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Distributions of parvalbumin, calbindin-D28k, and calretinin in the cerebrum of Chinese tree shrews (*Tupaia belangeri chinensis*): A high-resolution neuroanatomical resource

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ABSTRACT

The Chinese tree shrew has gained prominence as a model organism due to its phylogenetic proximity to primates, offering distinct advantages over traditional rodent models in biomedical research. However, the neuroanatomy of this species remains insufficiently defined, limiting its utility in neurophysiological and neuropathological studies. In this study, immunofluorescence microscopy was employed to comprehensively map the distribution of three calcium-binding proteins, parvalbumin, calbindin D-28k, and calretinin, across the tree shrew cerebrum. Serial brain sections in sagittal, coronal, and horizontal planes from 12 individuals generated a dataset of 3 638 cellular-resolution images. This dataset, accessible via Science Data Bank (<https://doi.org/10.57760/sciencedb.23471>), provides detailed region- and laminar-selective distributions of calcium-binding proteins valuable for the cyto- and chemoarchitectural characterization of the tree shrew cerebrum. This resource will not only advance our understanding of brain organization and facilitate basic and translational neuroscience research in tree shrews but also enhance comparative and evolutionary analyses across species.

Keywords: Tree shrew; Distribution; Parvalbumin; Calbindin; Calretinin; Resource

INTRODUCTION

The Chinese tree shrew (*Tupaia belangeri chinensis*) is a

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small, diurnal mammal endemic to South and Southeast Asia (Yao et al., 2024). Given their relatively short reproductive cycle, low maintenance costs, and close evolutionary relationship to primates (Fan et al., 2013, 2019; Xu et al., 2012; Ye et al., 2021), tree shrews have become increasingly prominent in biomedical research. Notably, tree shrews are now widely employed in modeling human diseases, offering an effective alternative to non-human primates while circumventing the logistical and ethical constraints associated with their use (Wei et al., 2025). They have been extensively applied across diverse research domains, including infectious diseases (Jia et al., 2025; Kayesh et al., 2021; Li et al., 2018), cancer (Lu et al., 2021), neurodegenerative and neuropsychiatric disorders (Li et al., 2024a, 2024b; Ni et al., 2020a; Pryce & Fuchs, 2017; Savier et al., 2021), and others (He et al., 2024; Wei et al., 2025; Xiao et al., 2017; Zheng et al., 2024). Comparative studies have also revealed a high degree of conservation in brain organization, connectivity, and function between tree shrews and primates, further supporting their relevance in translational neuroscience (Baldwin et al., 2016; Dimanico et al., 2021; Lee et al., 2016; Mustafar et al., 2018; Petry & Bickford, 2019; Schumacher et al., 2022; Sedigh-Sarvestani et al., 2021; Wang et al., 2023; Zhao et al., 2024).

Understanding the structural organization of the brain is foundational for elucidating its physiological roles and cognitive functions, as well as for uncovering the mechanisms underlying neurological disorders (Jeon et al., 2024). Despite the increasing use of tree shrews in neurobiology, detailed neuroanatomical references remain limited. Information on

Received: 18 April 2025; Accepted: 26 May 2025; Online: 27 May 2025

Foundation items: This research was supported by the Science and Technology Innovation (STI) 2030-Major Projects (2022ZD0205000 to L.L.), CAS "Light of West China" Program (xbzg-zdys-202404 to L.L.), Yunnan Revitalization Talent Support Program Yunling Scholar Project (to L.L.), and Yunnan Fundamental Research Projects (202305AH340006, 202301AS070060 to L.L., 202401AT070206 to X.C.)

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cytoarchitecture and chemoarchitecture, as well as afferent and efferent connections crucial for functional inference, is fragmentary, complicating interpretation of functional and comparative data (Huang et al., 2020; Ni et al., 2016, 2018b, 2021b, 2022; Nie et al., 2024; Petry & Bickford, 2019; Remple et al., 2006, 2007; Wong & Kaas, 2009). A stereotaxic brain atlas based on thionin and acetylcholinesterase staining has been established, providing an informative view of brain anatomy but lacking molecular and cellular specificity (Zhou & Ni, 2016), which has contributed to inconsistencies in regional delineation and nomenclature (Wong & Kaas, 2009).

Calcium-binding proteins (CaBPs) such as parvalbumin (PV), calbindin-D28k (CB), and calretinin (CR) are widely expressed across the mammalian brain (Defelipe, 1997; Medalla & Barbas, 2009). These proteins regulate intracellular calcium dynamics and modulate neuronal excitability (Camp & Wijesinghe, 2009). Each exhibits distinct expression profiles across neural circuits, reflecting specific neuronal phenotypes and functional specializations. PV is typically restricted to interneurons, whereas CB and CR are expressed in both inhibitory and certain excitatory neurons (Defelipe, 1997; Medalla & Barbas, 2009; Vanessa Becerra-Hernández et al., 2025). Due to their selective distribution, CaBPs are valuable markers for identifying neurochemical subtypes and delineating cytoarchitectonic boundaries. Furthermore, their expression patterns across different species can provide insights into lineage-specific traits and phylogenetic relationships (Balaram & Kaas, 2014; Hof et al., 1999). Extensive mapping of CaBP expression has been conducted in various brain regions across laboratory animals and humans (Bae et al., 2015; Barinka & Druga, 2010; Boccara et al., 2015; Ding & Van Hoesen, 2015; Hof et al., 1999; Lawrence et al., 2010; Medalla et al., 2023; Ohara et al., 2019; Wu et al., 2000; Zaborszky et al., 2005), contributing to studies of normal brain architecture and pathological alterations (Acharya et al., 2024; Grieco et al., 2023; Hof et al., 1999; Lawrence et al., 2010; Vanessa Becerra-Hernández et al., 2025). In tree shrews, immunoreactivity of PV, CB, and CR has been reported in restricted regions such as the hippocampus, striatum, and cerebellum (Keuker et al., 2003; Ni et al., 2018a, 2018b, 2021a; Rice et al., 2011; Wong & Kaas, 2009), but a comprehensive whole-brain map remains unavailable.

The present study systematically examined the immunoreactivity of PV, CB, and CR across the tree shrew cerebrum using immunofluorescence microscopy, along with neuronal nuclei antigen (NeuN) and cresyl violet (Nissl) staining. Serial sections in sagittal, coronal, and horizontal planes were obtained from 12 adult male and female individuals, yielding 3 638 high-resolution bright-field and fluorescent images. This dataset provides a comprehensive map of CaBP distribution at cellular resolution, facilitating neuroanatomical, neurodevelopmental, and neuropathological studies in tree shrews. Additionally, this resource strengthens comparative and evolutionary analyses across mammalian species, further establishing the tree shrew as a robust model for translational neuroscience.

MATERIALS AND METHODS

Subjects

Twelve adult Chinese tree shrews, including nine males and three females, aged 8–18 months and weighing 110–150 g,

were used in this study. The animals were individually housed in wire cages (44.0 cm long×38.0 cm wide×35.0 cm high) equipped with dark nesting boxes (36.0 cm long×16.0 cm wide×20.0 cm high) under standard laboratory conditions (temperature: 20–26°C, relative humidity: 40%–60%) and a 12-h light/dark cycle. Food and water were provided *ad libitum*. All experiments were conducted at the Kunming Institute of Zoology (Kunming, China) in accordance with the guidelines for the care and use of laboratory animals, as approved by the Institutional Animal Care and Use Committee of the Kunming Institute of Zoology IACUC-TE-2021-06-001.

Histological preparation

The tree shrew brains were extracted and processed as described in previous research (Li et al., 2023). In brief, tree shrews were euthanized using CO₂ and perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde (PFA) in 0.1 mol/L phosphate buffer. Brains were removed from the skull, fixed overnight in 4% PFA, and subjected to gradient dehydration in 20%, 30%, and 30% sucrose solutions sequentially for 2–3 weeks until sectioning. The olfactory bulb and majority of the cerebellum of each brain were removed prior to sectioning. The remaining part of the brain was sectioned using a cryostat (KEDEE, KD-2950) along the sagittal, coronal, or horizontal planes at a thickness of 40 µm. For coronal and horizontal sections, a cut was made in one hemisphere to distinguish between the left and right hemispheres. The brain sections were collected sequentially in phosphate-buffered saline (PBS) in 24-well plates and subsequently divided into three alternative staining sets: set 1 stained for Nissl, set 2 co-stained for NeuN and CB, and set 3 co-stained for PV and CR.

Nissl staining

Brain sections were mounted on microscope slides (CITOGlas, China) and soaked in distilled water for 2 min. They were then dehydrated through a graded series of ethanol solutions (70%, 80%, 90%, 100%, 100%, and 100%), cleared in xylene, and rehydrated in reverse order in the same set of ethanol baths before being soaked in a solution containing 70% ethanol and 0.5% acetic acid (HAc-EtOH solution) for 5 min, followed by staining with 0.1% cresyl violet solution (Sigma-Aldrich, USA, CAS: 10510-54-0, Cat# C5042-10g) for 5–10 min. Staining was differentiated by dipping the sections in HAc-EtOH solution. Finally, the sections were dehydrated in ethanol baths, cleared in xylene, and cover-slipped with neutral balsam.

Immunofluorescence

Double-labeling immunofluorescence experiments were conducted to assess the expression profiles of PV, CB, CR, and NeuN in individual brain sections. Free-floating brain sections were first blocked with 3% normal goat serum (NGS, Bytotime, China, Cat# C0265) in PBS on a shaker set at 80 r/min for 1 h at room temperature. Sections were then incubated with primary antibodies (Table 1) diluted in PBS containing 3% NGS and 0.5% Triton X-100 (dilutions: NeuN, 1:500; CB, CR & PV, 1:5 000) on a shaker at 4°C overnight. After three washes in PBS (10 min each), the sections were incubated with fluorescent dye-conjugated secondary antibodies (Table 2) diluted in PBS with 3% NGS and 0.5% Triton X-100 (dilution 1:500) on a shaker at room temperature for 2 h. Following another three washes in PBS (10 min each), the sections were mounted on microscope slides and cover-

Table 1 Primary antibodies for immunofluorescence staining

Immunogen	Host	Description	Producer	Cat#	RRID
Parvalbumin	Mouse	Monoclonal	Swant	235	AB_10000343
Calbindin D-28k	Mouse	Monoclonal	Swant	300	AB_10000347
Calretinin	Rabbit	Polyclonal	Swant	7697	AB_2619710
NeuN	Rabbit	Polyclonal	Servicebio	GB11138	AB_2868432

Table 2 Secondary antibodies for immunofluorescence staining

Immunogen	Host	Description	Producer	Cat#	Dye conjugated
Mouse IgG	Goat	Polyclonal	Proteintech	SA00009-1	Cy3®
Rabbit IgG	Goat	Polyclonal	Proteintech	SA00009-2	Cy3®
Mouse IgG	Goat	Polyclonal	Proteintech	SA00013-1	DyLight® 488
Rabbit IgG	Goat	Polyclonal	Proteintech	SA00013-2	DyLight® 488

slipped with anti-fade mounting medium.

