). (a) Median eminence and arcuate nucleus (Me/Arc). (b) Habenular nucleus (Hb). (c) Hippocampal CA3. (d and f) Cerebellar lobule X. (e and g) Lobule IX. (h) Deep cerebellar nucleus (medial nucleus) (DN). The insets show magnified views of the rectangular areas. Arrowheads indicate overlapping signals of 7B2 and astrocyte markers. 3v, third ventricle; gr, granular layer; lhb, lateral habenular nucleus; lu, stratum lucidum; mhb, medial habenular nucleus; mo, molecular layer; pc, Purkinje cell layer; py, pyramidal cell layer; ra, stratum radiatum; wm, white matter. Scale bars: 20 μm

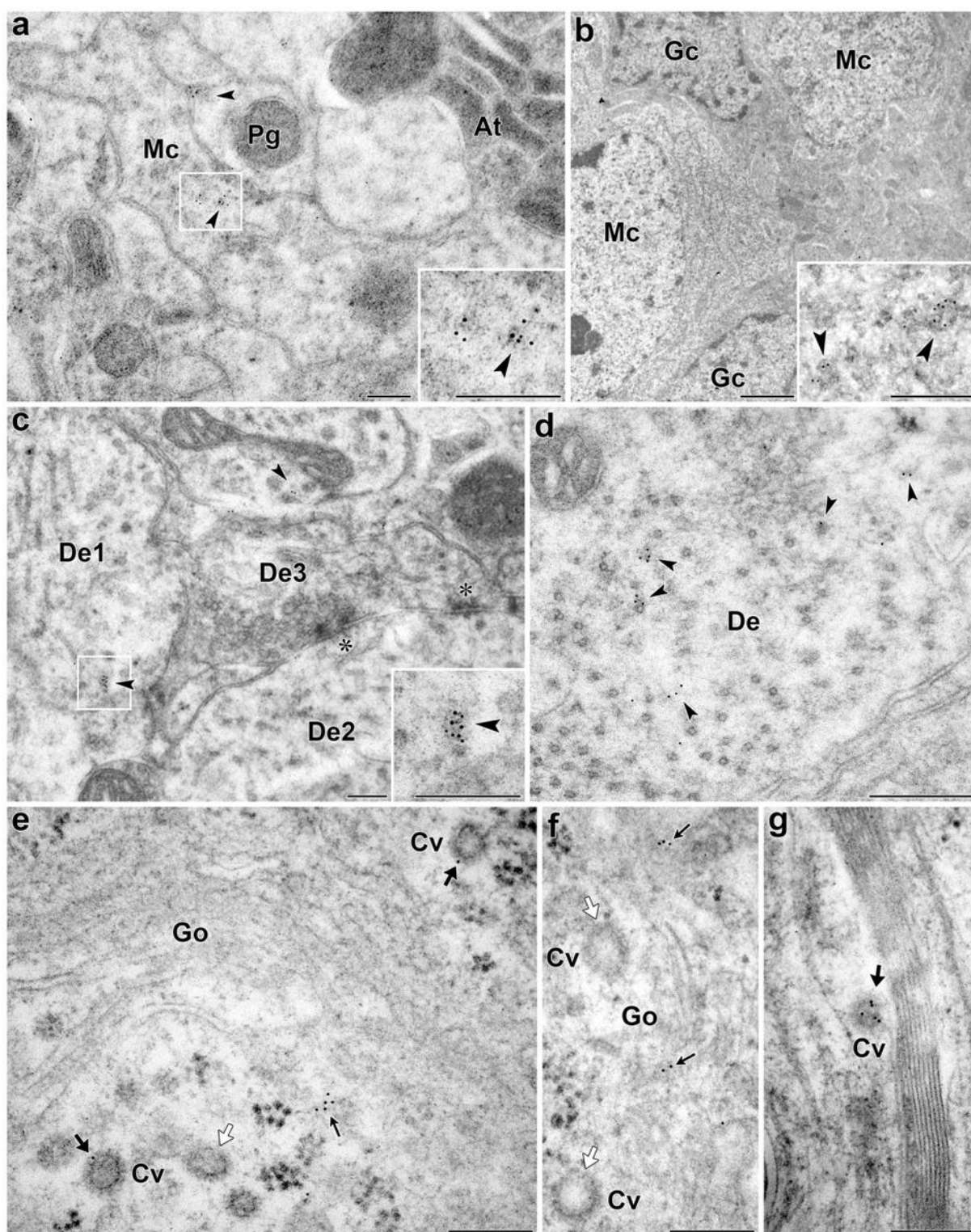


Fig. 8

Fig. 8 7B2 immunogold labeling in the olfactory bulb and medial habenular nucleus. **(a)** Immunogold-labeled LDCVs are observed in the dendrites of a mitral cell and a periglomerular cell within the olfactory glomerulus. **(b)** Low-magnification view of mitral cells and granule cells. Insets in **a** and **b** show magnified views of the mitral cell cytoplasm containing immunogold-labeled LDCVs. **(c)** External plexiform layer of the olfactory bulb. An

1 immunogold-labeled LDCV is observed within a dendrite (De1). The adjacent dendrite (De2) forms a reciprocal
2 synapse with another dendrite (De3) of a granule cell (Egger and Urban, 2006) or an EPL interneuron (Huang et
3 al. 2013). Asterisks indicate postsynaptic sites. **(d)** Accessory olfactory bulb. Black arrowheads in **a–d** indicate
4 immunogold-labeled LDCVs in the olfactory bulb. Immunogold-labeled LDCVs are observed in the dendrites of
5 mitral/tufted cells. **(e–g)** Medial habenular nucleus. Thick black arrows in **e** and **g** and thick white arrows in **e** and
6 **f** indicate immunogold-labeled and non-labeled coated vesicles, respectively. Thin black arrows in **e** and **f** indicate
7 immunogold-labeled Golgi membranes. At, axon terminals of the olfactory nerves; Cv, coated vesicle; De,
8 dendrite; Gc, granule cell; Go, Golgi apparatus; Mc, mitral cell; Pg, periglomerular cell. Scale bars: 200 nm; 2 μ m
9 **(b, except for inset)**