Digital photomicrography

Nissl-stained brain sections were imaged using a bright-field microscope equipped with a 10×, 0.4 NA objective (RRID:SCR_020343) (Olympus, BX61, Japan). Images were captured in arrays of fields of view (FOVs) at a resolution of 0.69 μm per pixel, then reconstructed and corrected for shading artifacts using OlyVIA v.3.2 (Build 21633, RRID:SCR_016167) (Olympus, Japan). Fluorescence microscopic images of brain sections were captured using a Confocal Tissue Cytometer equipped with a 10×, 0.3 NA objective (TissueGnostics, TissueFAXS PLUS, Austria). Images were acquired in arrays of FOVs at a resolution of 0.65 μm per pixel and reconstructed using TissueFAXS Viewer v.7.1 (Build 6245.0119) (TissueGnostics, Austria). To optimize signal visibility, brightness and contrast were adjusted evenly across the entire image if needed. Each brain section image was exported at a resolution of 72 pixels per inch (for Nissl staining) or 96 pixels per inch (for fluorescence staining) in .jpg/.png format with a scale bar.

Indexing of image data

To facilitate systematic visualization of all brain sections from each individual, low-resolution images were compiled into indexed slides using PowerPoint (RRID:SCR_023631) (Microsoft, USA), with one section per slide. Each slide was labeled with the corresponding section ID, staining method, and relevant notes. Missing or damaged sections were left blank, and misaligned or inverted sections were corrected based on anatomical landmarks observed in adjacent sections. Corrections were made using Photoshop v.21.2.1 (RRID:SCR_014199) (Adobe, USA) and fully documented. Sections stained with recycled antibodies were marked with a dark background. Image dimensions and orientation were adjusted to ensure a consistent and smooth representation of the brain.

Western blot analysis

Fresh brain tissue was collected without perfusion and immediately frozen at −80°C. Proteins were then extracted using radio immunoprecipitation assay (RIPA) lysis buffer (Sigma-Aldrich, USA, Cat # R0278) supplemented with protease and phosphatase inhibitors (MedChem Express, USA, Cat # HY-K0010 & HY-K0022). The extracted protein samples were combined with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer and boiled at 95°C for 5–10 min to denature the proteins. The denatured samples were then loaded onto 4%–20% SDS-PAGE gels (Epizyme, China, Cat # LK213) for

separation and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in a 5% bovine serum albumin (BSA) solution in TBST buffer (Tris-buffered saline with Tween-20: 2.42 g/L Tris base, 8 g/L NaCl, 0.1% Tween-20 (v/v), pH 7.6) at room temperature for 2 h. The blocking solution was then discarded, and the membranes were incubated with primary antibodies (anti-CB, anti-PV, anti-CR, anti-NeuN, anti-GAPDH (Affinity Biosciences, USA, Cat # T0004, RRID: AB_2833041)) diluted 1:1 000 overnight at 4°C. Following primary antibody incubation, the membranes were washed with TBST buffer for 5–10 min 3–5 times, then incubated with horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse IgG and goat anti-rabbit IgG (Jackson ImmunoResearch, USA, Cat# 115-035-003 & 111-035-003)) diluted 1:5 000 at room temperature for 1 h. Finally, the membranes were again washed with TBST buffer and developed using an enhanced chemiluminescence kit (Tanon, China, Cat # B180-5001) with a Tanon 5200 Chemiluminescent Imaging System.

Quantitative analyses

CB-immunoreactive (-ir), PV-ir, and CR-ir neuron density in layer 4 of primary visual cortex (V1): TS098 (juvenile) and TS116 (no V1 sections) were excluded from the analyses. For each animal, three sections spaced 360–720 μm apart were selected. For each section, layer 4 of V1 was outlined and its size (in mm²) was measured using ImageJ (RRID:SCR_003070) (Schneider et al., 2012). The densities of CB-ir, PV-ir, and CR-ir neurons were calculated by dividing the number of corresponding immunoreactive neurons in layer 4 by its size.

CR-ir neuron density in the hilus: The four animals with sagittally sectioned brains were included in this analysis. For each brain, three sections spaced 360–480 μm apart, with the hippocampus separated into dorsal and ventral parts, were selected. For each section, the dorsal and ventral hilar regions were outlined, and their sizes were measured using ImageJ. The density of CR-ir neurons in each region was calculated by dividing the number of CR-ir neurons by the size of the region.

Results are presented as mean±standard error of the mean (SEM). Independent and related samples were compared using *t*-test and paired *t*-test, respectively. Significance was set to *P*<0.05 and was given for two-tailed tests. Statistical analyses were performed using GraphPad Prism v9 (RRID:SCR_002798) (GraphPad, USA).

Data availability and access

The exported brain section images (one animal per folder, including a ppt file with all images as an index) are available

via the Science Data Bank (<https://doi.org/10.57760/sciencedb.23471>). Images with resolution or brightness settings differing from the current release will be available in future versions, or from the corresponding author upon request.

RESULTS

Data collection and organization

A structured pipeline was established for data collection, encompassing whole-brain sectioning, brain-wide Nissl and immunofluorescence staining, and bright-field and fluorescent microscopy with downstream image processing (Figure 1A). To achieve a comprehensive representation of CaBP expression in the tree shrew brain, the immunoreactivity of PV, CB, CR, and the neuronal nuclear marker NeuN was examined across horizontal (Figure 2), sagittal (Figure 3), and coronal (Figure 4) sections. Adjacent sections were stained with Nissl to facilitate anatomical annotation at each level with

reference to the brain atlas (Zhou & Ni, 2016).

To ensure broad spatial coverage of the cerebrum and minimize loss of chemoarchitectural information during sectioning, staining, or imaging, each anatomical plane included four replicates, with one female per plane to capture potential sexual dimorphism in the brain. Plane assignments were randomized. Horizontal and sagittal sections spanned the entire dorsoventral and mediolateral extents of the cerebrum, respectively, while coronal sections extended from approximately 1 mm posterior to the rostral tip of the neocortex to its caudal end. The proportion of damaged or missing sections remained low, ranging from 0% to 2.34% across individuals.

In total, 3 638 brain sections from 12 tree shrews (aged 13.75 ± 0.87 months) were processed, generating 4 462 GB of raw image data at a resolution of 0.69 μm (bright-field) or 0.65 μm (fluorescent) in x-y dimensions (Table 3). Image brightness and contrast were manually optimized for signal clarity before export. The current version of the dataset

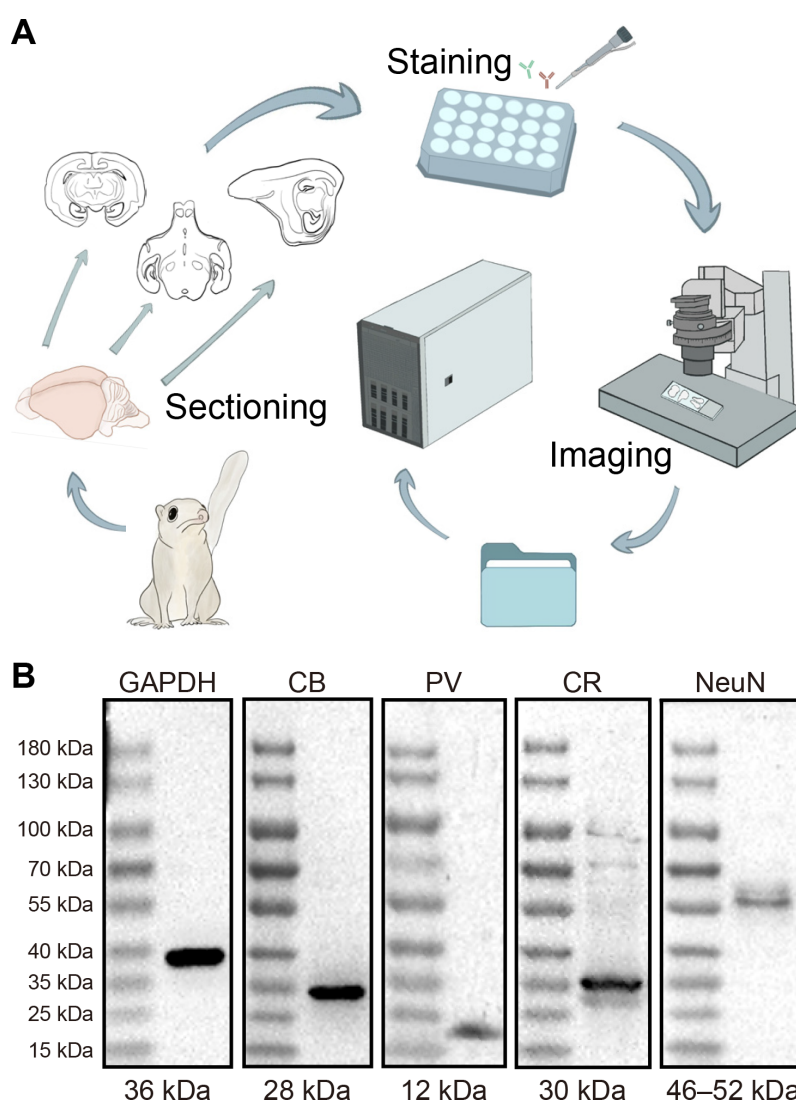


Figure 1 Data collection pipeline and validation of primary antibody specificity

A: Schematic representation of experimental workflow. Tree shrew brains were first sectioned in sagittal, coronal, or horizontal planes, followed by Nissl or immunohistochemical staining. Stained sections were imaged under bright-field or fluorescence microscopy and archived on a central server. B: Western blot analysis showing specific binding of primary antibodies to GAPDH (control), CB, PV, CR, and NeuN in tree shrew brain lysates. Expected molecular weights are marked below each band. Note that protein markers migrated slightly faster than the lysate, resulting in slightly higher molecular weights. Minor nonspecific bands were observed with the polyclonal antibodies against CR and NeuN.

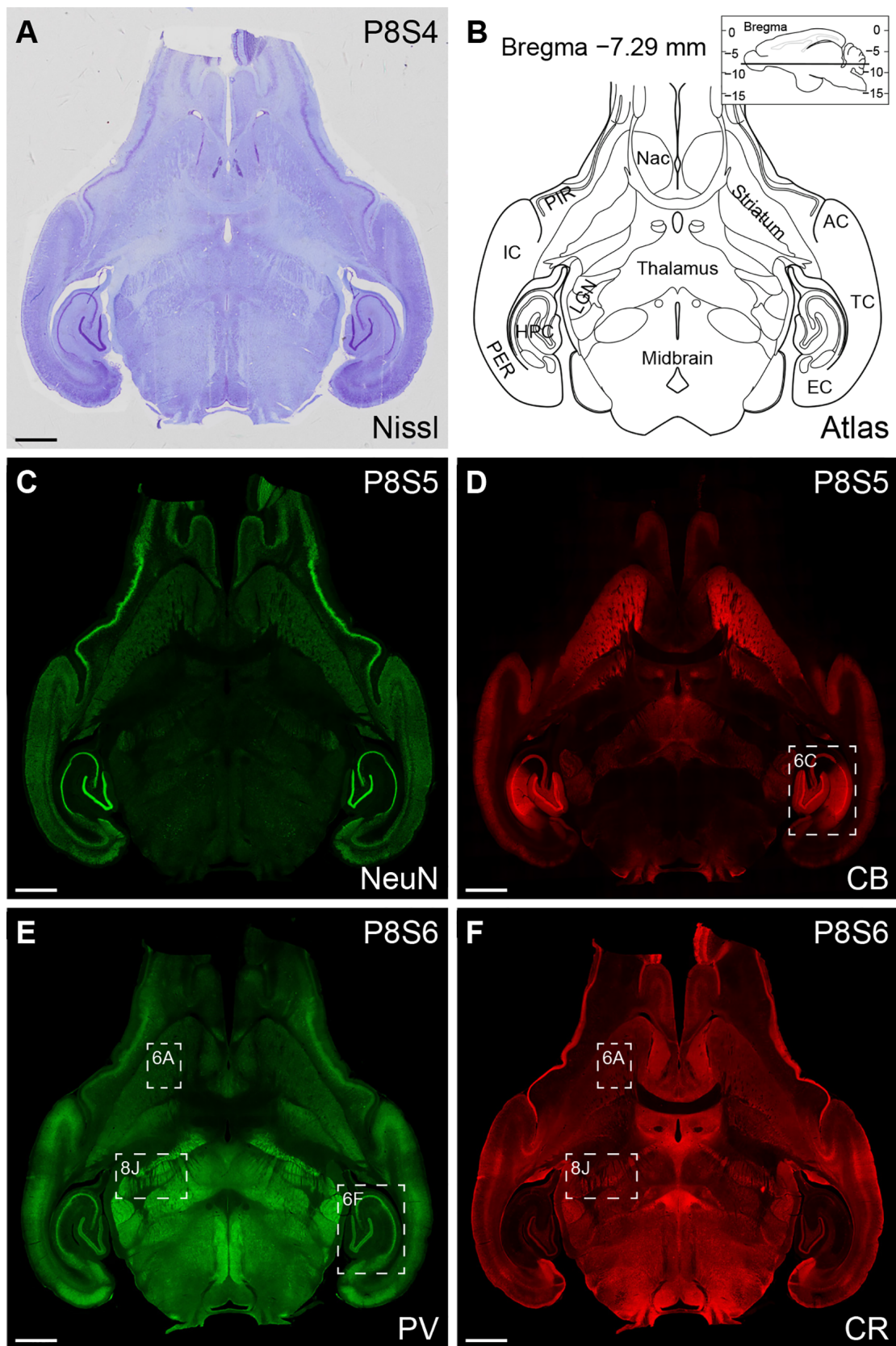


Figure 2 Representative staining of horizontal sections

Low-magnification images of three consecutive sections from TS116, with section IDs indicated on the top-right of each panel. A: Brain section stained with Nissl. B: Schematic atlas at 7.29 mm ventral to Bregma, adapted with permission from The Tree Shrew (*Tupaia belangeri chinensis*) Brain in Stereotaxic Coordinates (Zhou & Ni, 2016). C, D: The same section stained with antibodies against NeuN (C) and calbindin (D). E, F: The same section stained with antibodies against parvalbumin (E) and calretinin (F). Boxed regions are shown at higher magnifications in the figures indicated. AC, auditory cortex; EC, entorhinal cortex; HPC, hippocampus; IC, insular cortex; LGN, lateral geniculate nucleus; Nac, nucleus accumbens; PER, perirhinal cortex; PIR, piriform cortex; TC, temporal cortex. Scale bars: 2 mm.

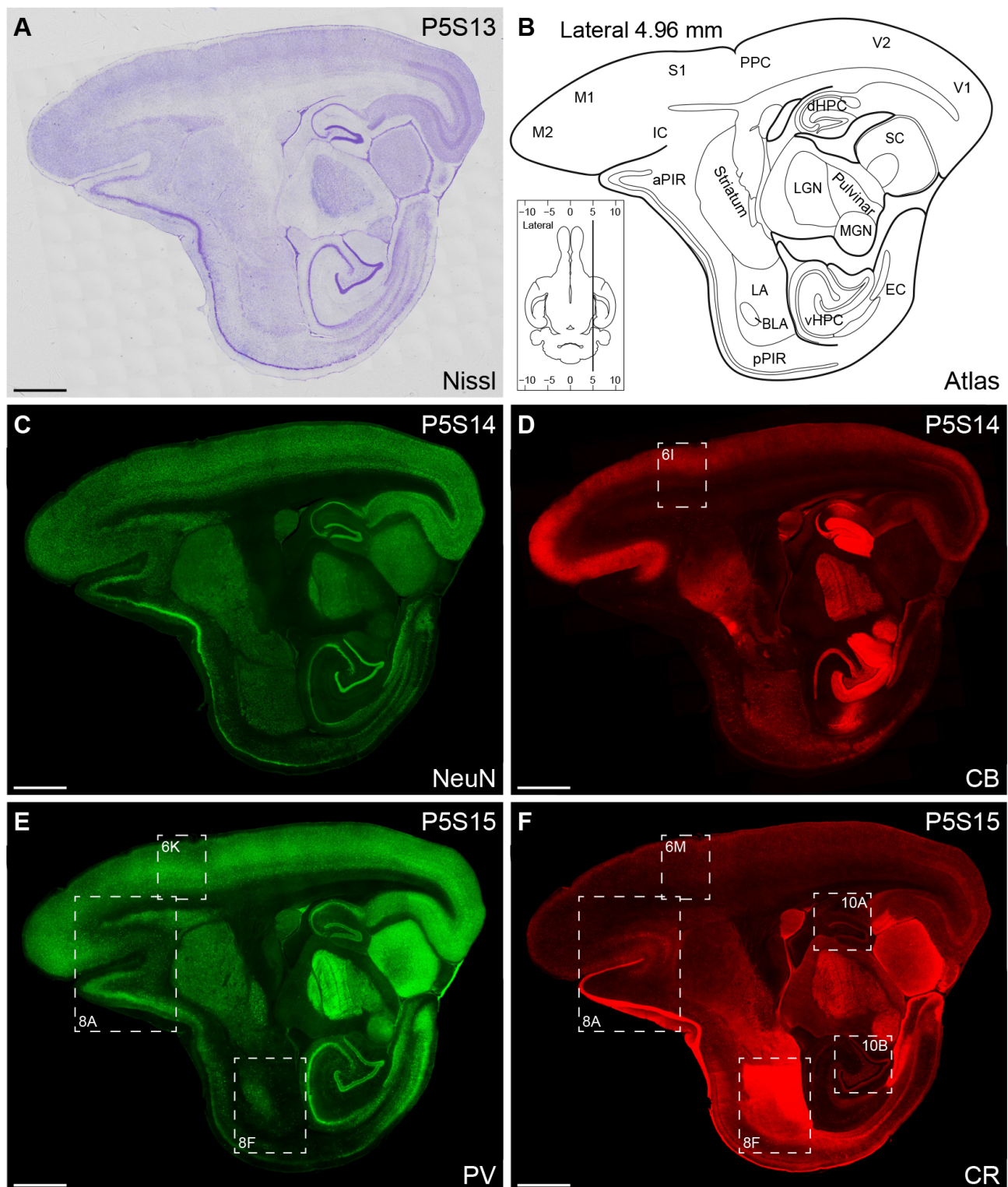


Figure 3 Representative staining of sagittal sections

Low-magnification images of three consecutive sections from TS031, with section IDs indicated on the top-right of each panel. A: Brain section stained with Nissl. B: Schematic atlas at 4.96 mm lateral to midline, adapted with permission from The Tree Shrew (*Tupaia belangeri chinensis*) Brain in Stereotaxic Coordinates (Zhou & Ni, 2016). C, D: The same section stained with antibodies against NeuN (C) and calbindin (D). E, F: The same section stained with antibodies against parvalbumin (E) and calretinin (F). Boxed regions are shown at higher magnifications in the figures indicated. BLA, basolateral amygdaloid nucleus; LA, lateral amygdaloid nucleus; EC, entorhinal cortex; dHPC, dorsal hippocampus; vHPC, ventral hippocampus; IC, insular cortex; LGN, lateral geniculate nucleus; MGN, medial geniculate nucleus; M1, primary motor cortex; M2, secondary motor cortex; aPIR, anterior piriform cortex; pPIR, posterior piriform cortex; PPC, posterior parietal cortex; S1, primary somatosensory cortex; SC, superior colliculus; V1, primary visual cortex; V2, secondary visual cortex. Scale bars: 2 mm.

contained images exported at a resolution of 72 dpi (bright-field) or 96 dpi (fluorescent). Higher-resolution versions will be

released in future updates or are available from the corresponding author upon request.