10

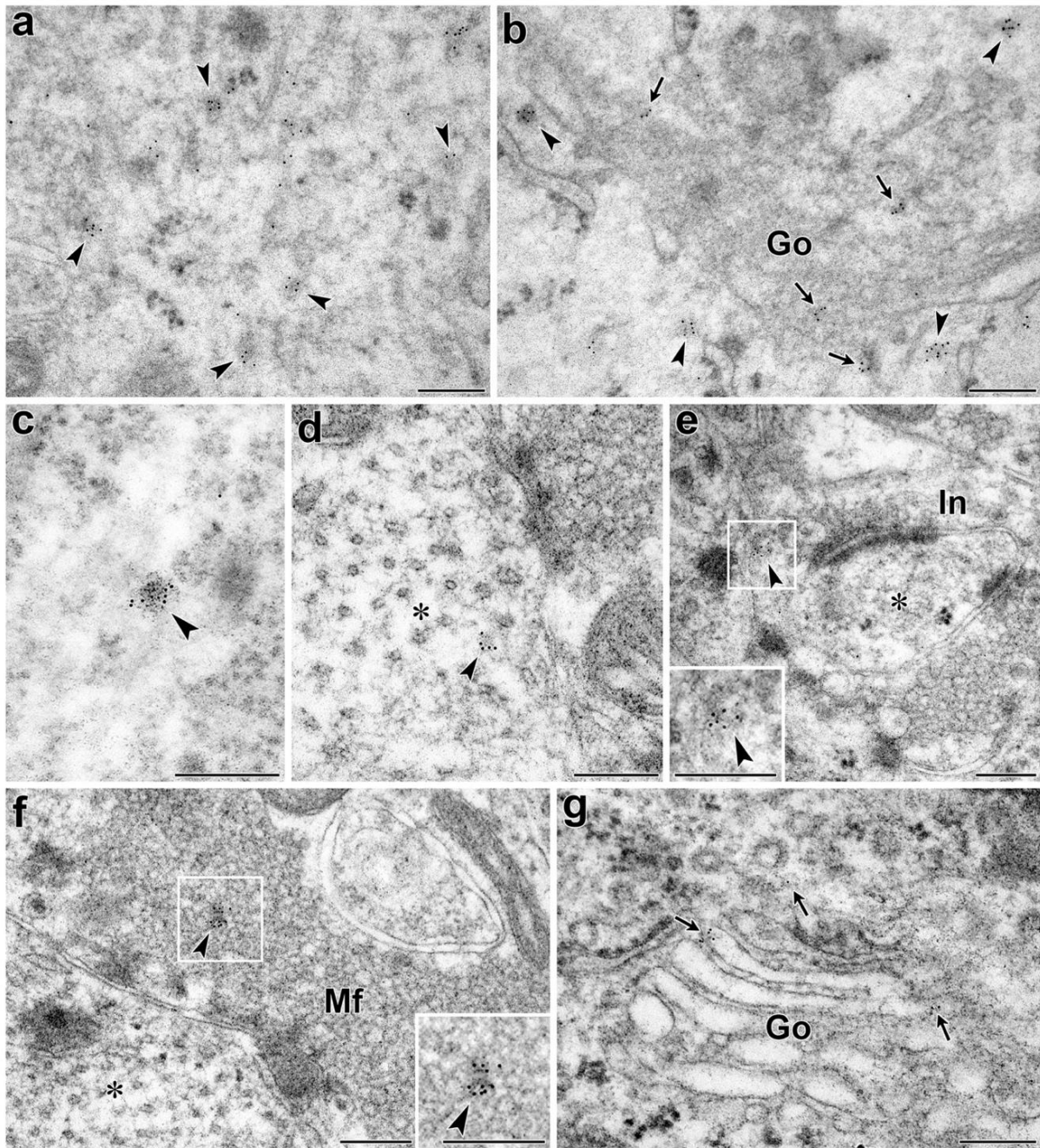


Fig. 9

Fig. 9 Immunogold labeling for 7B2 in the hippocampus. (a) Cytoplasm of a pyramidal cell and (b) an astrocyte in the CA1 region. (c) Cytoplasm of a pyramidal cell in the CA3 region. (d) Dendrite in the molecular layer of DG. (e) An immunogold-labeled LDCV is present in the synaptic region of a presumptive interneuron (In). (f) An immunogold-labeled LDCV is present in the large mossy fiber terminal (Mf) in the CA3 region. (g) Golgi apparatus (Go) in the cytoplasm of a granule cell in the DG. Black arrowheads in a–f indicate 7B2-labeled LDCVs. Arrows in b and g indicate 7B2 labeling on the vesicular membranes in the vicinity of the Golgi apparatus in an

- 1 astrocyte (**b**) and on the Golgi cisternal lumen and Golgi vesicles in a granule cell (**g**), respectively. Asterisks in
- 2 **d–f** indicate dendrites. Insets in **e** and **f** show magnified views of the LDCVs. Scale bars: 200 nm
- 3