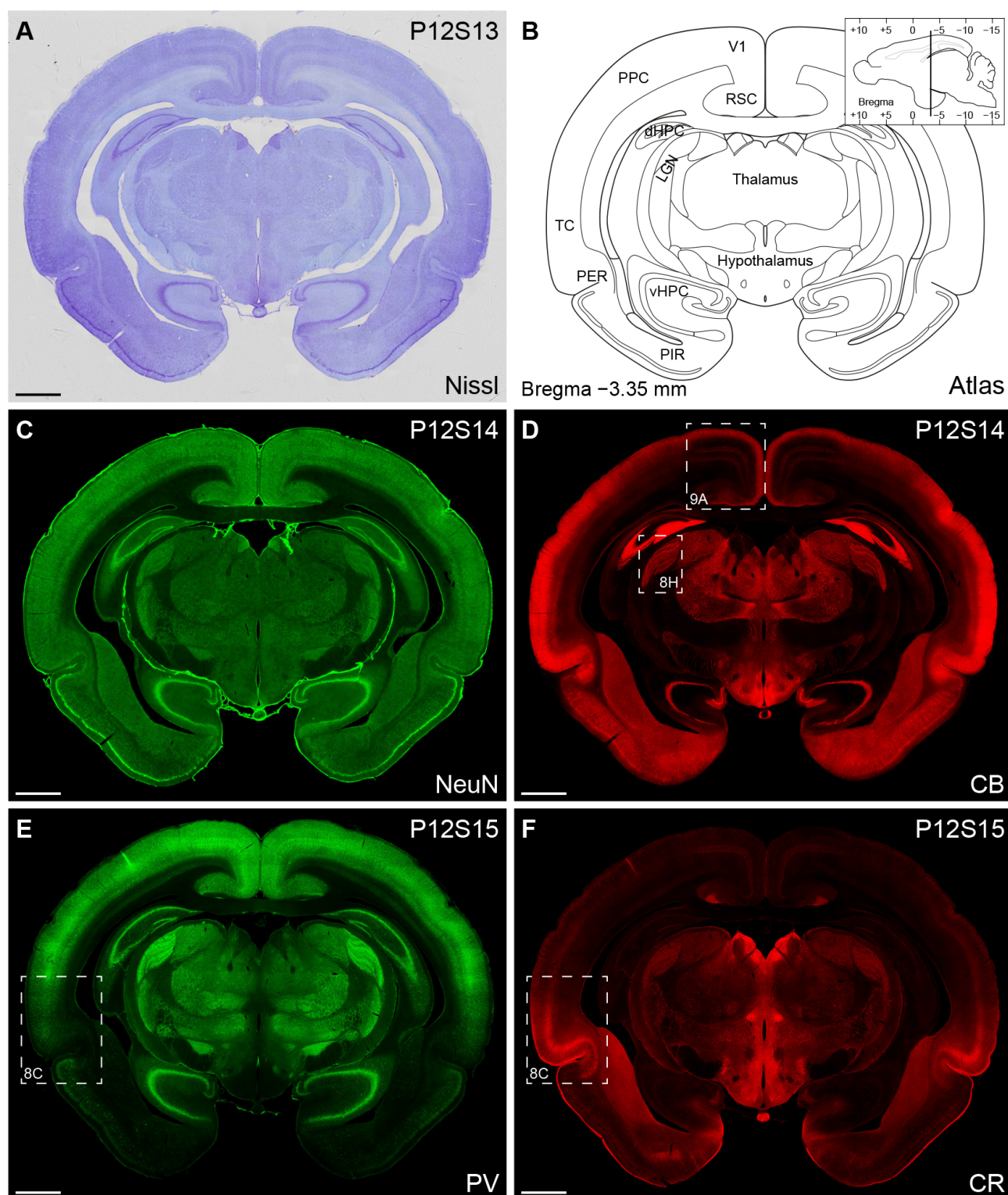


Figure 4 Representative staining of coronal sections

Low-magnification images of three consecutive sections from TS073, with section IDs indicated on the top-right of each panel. A: Brain section stained with Nissl. B: Schematic atlas at 3.35 mm posterior to Bregma, adapted with permission from The Tree Shrew (*Tupaia belangeri chinensis*) Brain in Stereotaxic Coordinates (Zhou & Ni, 2016). C, D: The same section stained with antibodies against NeuN (C) and calbindin (D). E, F: The same section stained with antibodies against parvalbumin (E) and calretinin (F). Boxed regions are shown at higher magnifications in the figures indicated. dHPC, dorsal hippocampus; vHPC, ventral hippocampus; LGN, lateral geniculate nucleus; PER, perirhinal cortex; PIR, piriform cortex; PPC, posterior parietal cortex; RSC, retrosplenial cortex; TC, temporal cortex; V1, primary visual cortex. Scale bars: 2 mm.

To enable efficient serial viewing of brain sections, indexed PowerPoint (.ppt) files were created for each individual, with low-resolution images from the same brain arranged sequentially, one per slide. Each slide was labeled with the

corresponding section ID, staining protocol, and additional notes. All sections were manually reviewed to confirm correct order based on anatomical landmarks. Misplaced sections were repositioned to their correct locations and marked with

Table 3 Overview of image dataset from 12 animals

ID	Sex	Age (m)	Plane	No. of sections	Coverage in atlas (mm)	Raw data size (GB)
TS011	F	15	Hor	312 (0)	Entire DV extent	546
TS015	F	15	Cor	406 (2)	Bregma -10.51–7.23	490
TS031	M	10	Sag	216 (0)	Lateral 0.17–end	279
TS046	F	12	Sag	216 (0)	Lateral 0.58–end	213
TS050	M	13	Hor	310 (2)	Bregma -13.13–0	453
TS054	M	14	Cor	375 (9)	Bregma -10.64–6.03	526
TS066	M	16	Sag	239 (1)	Entire ML extent	314
TS070	M	17	Hor	332 (2)	Entire DV extent	449
TS072	M	16	Sag	239 (1)	Entire ML extent	257
TS073	M	18	Cor	429 (1)	Bregma -10.24–8.17	519
TS098	M	8	Cor	312 (0)	Bregma -9.97–5.36	327
TS116	M	11	Hor	252 (0)	Bregma -4.79–end	289

Table shows information of tree shrews used in this study. Numbers shown in brackets are number of missing/destroyed sections. M, male; F, female; Hor, horizontal; Cor, coronal; Sag, sagittal; DV, dorsoventral; ML, mediolateral.

red section IDs. Missing or damaged brain sections were left blank to indicate their absence. Flipped sections or fragments were manually corrected using Photoshop when necessary, with the original view retained as a thumbnail on the same slide (Figure 5A). Image dimensions and orientation were manually adjusted and aligned with adjacent sections to ensure a coherent and smooth representation of the brain. Exported image files were named according to their corresponding slide numbers in the index (Figure 5B). The complete image dataset, totaling approximately 50 GB, is publicly available at <https://doi.org/10.57760/sciencedb.23471>.

Validation of antibody specificity

Although antibody binding affinity and specificity have been tested by the manufacturers, experimental verification was required, as these antibodies had not previously been used in tree shrews. Western blot analysis using lysates from tree shrew brain tissue demonstrated specific binding for monoclonal antibodies targeting CB and PV. In contrast, the polyclonal antibody against CR exhibited minor nonspecific bands (Figure 1B). The presence of two closely positioned bands detected by the NeuN antibody was consistent with observations in rodents (see antibody datasheet). Control sections processed without primary antibodies showed only weak auto-immunofluorescent signals, which did not significantly influence the interpretation of immunofluorescent labeling (Supplementary Figure S1).

To further confirm antibody specificity, the expression patterns of CB, PV, and CR in the tree shrew brain were compared with those reported in previous studies. The immunoreactivity pattern of CB in the neocortex, hippocampus, striatum, and thalamus closely corresponded with earlier findings (Ni et al., 2018a). In the striatum, the high density of PV-ir neurons and moderate density of smaller CR-ir neurons aligned with published data (Ni et al., 2018a, 2021b) (Figure 6A, B; Supplementary Figure S2A–C).

In the hippocampal formation (Supplementary Figure S2D–F), strong CB expression was observed in the cell bodies of dentate granule cells and CA1 pyramidal neurons, with lower expression in their processes, consistent with previously described patterns (Keuker et al., 2003). In contrast, very few CB-ir neurons were detected in CA3, CA2, and the subiculum (Figure 6C–E). PV-ir interneurons were primarily located above and below the dentate granule cell and hippocampal pyramidal cell layers, with short or long dendrites extending into the molecular layer of the dentate

gyrus (DG) and strata radiatum of the CA regions, respectively (Figure 6F, G). Notably, a higher density of PV-ir interneurons and accompanying fibers was present in the CA2 region relative to adjacent CA subfields (Figure 6H), precisely matching earlier observations (Keuker et al., 2003). Together, these molecular and anatomical results confirm the specificity of the CB, PV, and CR antibodies in the tree shrew brain.

Regional and laminar-selective expression of CaBPs

The distribution patterns of the three CaBPs were first assessed across cortical and subcortical regions of the tree shrew brain. Within the neocortex, analysis focused on the primary somatosensory cortex (S1, Figure 6I–N; Supplementary Figure S2G, H) and prefrontal cortex (Figure 7; Supplementary Figure S2I, J). CB-ir neurons were predominantly localized to layers 2 and 3 in both regions, with sparse representation in deeper layers (Figure 6I, J; Figure 7D, G, H). Notably, strong CB expression in layer 3 of the prefrontal cortex differed from patterns reported in mice (Van De Werd et al., 2010). Beyond the cortex, CB was also highly expressed in the striatum, amygdala (Figures 2D, 3D), and selected nuclei of the thalamus and hypothalamus (Figure 4D). In contrast, CB expression was relatively low in the anterior piriform cortex and superior colliculus (Figure 3D).

PV-ir neurons in S1 were prevalent in the middle to lower layers (Figure 6K, L). In the prefrontal cortex, PV expression exhibited a dorsoventral gradient, with decreasing intensity from the prelimbic to infralimbic subdivisions (Figure 7E, I, J), consistent with observations in mice (Courcelles et al., 2024; Kim et al., 2017). In subcortical regions, PV-ir neurons were abundant in the thalamus (Figures 2E, 4E) and the superficial gray layer of the superior colliculus (Figure 3E). The piriform cortex exhibited relatively low PV expression, particularly in its posterior portion (Figure 3E).

CR-ir neurons were less abundant in the neocortex compared to PV- and CB-expressing populations. These vertically oriented neurons were present across all layers of the cortex, with the highest concentration at the border between layers 1 and 2, and a gradual decrease toward deeper layers in S1 (Figure 6M, N). In the prefrontal cortex, the laminar distribution of CR-ir neurons resembled that in S1, with the exception of increased density in layer 4, particularly within the infralimbic cortex (Figure 7F, K, and L). In subcortical regions, dense populations of CR-ir neurons were detected in the amygdala (Figure 3F), nucleus accumbens (Figure 2F), superior colliculus (Figure 3F), and hypothalamus

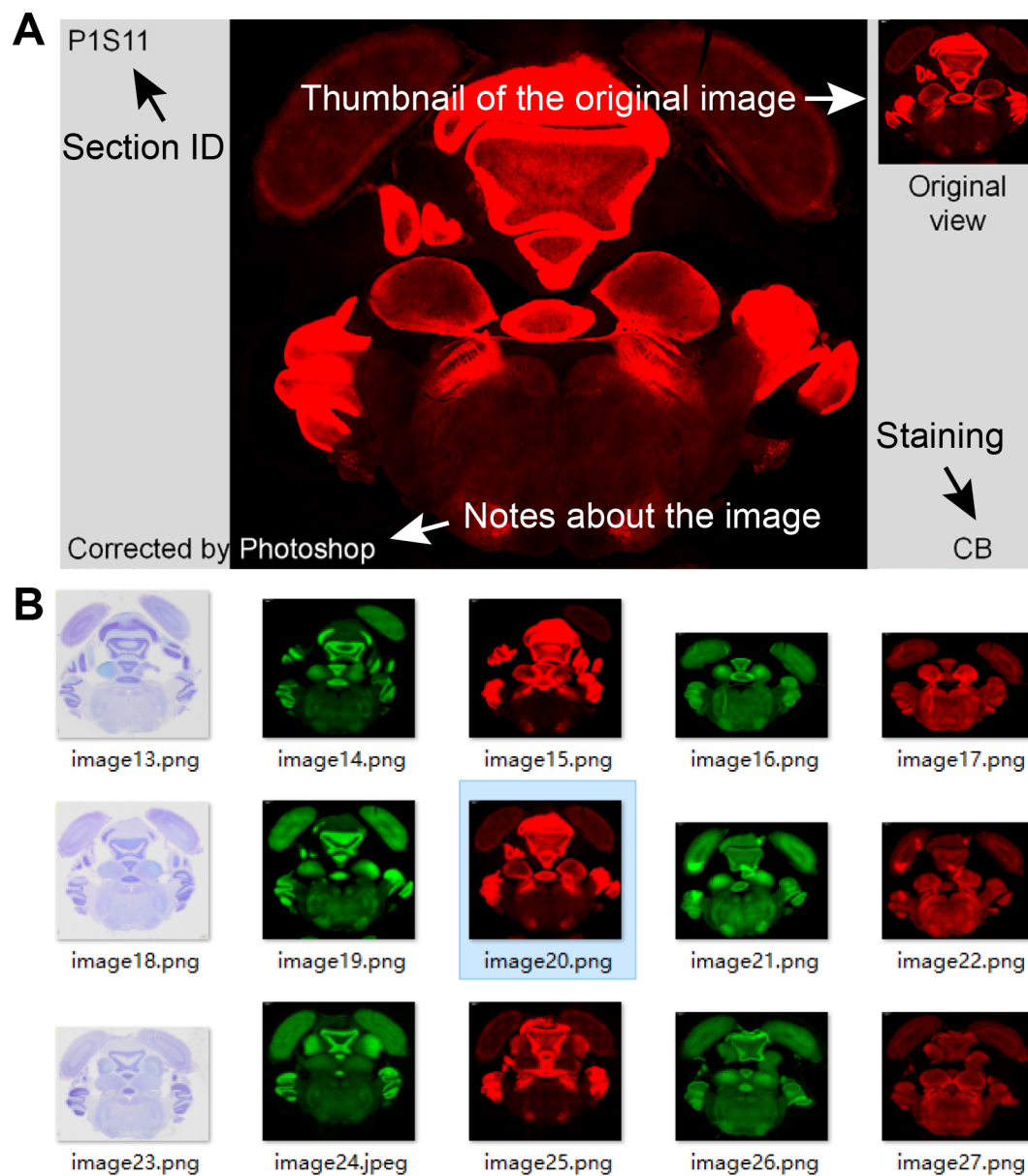


Figure 5 Organization of index files and images

A: Representative page from the index file of TS015 showing the reconstructed brain image centrally displayed with relevant metadata indicated at the corners. Top left: Section ID. If a section has been repositioned, the ID is shown in red. P, plate; S, section. Top right: Thumbnail of original image view. Modifications, such as rotation or flipping, are indicated here. In this example, the midbrain was flipped left-to-right and corrected. Bottom left: General notes describing image quality or anomalies (e.g., “patchy staining” or “flawed noise correction”). Bottom right: Type of staining. A dark background indicates that a recycled antibody was used. Note the saturated CB signals in the cerebellum. B: Exported image files, named to match the corresponding page numbers in the index.

(Figure 4F).

Overall, the spatial distribution of CaBP-immunofluorescent signals in the tree shrew was largely consistent with patterns described in rats, monkeys, and humans (Barinka & Druga, 2010; Hof et al., 1999; Vanessa Becerra-Hernández et al., 2025). No apparent inter-individual variation was observed in the distribution patterns of the three CaBPs.

CaBP expression facilitates delineation and parcellation of distinct brain regions

The selective expression patterns of CaBPs were examined with a focus on cortical and subcortical regions of the tree shrew brain that are challenging to delineate using conventional Nissl or NeuN staining. Similar to the infralimbic cortex, both the insular and perirhinal cortices were clearly

distinguished from neighboring auditory, temporal, and motor cortices by the dense presence of CR-ir neurons in layer IV and markedly reduced PV immunoreactivity (Figure 8A–E). This expression profile parallels findings in rodents and nonhuman primates (Barinka et al., 2012; Beaudin et al., 2013; Burwell et al., 1995; Courcelles et al., 2024; Pitkänen & Amaral, 1993; Salaj et al., 2021; Uva et al., 2004). In contrast, CB, NeuN, and Nissl staining in these regions showed largely homogeneous patterns, limiting their utility for regional delineation (Supplementary Figure S3A–F).

CaBP expression also facilitated the parcellation of subcortical structures. For example, while no clear anatomical borders were visible in Nissl, NeuN, and CB staining (Supplementary Figure S3G–I), the basolateral amygdaloid nucleus, characterized by high PV-ir signals and relatively low

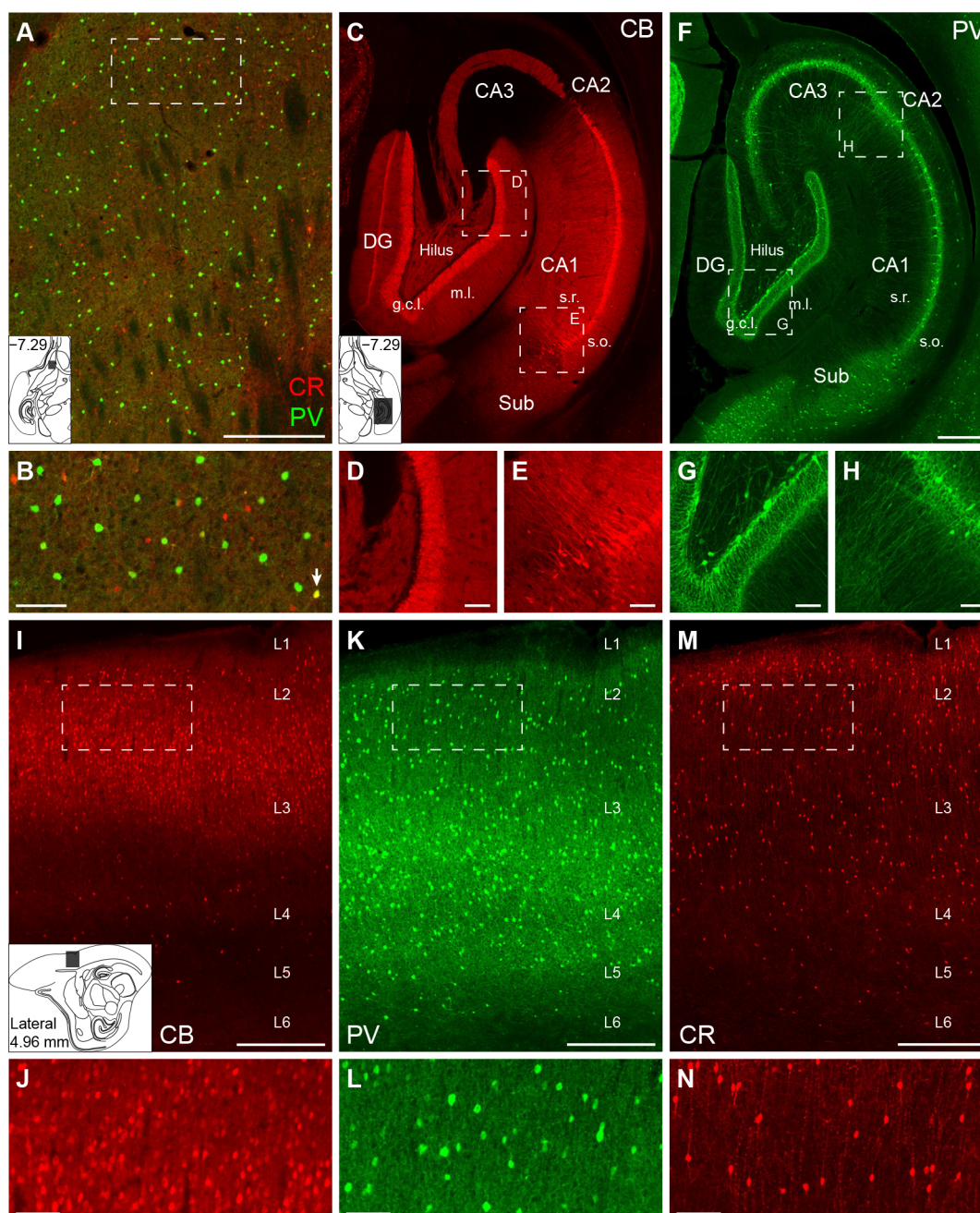


Figure 6 Distribution of CaBPs in the tree shrew is consistent with published literature

A: Horizontal section showing merged PV and CR signals in striatum, with scattered PV-ir and CR-ir neurons. Dark box (bottom left) indicates the anatomical location, with distance from Bregma labeled in mm. B: High-magnification view of boxed region in A, showing 20 PV-ir neurons and 10 CR-ir neurons. Note dual-labeled neuron (arrow) in the lower right and the generally larger somata of PV-ir neurons compared to CR-ir neurons. C: Horizontal section showing CB expression in the hippocampus. Inset shows section location. D, E: High-magnification views of boxed regions in C, showing CB expression in dentate granule cells (D) and pyramidal cells at the distal end of CA1 (E). F: Horizontal section showing PV-ir interneurons in the hippocampus. G, H: High-magnification views of boxed regions in F, showing PV-ir neurons and their processes in the dentate gyrus (G) and CA2 (H). I: Sagittal section showing CB-ir neurons in S1. Cortical layers are labeled at right. Inset indicates section location. J: High-magnification view of boxed region in I, showing dense distribution of CB-ir neurons in layers 2 and 3. K: Adjacent section showing distribution of PV-ir neurons in the same cortical region as in I. L: High-magnification view of boxed region in K, showing scattered PV-ir neurons in superficial layers. Note that these neurons have larger somata compared to neurons in J. M: Vertically oriented CR-ir neurons in the same view as in K. N: High-magnification view of boxed region in M, showing neurons with processes extending almost perpendicularly to the cortical surface. DG, dentate gyrus; g.c.l., granule cell layer; m.l., molecular layer; s.o., strata oriens; s.r., strata radiatum; Sub, subiculum. Scale bars: 0.5 mm for A, C, F, I, K, and M; 100 μ m for B, D, E, G, H, J, L, and N.

CR-ir signals, could be distinguished from the lateral amygdaloid nucleus, which contained sparsely scattered PV-ir neurons and a high density of CR-ir neurons (Figure 8K, L).

Moreover, the six layers of the dorsal lateral geniculate

nucleus (LGN), a notable anatomical feature shared by tree shrews and primates (Takahata & Kaas, 2017), were better visualized in CB immunostaining than in other staining methods (Figure 8F, G; Supplementary Figure S4A–D).