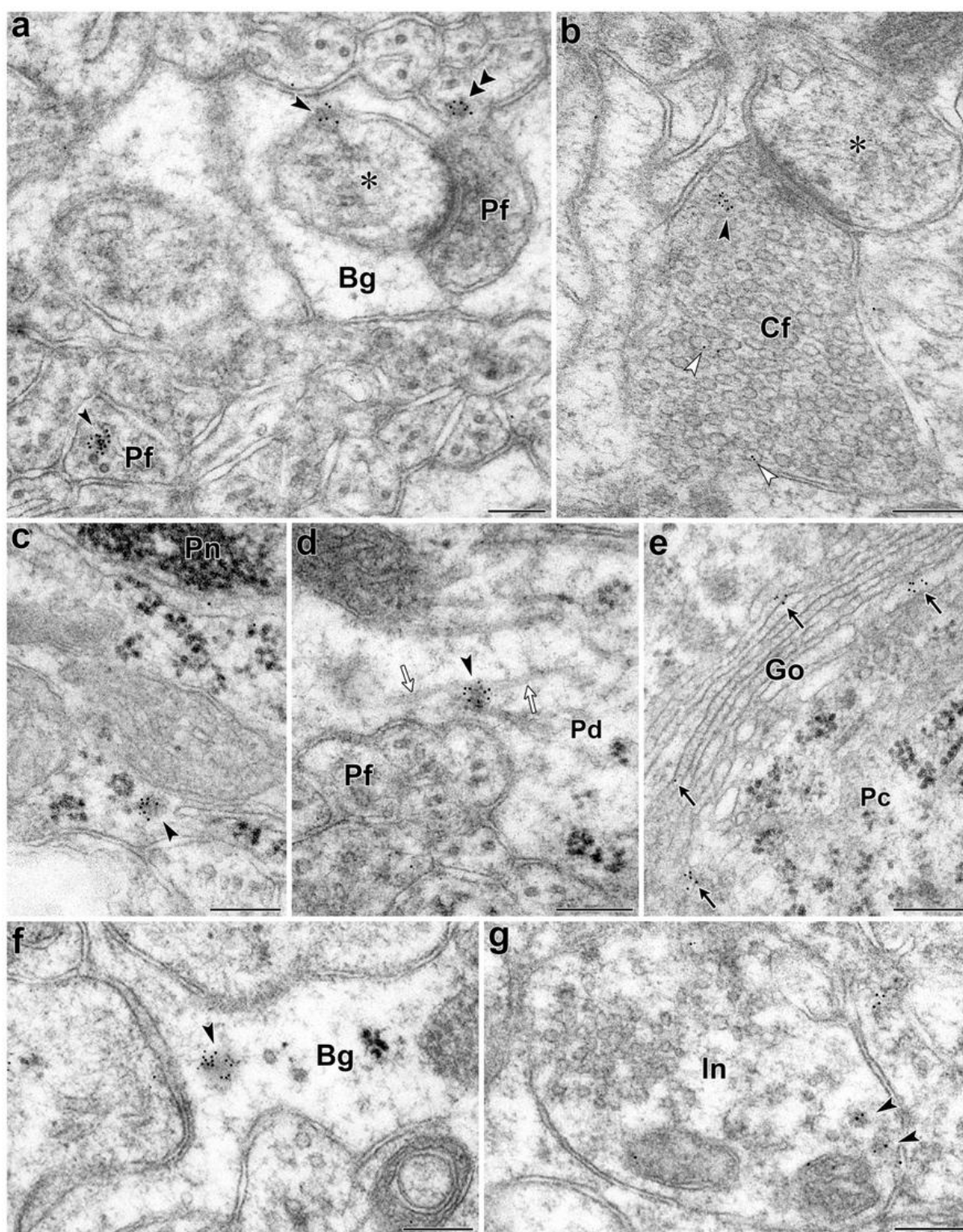


Fig. 10

Fig. 10 7B2 immunogold labeling in the cerebellar molecular layer and Purkinje cells. Black arrowheads in **a–d**, **f**, and **g** indicate immunogold-labeled LDCVs. White arrowheads in **b** indicate immunogold-labeled synaptic vesicles. (**a**) Immunogold-labeled LDCVs are present in parallel fibers and at the apposition between a Purkinje cell dendritic spine and an adjacent Bergmann glial process. In the same field, a profile suggestive of exocytosis