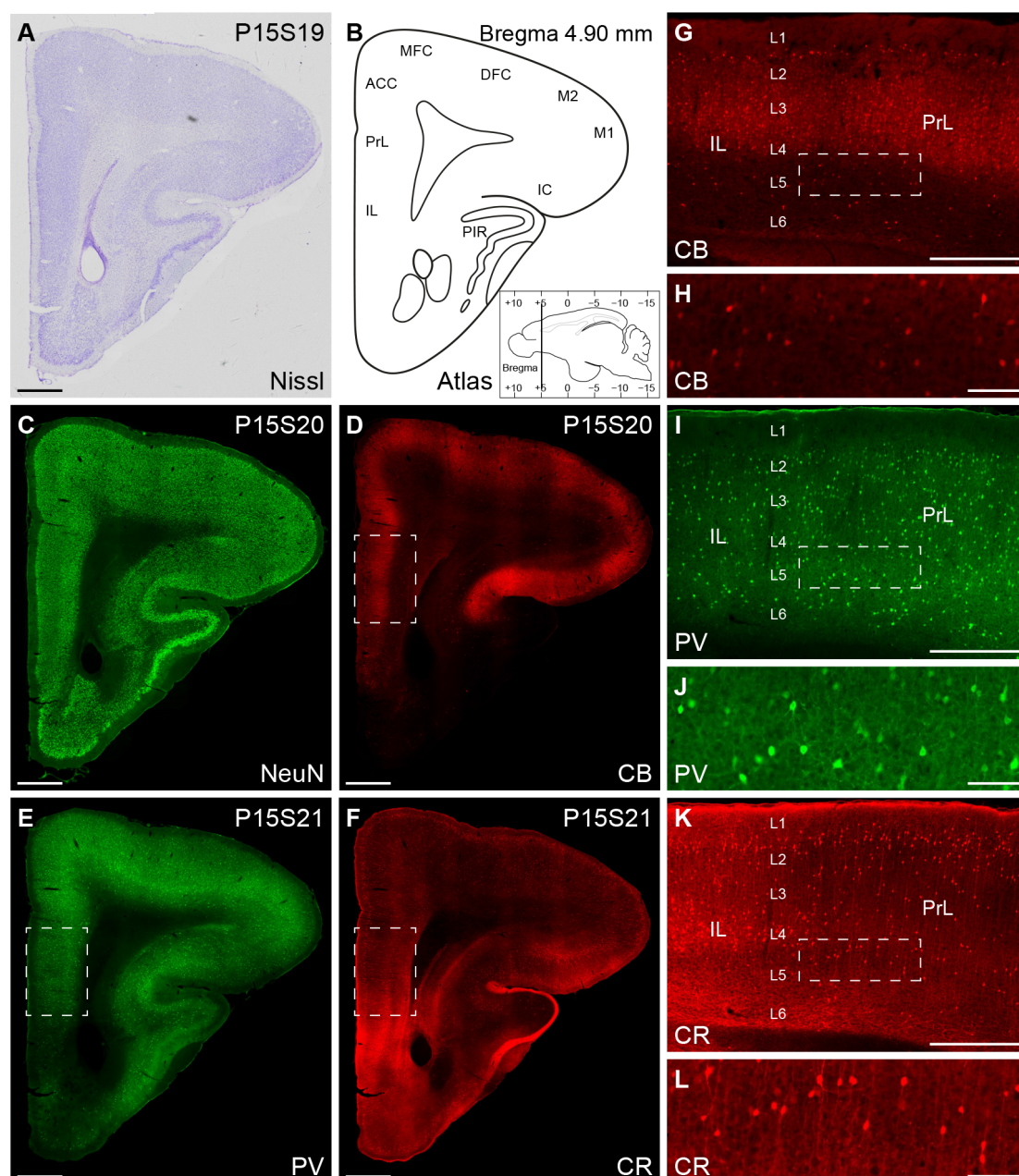


Figure 7 Expression pattern of CaBPs in the prefrontal cortex

A–F: Low-magnification images of three consecutive sections from the right hemisphere of TS054, with section IDs indicated on the top-right of each panel. A: Brain section stained with Nissl. B: Schematic atlas at 4.90 mm anterior to Bregma, adapted with permission from The Tree Shrew (*Tupaia belangeri chinensis*) Brain in Stereotaxic Coordinates (Zhou & Ni, 2016). C, D: The same section stained with antibodies against NeuN (C) and calbindin (D). E, F: The same section stained with antibodies against parvalbumin (E) and calretinin (F). G, I, K: High-magnification views of boxed regions in D–F, with layers indicated. Images are rotated 90° clockwise. Note the strong CR expression in layer 4 of the infralimbic cortex. H, J, L: Magnified views of boxed regions in G, I, and K showing neural morphology in deep layers. ACC, anterior cingulate cortex; DFC, dorsal frontal cortex; MFC, medial frontal cortex; PrL, prelimbic cortex; IL, infralimbic cortex; M1, primary motor cortex; M2, secondary motor cortex; IC, insular cortex; PIR, piriform cortex. Scale bars: 1 mm for A–F; 0.5 mm for G, I and K; 100 μm for H, J, and L.

Furthermore, the magnocellular and parvocellular subdivisions of the ventral LGN (Agarwala et al., 1992) were distinguishable based on PV and CR distributions, with the former selectively marked by densely scattered PV-ir neurons and the latter by high CR and low PV double-stained neurons (Figure 8H–J; Supplementary Figure S4E–G).

These observations collectively suggest that the region- and layer-specific distribution of CaBPs offers critical molecular contrast for anatomical differentiation, enabling refined delineation and parcellation of both cortical and subcortical

structures in the tree shrew brain.

Sex- and age-dependent expression of CaBPs

The inclusion of brain images from female and juvenile tree shrews enabled the exploration of potential sex- and age-related differences in CaBP expression patterns. The visual system of the tree shrew shares multiple organizational features with that of primates. Notably, layer 4 of V1 can be further subdivided into two distinct sublayers, each receiving inputs from separate combinations of LGN layers that convey information from the ipsilateral and contralateral eyes

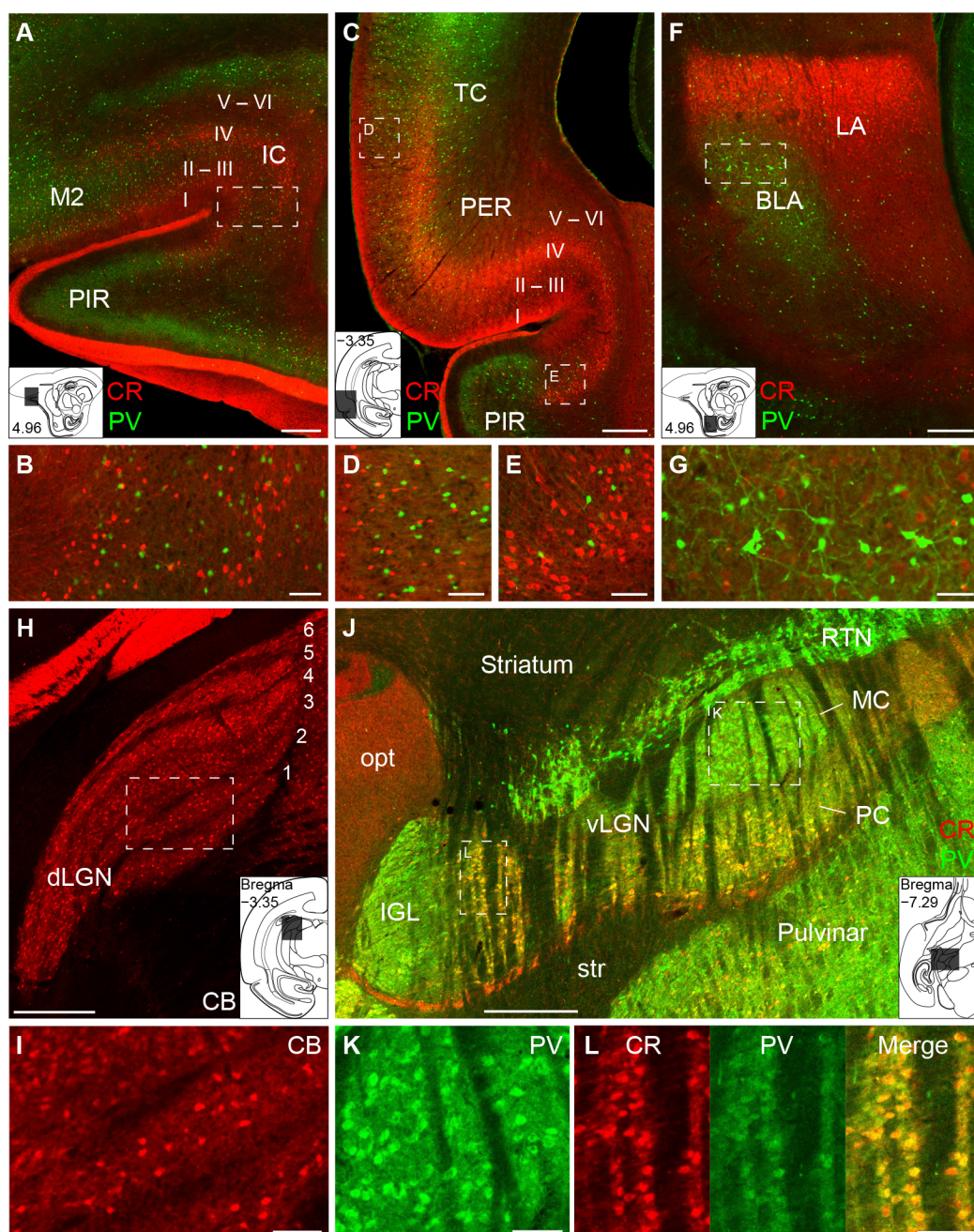


Figure 8 Example of regions selectively delineated by PV and CR expression

A–E: Sagittal and coronal sections showing dense CR-ir neurons in layer IV of the insular cortex (A) and perirhinal cortex (C), which clearly separated both regions from their neighbors. Cortical layers are indicated in Roman numerals. B, D, E: High-magnification views of boxed regions in A and C, showing PV-ir and CR-ir neurons in the insular cortex (B), superficial layers (D), and layer IV (E) of the perirhinal cortex. F: Sagittal section showing merged PV and CR signals defining the borders between the basomedial and basolateral amygdaloid nuclei. G: High-magnification view of boxed region in F, showing neurons strongly expressing PV. H: Coronal section showing CB expression in the dorsal LGN, with layers indicated on the right. I: High-magnification view of boxed region in H, showing CB-ir neurons in layers 1–3 of the LGN. J: Horizontal section showing merged PV and CR signals in the ventral LGN and surrounding structures, with PV-ir neurons in the magnocellular division and high-CR and low-PV double positive neurons in the parvocellular division. K, L: High-magnification views of boxed regions in J, showing PV-ir neurons in the MC (K) and CR-ir neurons with low PV expression (L). Insets show locations of the images in the brain, with numbers indicating distances from Bregma in mm. IC, insular cortex; M2, secondary motor cortex; PIR, piriform cortex; PER, perirhinal cortex; TC, temporal cortex; BLA, basolateral amygdaloid nucleus; LA, lateral amygdaloid nucleus; dLGN, dorsal lateral geniculate nucleus; vLGN, ventral lateral geniculate nucleus; IGL, intergeniculate leaf; MC, magnocellular division; PC, parvocellular division; RTN, reticular thalamic nucleus; opt, optic tract; str, superior thalamic radiation. Scale bars: 0.5 mm for A, C, F, H, and J; 100 μm for B, D, E, G, I, K, and L.

(Balaram & Kaas, 2014; Ding, 2024; Murphy & Monteiro, 2024; Takahata & Kaas, 2017).

In contrast to other neocortical regions (Figures 6I–N, 7), CB expression in V1 was markedly reduced in layer 3.

Instead, two narrow, well-defined CB-ir bands were observed within layer 4 of V1, separated by a narrow CB-free cleft (Figure 9A, B). This pattern closely resembles the activation profile of c-fos following visual stimulation (Takahata & Kaas,

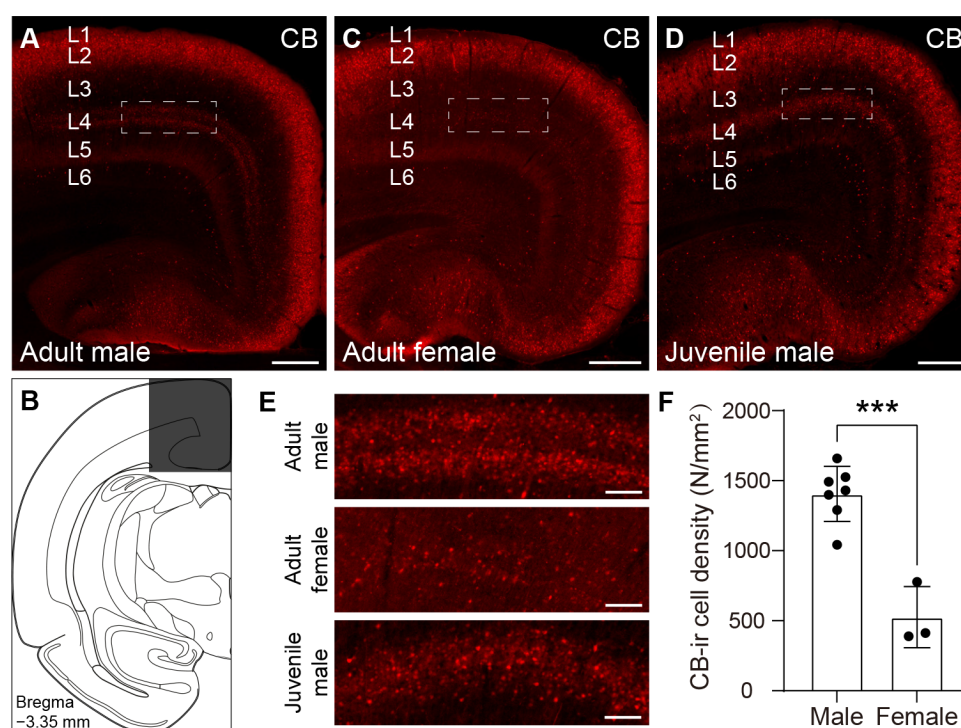


Figure 9 Sex- and age-dependent CB expression in the visual cortex

A: Representative image showing CB expression in V1 of adult male tree shrews, with cortical layers indicated. B: Schematic of coronal section showing the location of the image in A within the brain. C: V1 of adult female (TS015, section P7S8) showing minor expression of CB in layer 4. D: V1 of juvenile male (TS098, section P5S23) with a single CB-ir band in layer 4. E: High-magnification views of boxed regions in A, C, and D, showing distinct distribution of CB-ir neurons in layer 4 of V1 between sexes and developmental stages. F: Bar plot (mean±SEM) showing significant differences in the density of CB-ir neurons in layer 4 of V1 between adult male and female tree shrews (*t*-test, seven males, three females, $t=6.306$, $P<0.001$). Scale bars: 100 μ m for E and 0.5 mm for A, C, and D. ***: $P<0.001$.

Table 4 Colocalization of PV and CR-labeled neurons

Brain region	PV-ir neurons	CR-ir neurons	Double labeled	% double labeled PV-ir	% double labeled CR-ir
PFC	420	272	1	0.24	0.37
S1	532	377	11	2.07	3.58
V1	764	370	13	1.70	3.51
IC	447	558	2	0.45	0.36
PER	468	691	1	0.21	0.14
HPC	152	129	2	1.32	1.55
Striatum	368	264	9	2.45	3.41
BLA	81	109	0	0	0
dLGN	86	178	30 (2)	34.88 (2.33)	16.85 (1.12)
PC of vLGN	29	59	12 (2)	41.38 (6.90)	20.34 (3.39)
Pulvinar	160	513	39 (6)	24.38 (3.75)	7.60 (1.17)

Table shows number of single-labeled (PV-ir or CR-ir) and double-labeled (PV & CR) neurons in various brain regions, along with their respective proportions. Numbers shown in brackets indicate number/proportion of CR-ir neurons also strongly labeled by PV. PFC, prefrontal cortex; S1, primary somatosensory cortex; V1, primary visual cortex; IC, insular cortex; PER, perirhinal cortex; HPC, hippocampus; BLA, basolateral amygdala; dLGN, dorsal lateral geniculate nucleus; PC, parvocellular division; vLGN, ventral lateral geniculate nucleus.

2017), suggesting its potential involvement in visual processing. Interestingly, the well-separated CB-ir bands were less prominent in adult females, while juvenile males exhibited only a single broad CB-ir band within layer 4 (Figure 9C–F). In contrast, no sex- or age-related differences were detected in layer 4 of V1 using other staining methods (Supplementary Figure S5).

An internal review of all laboratory materials confirmed this pattern: a double-layered CB-ir band was detected in V1 of 18 out of 19 male tree shrews aged 10 months or older. In contrast, all four male tree shrews aged 1, 3, 6, and 8 months displayed a single CB-ir band, and the band was barely

discernible in all five females examined. These observations suggest that CB expression in layer 4 of V1 varies by sex and undergoes postnatal refinement. The emergence of a split in the CB-ir band before adulthood may reflect postnatal maturation of visual function, a process likely driven and shaped by experience-dependent neural plasticity (Murphy & Monteiro, 2024). The inclusion of female and juvenile tree shrews in the dataset provides a framework for the exploration of sexual dimorphism and postnatal development in neuronal function.

Colocalization of PV-ir and CR-ir neurons in the tree

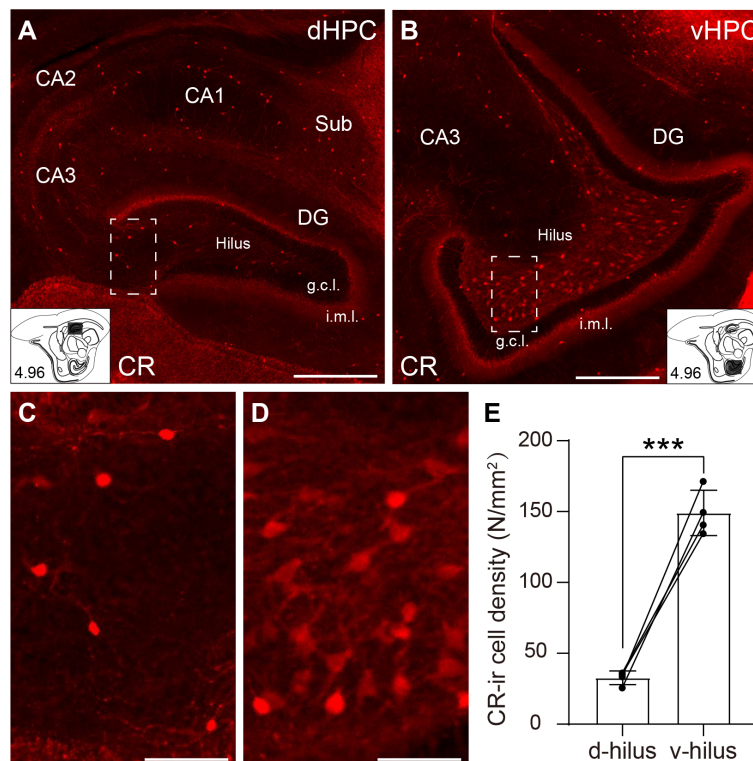


Figure 10 Selective CR expression in ventral dentate mossy cells

A, B: Distribution of CR-ir neurons in the dorsal hippocampus (A) and ventral dentate gyrus (B) from the same sagittal section, showing many CR-ir neurons in the ventral hilus. Insets show locations of images in the brain, with numbers indicating distances in mm from midline. C, D: High-magnification views of boxed regions in A and B, showing CR-ir neurons in the dorsal hilus (C) and ventral hilus (D). Note: CR-ir neurons in the ventral hilus are larger than those in the dorsal part. E: Bar plot (mean±SEM) showing density of CR-ir neurons in the hilus of both the dorsal and ventral hippocampus (paired *t*-test, four animals, $t=13.090$, $P=0.001$). dHPC, dorsal hippocampus; vHPC, ventral hippocampus; DG, dentate gyrus; g.c.l., granule cell layer; i.m.l., inner molecular layer; Sub, subiculum. Scale bars: 0.5 mm for A and B; 100 μ m for C and D. ***: $P<0.001$.

shrew brain

While CB, PV, and CR immunoreactivity generally displays minimal overlap in rodent and primate brains, the degree of overlap varies significantly across different brain regions (Camp & Wijesinghe, 2009; Hof et al., 1999; Vanessa Becerra-Hernández et al., 2025). For example, in V1, the proportion of neurons co-expressing multiple CaBPs remains below 7.5% across rats, monkeys, and humans (Gonchar & Burkhalter, 1997; Hendry et al., 1989; Leuba & Saini, 1997). In contrast, the basolateral amygdala shows higher colocalization of PV and CB, with at least 23% of PV-ir neurons also expressing CB (Mascagni et al., 2009; McDonald & Betette, 2001; Pantazopoulos et al., 2006), while CR overlaps with either marker only infrequently (McDonald, 2021; McDonald & Mascagni, 2001; Pantazopoulos et al., 2006).

The present dataset includes double-label immunofluorescence staining of PV and CR in the same sections, allowing for detailed assessment of colocalization in the tree shrew brain (Table 4). In general, minimal overlap (<4%) was observed in cortical regions, including the prefrontal cortex (Supplementary Figure S6A), S1 (Supplementary Figure S6B), V1 (Supplementary Figure S6C), insular cortex (Figure 8A, B), and perirhinal cortex (Figure 8C–E). Similar findings were noted in the striatum (Figure 6A, B), hippocampus (Supplementary Figure S6D), and amygdala (Figure 8F, G), consistent with patterns reported in other species (Gonchar & Burkhalter, 1997; Hendry et al., 1989; Hermanowicz-Sobieraj et al., 2018;

Kubota et al., 1994; Leuba & Saini, 1997).

In contrast to the robust and distinct PV or CR expression observed in the aforementioned brain regions, PV immunoreactivity was relatively weak in the somata of thalamic neurons, including those in the parvocellular division of the ventral LGN (Figure 8J, L), as well as the dorsal LGN and pulvinar (Supplementary Figure S6F). This low-intensity labeling led to an apparent increase in CR and PV signal overlap (Table 4). However, due to the low PV signal intensity in these regions, establishing reliable thresholds for cell classification was not feasible, precluding accurate quantification. Additionally, CR-ir neurons that were also strongly labeled by PV were rare (Table 4, numbers in brackets), consistent with findings in the human brain (Leuba & Saini, 1997). In summary, the overall pattern of PV-ir and CR-ir neuron colocalization in the tree shrew brain did not significantly deviate from that observed in rats, monkeys, and humans. This consistency aligns with the phylogenetic position of the tree shrew between rodents and primates.

CaBP chemoarchitecture varies across mammalian species

Although CaBP expression follows broadly conserved principles across mammals, interspecies differences in spatial distribution remain evident. In the final analysis, CR expression was examined in mossy cells of the DG, a cell population defined by its extensive excitatory connections with granule cells and inhibitory interneurons and known to support hippocampus-dependent mnemonic functions (Scharfman, 2016). In most rodent and primate species, CR expression in

mossy cells originates at the ventral/temporal/uncal pole of the DG and extends variably along the hippocampal longitudinal axis toward the dorsal/septal/tail end (Fabene et al., 2001; Fujise et al., 1998; Kwak et al., 2005; Liu et al., 1996; Maliković et al., 2023; Murakawa & Kosaka, 1999; Seress et al., 2008). In contrast, CR expression is largely absent from mossy cells in carnivores and artiodactyls (Hof et al., 1996; Maliković et al., 2023; Pillay et al., 2021).