1 is seen in a parallel fiber, as indicated by an indistinct plasma membrane (double black arrowheads). **(b)** In a
2 climbing fiber terminal, an immunogold-labeled LDCV is present, and one or two immunogold particles are also
3 associated with small synaptic vesicles. Asterisks in **a** and **b** indicate Purkinje cell dendritic spines. **(c)** An
4 immunogold-labeled LDCV is present in the cytoplasm of a Purkinje cell. **(d)** An immunogold-labeled LDCV is
5 seen in direct association with a microtubule (white arrows) in the cytoplasm of a Purkinje cell. **(e)** Immunogold
6 labeling is present within Golgi cisternae and along Golgi membranes (black arrows). **(f)** An immunogold-labeled
7 LDCV is present in a Bergmann glial process. **(g)** Immunogold-labeled LDCVs are present in a presumptive
8 inhibitory interneuron terminal, identified on the basis of the morphology of its synaptic vesicles. Bg, Bergmann
9 glia; Cf, climbing fiber; In, interneuron terminal; Pc, Purkinje cell cytoplasm; Pd, Purkinje cell dendrite; Pf,
10 parallel fiber; Pn, Purkinje cell nucleus. Scale bars: 200 nm

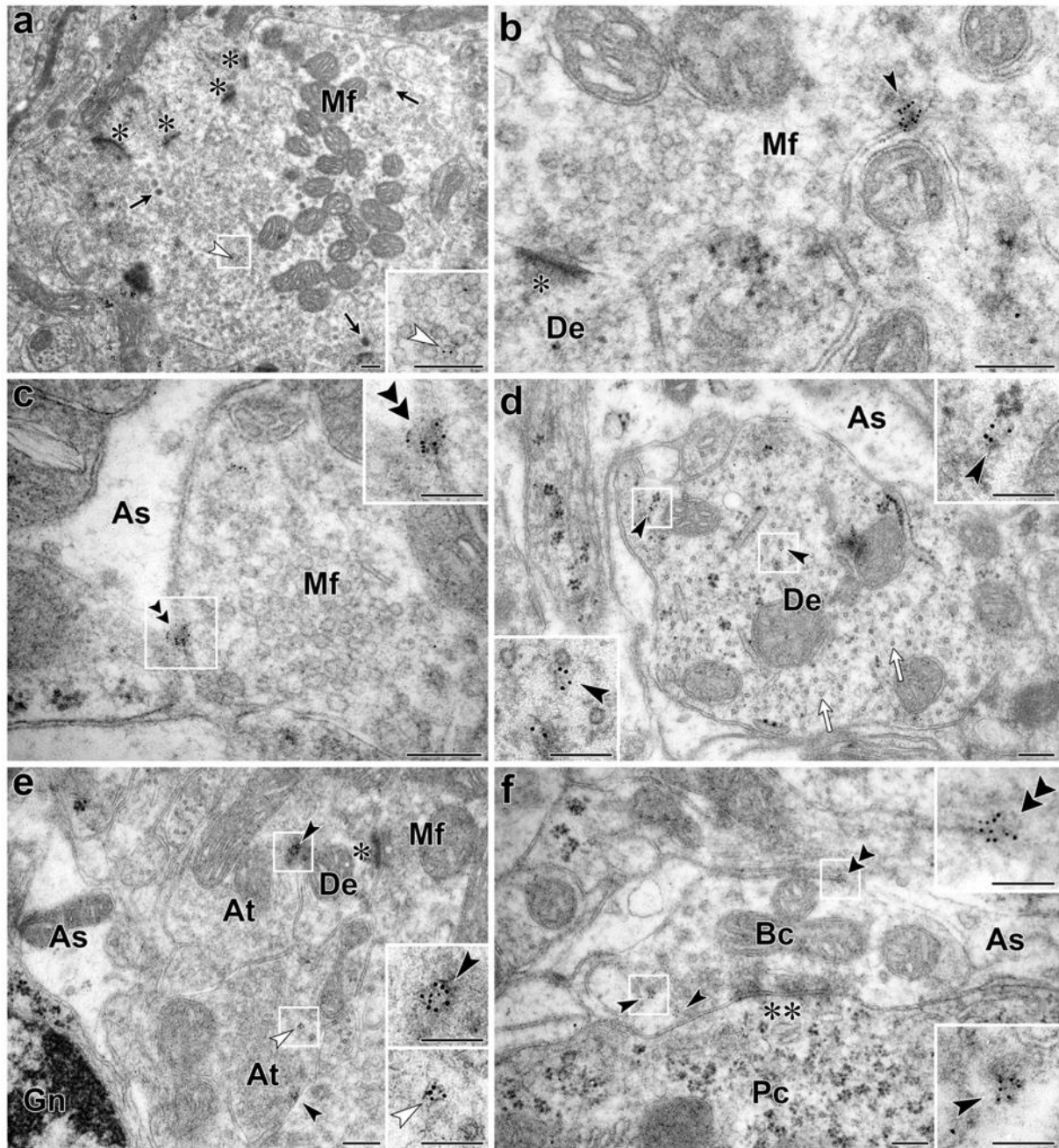


Fig. 11

Fig. 11 7B2 immunogold labeling in the cerebellar granular layer and the basket cell pinceau. **(a)** Cerebellar glomerulus in lobule V. In the mossy fiber terminal (Mf), a few immunogold particles are observed on synaptic vesicles (white arrowheads). Black arrows indicate unlabeled LDCVs. **(b)** Cerebellar glomerulus in lobule X. An immunogold-labeled LDCV is indicated by black arrowheads. Asterisks in **a** and **b** indicate synapses between Mf and granule cell dendrites. **(c)** A profile suggestive of exocytotic release is observed, presumed from an astrocytic process (As) (double black arrowheads). **(d)** Immunogold-labeled LDCVs in the presumptive dendritic shaft of a Golgi cell, measuring approximately 2 μm in diameter, containing multiple mitochondria and numerous microtubule cross-sections, and ensheathed by astrocytic glial processes (black arrowheads). White arrows indicate

1 cross-sections of microtubules. (e) Cerebellar glomerulus. An immunogold-labeled LDCV (black arrowhead) and
2 a small vesicle (white arrowhead) are observed in presumptive axon terminals of Golgi cells. (f) Immunogold-
3 labeled LDCVs in the axon terminal of a basket cell, a component of the pinceau formation (black arrowhead),
4 and in the space between an astrocyte process and the axon terminal (double black arrowheads). Double asterisks
5 indicate a Gray type II inhibitory synapse on the Purkinje cell body. As, astrocyte; At, axon terminal; Bc, basket
6 cell; De, dendrite; Gn, granule cell nucleus; Pc, Purkinje cell cytoplasm. Insets in **c–f** show magnified views of
7 LDCVs and small vesicles. Scale bars: 200 nm; 100 nm (insets)

8

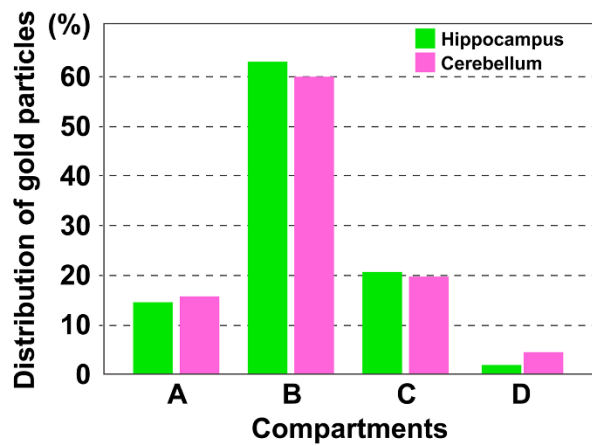


Fig. 12

Fig. 12 Quantitative analysis of immunogold particle distribution across subcellular compartments in the hippocampus and cerebellar molecular layer. Gold particles were classified into four compartments: A, neuronal axon terminals and synaptic active zones; B, neuronal cytoplasm and dendrites; C, astrocytes and glial processes; D, others (myelin sheaths, mitochondria, extracellular space). Data are expressed as percentages of total gold particles analyzed (green bars, hippocampus, $n = 1,023$ particles from 75 micrographs; red bars, cerebellar molecular layer, $n = 3,588$ particles from 155 micrographs). The total number of particles is the sum of the number of particles from the three animals combined.

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