In the tree shrew DG, CR-ir neurons were sparsely distributed in the dorsal hilus but densely distributed in the ventral hilus (Figure 10A, B). Although CR was also expressed in subsets of hilar interneurons, most CR-ir neurons in the ventral hilus were identified as mossy cells based on their larger somatic profiles (Figure 10C, D) and the presence of clear CR-ir signals in the inner molecular layer (Figure 10B), where mossy cell axons innervate granule cells (Seress et al., 2008). The density of CR-ir neurons in the ventral hilus was significantly higher than that in the dorsal hilus (Figure 10E), suggesting a higher proportion of CR-expressing mossy cells in the ventral DG. These findings indicate that CR distribution in the tree shrew DG resembles that observed in most rodents and monkeys, suggesting both morphological and potentially functional homologies. However, the extent to which CR expression in mossy cells along the hippocampal longitudinal axis reflects phylogenetic relationships among mammalian species remains undetermined.

DISCUSSION

This study presents a high-resolution, brain-wide dataset mapping the immunoreactivity of PV, CB, and CR in consecutive sections across three anatomical planes of the tree shrew cerebrum. By capturing the region- and layer-specific distribution of these CaBPs at cellular resolution, the dataset offers a versatile resource for advancing neuroanatomical, functional, and translational studies in this increasingly important primate-adjacent model.

Potential contributions of the dataset

First, the dataset maps spatially distinct CaBP expression patterns that correspond to specific neuronal populations, enabling refined morphological and chemoarchitectural descriptions of brain regions. Such molecular precision facilitates the anatomical delineation and parcellation of specific regions (Figure 8), as previously reported in the hippocampus and cerebellum (Keuker et al., 2003; Ni et al., 2018a). This work will facilitate a more comprehensive understanding of brain organization in tree shrews, particularly in regions implicated in neurological disorders. For example, the cyto- and chemoarchitecture of the entorhinal cortex, a site of tauopathy in Alzheimer's disease (AD) (Braak & Braak, 1991), can be characterized by CaBP-based signatures derived from the present dataset (manuscript in preparation). This molecular definition will significantly enhance the anatomical accuracy of early AD models in tree shrews and provide a reference for pathological and mechanistic investigations targeting A β accumulation, tau aggregation, and neurodegeneration, all of which have been observed in aged tree shrew brains (Li et al., 2024b) but not in aged rats (Yamashita et al., 2012).

Second, this dataset serves as a foundation for future functional mapping and circuit-level analyses. The CaBP-defined distribution of inhibitory neuron subtypes can guide neural tracing and colocalization experiments to explore

anatomical connections and excitation-inhibition dynamics across brain regions. This framework parallels approaches in rodents and nonhuman primates (Mascagni et al., 2009; McDonald & Mascagni, 2001; Xu et al., 2010) and now extends such capabilities to tree shrews. In addition, the dataset enables integration of activity-dependent markers such as *c-fos* to chart response to various sensory stimuli and neural manipulations in tree shrews (Ni et al., 2020b; Poveda & Kretz, 2009; Takahata & Kaas, 2017). This approach allows the linking of structural data and functional activity, providing a comprehensive view of how different brain regions contribute to sensory processing, motor control, and high-order cognitive functions. Such studies will help establish connections between structural and functional atlases of the tree shrew brain, paving the way for a deeper understanding of the neural basis of behavior and cognition.

Third, this dataset enables the exploration of sex- and age-related differences in CaBP expression (Figure 9), offering critical insights into sexual dimorphism in brain architecture (Ni et al., 2022) and the postnatal maturation of neuronal circuitry (Xiong et al., 2024). By establishing a baseline for CaBP expression patterns across cortical and subcortical regions, the dataset supports more comprehensive and targeted investigations into how these molecular profiles evolve with age and differ between the sexes. Such analyses are essential for understanding dynamic shifts in brain structure and function across developmental and biological contexts.

Fourth, this dataset supports cross-species comparisons of brain chemoarchitecture, highlighting morphologically and potentially functionally conserved features between tree shrews and other mammals, particularly primates (Balaram & Kaas, 2014; Mustafar et al., 2018; Yamashita et al., 2012). This comparative framework enhances the relevance of tree shrews in neurophysiological and neuropathological research, including models of stress, AD, and Parkinson's disease (Li et al., 2024a, 2024b; Zambello et al., 2010). Moreover, the dataset facilitates the identification of both conserved and divergent features in brain organization, refining evolutionary interpretations and bridging the translational gap between rodent and primate systems.

Antibody specificity and immunofluorescence staining quality

Although the primary antibodies employed in this study have not been previously validated in tree shrews, they have been extensively used in rodents, nonhuman primates, and humans with established specificity (Boccaro et al., 2015; Cui et al., 2023; Ding & Van Hoesen, 2015; McDonald, 2021; Medalla et al., 2023; Xie et al., 2021). Western blot analysis of tree shrew brain lysates confirmed that each antibody selectively recognized bands of PV, CB, CR, or NeuN (Figure 1B). To further confirm their specificity in tree shrews, the immunostaining patterns obtained from specific brain regions were compared with previously published data. The regional expression of CaBPs in the hippocampus (Figure 6C–H) (Keuker et al., 2003), striatum (Figure 6A, B) (Ni et al., 2018b, 2021a; Rice et al., 2011), and cerebellum (TS066 dataset) (Bhagwandin et al., 2024; Ni et al., 2018a) demonstrated high concordance with published findings, thus confirming the selective binding of these antibodies to their target proteins in tree shrews. In addition, overall expression patterns of CaBPs were compared with those reported in rodents and primates (Figures 6I–N, 7) (Hof et al., 1999; Wong & Kaas, 2009). The

observed distribution patterns were broadly conserved, with only minor interspecies differences in laminar and regional expression (Leuba & Saini, 1997; Van De Werd et al., 2010).

Overall, the antibodies used in this study performed well, yielding high-quality staining results across multiple samples and brain regions. Most antibodies provided clear and specific labeling, facilitating detailed visualization of the target proteins and neuronal morphology. However, staining with the NeuN antibody exhibited batch-to-batch differences in staining intensity and specificity. In particular, one batch failed to produce reliable results in sample TS050, indicating a lack of consistency in antibody performance and emphasizing the need for rigorous quality control in immunofluorescence experiments. Recycled antibody usage was also evaluated in a subset of animals (TS011, TS015, TS031, TS054, TS072), producing comparable results for NeuN and CB but not for PV and CR. These discrepancies may reflect variability in antibody stability or loss of binding affinity after the initial application. These findings emphasize the necessity of stringent quality control measures in immunofluorescence studies, including batch testing and caution in antibody recycling. Ensuring consistent and reliable staining results is essential for maintaining reproducibility and interpretability in high-resolution neuroanatomical mapping.

Exclusion of the cerebellum

The present dataset provides detailed maps of PV, CB, and CR expression across the majority of cortical and subcortical regions in the tree shrew brain, with the exception of the cerebellum. This omission was due to technical constraints encountered during image acquisition and reconstruction. The methodology involved the reconstruction of large images from multiple FOVs; however, the extremely high expression of CB in the cerebellum resulted in image saturation, rendering the reconstructed images unsuitable for quantitative or structural analysis (Figure 5A). As a result, cerebellar sections were excluded from the final dataset. Despite this exclusion, prior studies have provided substantial anatomical characterization of the tree shrew cerebellum and partial delineation of the expression patterns of PV, CB, and CR (Bhagwandin et al., 2024; Ni et al., 2018a). These earlier findings complement the current dataset, providing a more complete picture of CaBP expression in the tree shrew brain. Addressing these technical challenges associated with high-signal regions in future imaging protocols may allow for the integration of cerebellar data into a comprehensive atlas of CaBP expression.

Limitations of the current dataset

Although the present dataset provides high-resolution two-dimensional (2D) maps of CaBP expression in the tree shrew cerebrum, three primary limitations restrict its broader utility and integration with existing neuroanatomical resources.

First, the current image set lacks standardized anatomical annotations. While every third section was stained with Nissl to visualize cytoarchitectonic features and enable alignment with the reference atlas (Zhou & Ni, 2016), variability in cutting angles across brains complicates accurate spatial mapping. The discrepancy between the sectioning planes and those of the atlas may lead to misinterpretations of the data. Nevertheless, this dataset is intended as a dynamic and evolving resource. Future versions will incorporate manual annotations and cross-reference representative sections with standard brain templates to improve interpretability and facilitate comparative analyses.

Second, the CaBPs immunolabeled in this study were predominantly expressed in inhibitory interneurons and certain excitatory neurons (Defelipe, 1997). While these markers have proven valuable for delineating and parcellating brain structures, they offer limited morphological and chemical information regarding excitatory projection neurons, which are central to long-range connectivity and cortical integration of sensory, motor, and cognitive functions (Florio, 2025). To address this gap, future work will apply neurotransmitter-specific markers to systematically label principal neuron populations across various cortical and subcortical regions (Rees et al., 2017), thereby expanding the molecular resolution of the atlas.

Third, accurate three-dimensional (3D) modeling from 2D atlas images requires block-face references to guide rigid multiscale registration for each brain section, a prerequisite for accurate alignment (Jeon et al., 2024). Given the absence of block-face images, our methodology cannot support 3D reconstruction of the tree shrew brain. This limitation significantly restricts the comprehensive visualization of how CaBPs are spatially expressed throughout the entire brain volume, which is essential for understanding the complex spatial relationship of different CaBP-expressing neurons.

Despite these limitations, the dataset remains a valuable foundational resource for investigating the region- and layer-specific architecture of CaBP expression in the tree shrew brain. Future studies employing advanced 3D imaging techniques, such as volumetric imaging with synchronized on-the-fly-scan and readout (ViSoR) (Wang et al., 2019) and fluorescent micro-optical sectioning tomography (fMOST) (Zhong et al., 2021), in combination with neurotransmitter-specific immunofluorescence, may provide a more holistic view of the cyto- and chemoarchitecture of the tree shrew brain in three dimensions at synaptic resolution. Such advancements will not only enhance our understanding of the structural organization of the tree shrew brain but also catalyze future neurophysiological and neuropathological research in this species.

DATA AVAILABILITY

The exported brain section images (one animal per folder, including a .ppt file with all images indexed) are available via the Science Data Bank (<https://doi.org/10.57760/sciencedb.23471>). Images with a resolution or brightness differing from the current version will be available in future versions, or from the corresponding author upon request.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

L.L. conceived and designed the study. R.Z. prepared the tissue samples and performed Nissl and immunofluorescence staining. R.Z., J.L.L., H.Y.Y., and X.N.Z. imaged the brain sections and organized the image data. L.L. verified the organized image data. J.L.L. performed the western blot experiments. Y.F.Y. quantified the CB, PV, and CR-ir signals. J.L.L., Y.F.Y., and L.L. created the figures. L.L. and X.C. wrote the manuscript. All authors read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

The authors express their gratitude to Cheng-Ji Li and Jia-Xue Jia from our research group, as well as staff members of the National Research Facility

for Phenotypic & Genetic Analysis of Model Animals (Primate Facility) (<https://cstr.cn/31137.02.NPRC>), for providing technical support and assistance in data collection and analysis. The authors also thank Robert Konrad Naumann from the Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences, for valuable suggestions regarding primary antibodies.

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