

Distribution and Neuronal Expression of Phosphatidylinositol Phosphate Kinase II γ in the Mouse Brain

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ABSTRACT

The role of cellular phosphatidylinositol 5-phosphate (PtdIns5P), as a signalling molecule or as a substrate for the production of small, compartmentalized pools of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂], may be dependent on cell type and subcellular localization. PtdIns5P levels are primarily regulated by the PtdIns5P 4-kinases (type II PIP kinases or PIP4Ks), and we have investigated the expression and localization in the brain of the least-studied PIP4K isoform, PIP4K γ . In situ hybridization and immunohistochemistry, using antisense oligonucleotide probes and a PIP4K γ -specific antibody, revealed that this isoform has a restricted CNS expression profile. The use of antibodies to different cell markers showed that this expression is limited

to neurons, particularly the cerebellar Purkinje cells, pyramidal cells of the hippocampus, large neuronal cell types in the cerebral cortex including pyramidal cells, and mitral cells in the olfactory bulb and is not expressed in cerebellar, hippocampal formation, or olfactory bulb granule cells. In neurons expressing this enzyme, PIP4K γ has a vesicular distribution and shows partial colocalization with markers of cellular compartments of the endomembrane trafficking pathway. The PIP4K γ isoform expression is established after day 7 of postnatal development. Overall, our data suggest that PIP4K γ may have a role in neuron function, specifically in the regulation of vesicular transport, in specific regions of the developed brain. *J. Comp. Neurol.* 517: 296–312, 2009. © 2009 Wiley-Liss, Inc.

Indexing terms: phosphoinositide; PtdIns5P 4-kinase; PIP4K; Purkinje; pyramidal; neuronal expression

Polyphosphoinositides (PIs) are emerging as important contributors to many physiological functions in cells and, as minor phospholipid components of cellular membranes, have important roles in signalling pathways. These are involved in diverse functions such as cell migration and proliferation, ion channel regulation, and membrane trafficking (for reviews see Di Paolo and De Camilli, 2006; Gamper and Shapiro, 2007; Irvine, 2003; Pendaries et al., 2005; Suh and Hille, 2005; Yin and Janmey, 2003).

The canonical pathway to the production of phosphatidylinositol 4,5-bisphosphate [(PtdIns(4,5)P₂)] in the cellular PI-cycle involves the 5-kinase activity of the type I PtdInsP kinases (PIP5Ks) using phosphatidylinositol 4-phosphate (PtdIns4P) as substrate (Ishihara et al., 1998). However, since the discovery of phosphatidylinositol 5-phosphate (PtdIns5P) in cells (Rameh et al., 1997), another potential route has been found via the 4-kinase activity of type II PtdInsP kinases (here referred to as PIP4Ks, consistent with their substrate specificity, although it should be noted that gene nomenclature still refers to them as PIP5K2s). The PIP4Ks are present as α , β , and γ isoforms in vertebrates, and there is evidence that each isoform has a specific compartmentalization in cells that could be intrinsic to its function. PIP4K β is present in the

nucleus (Bunce et al., 2008; Ciruela et al., 2000; Richardson et al., 2007), where it may be involved in stress responses (Gozani et al., 2003; Jones et al., 2006; Zou et al., 2007). PIP4K α , the most active isoform, is predominantly cytosolic (Hinchliffe et al., 1999; Wilcox and Hinchliffe, 2008) and can be recruited to the plasma membrane by interaction with a PIP5K (Hinchliffe et al., 2002).

The regulation of PtdIns(4,5)P₂ levels in the brain has been associated with numerous diseases, such as cancer, bipolar disorder, and channelopathies (for reviews see Endersby and

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Baker, 2008; Halstead et al., 2005; Jentsch et al., 2004). Phosphoinositide regulation of a wide variety of neuronal ion transporters (Gamper and Shapiro, 2007; Suh and Hille, 2005) suggests that aberrant PtdIns(4,5)P₂ metabolism may be involved in epilepsy and Alzheimer's disease (Jentsch et al., 2004; Landman et al., 2006) and may contribute to symptoms of Down's syndrome (Voronov et al., 2008). Specific genetic mutations have also linked PIP4K α with schizophrenia (Fedorenko et al., 2008), and disruption of the *PIP5K2B* gene is a common feature in neuroblastoma (Schleiermacher et al., 2005). All of the PIP4K and PIP5K isoforms are expressed in the brain (Akiba et al., 2002), and the PIP5K γ splice variants have been most extensively studied, having a major role in synaptic vesicle cycling (Di Paolo et al., 2004; Nakano-Kobayashi et al., 2007; Wenk et al., 2001). PIP5K is also involved in neuronal maintenance (Giudici et al., 2004), development and migration (Coulson et al., 2008; Wang et al., 2007), and neurite remodelling (van Horck et al., 2002), suggesting that polyphosphoinositides play significant roles in both neurogenesis and neuronal function, including learning and memory. The observed interaction of PIP4K and PIP5K isoforms (Hinchliffe et al., 2002) may also result in the recruitment of specific PIP4Ks required to maintain distinct phosphoinositide pools within different cellular compartments.

The PIP4K γ isoform has not yet been associated with a cellular function and is expressed predominantly in the kidney and brain (Akiba et al., 2002; Clarke et al., 2008; Itoh et al., 1998). In the present study, we have extensively investigated the localization of PIP4K γ in the brain by using a specific antibody to this isoform and found that it is remarkably confined to specific neuronal populations, where it is localized to vesicular structures. Developmental studies imply that this enzyme may be involved in neuronal function rather than in neurogenesis. Overall, our results suggest that PIP4K γ may have a specialized function in vesicle trafficking in specific neuronal cells.

MATERIALS AND METHODS

PIP4K γ cloning and expression

The *PIP5K2C* gene was amplified from a whole human brain marathon-ready cDNA library (Clontech, Mountain View, CA) and cloned into the plasmid expression vectors pET-32a (Novagen, Madison, WI) and pEGFP-C1 (Clontech) as previously described (Clarke et al., 2008). Full-length recombinant PIP4K γ was obtained by enterokinase cleavage of protein purified by TALON metal affinity resin (Clontech) from cell culture lysates of *Escherichia coli* BL21(DE3)pLysS transformed with the bacterial expression construct.

Endotoxin-free pEGFP-C1 plasmid constructs, purified from bacterial clones (Qiagen purification kit, Huntsville, AL), were used for eukaryotic expression. HeLa cells, maintained in DMEM (Gibco, Paisley, United Kingdom) supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μ g/ml streptomycin were transiently transfected for 24 hours using TransFectin reagent (Bio-Rad, Hercules, CA), following the manufacturer's protocol.

Tissue preparation

Animal care was in accordance with institutional and national guidelines, and all procedures were performed in accordance with Home Office guidelines, Animals (Scientific

Procedures) Act of 1986 under Home Office Project licence 80/1747. Samples were collected from P1, P7, P14, P21, P28, and adult male CD1 mice. Tissues used for cDNA library construction and Western blotting lysates were collected post-mortem from six animals and immediately frozen on dry ice. For imaging experiments, brains were removed from three mice that had been terminally anesthetized intraperitoneally with sodium pentobarbital and perfused transcardially with phosphate-buffered saline followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. After cryoprotection in 0.1 M phosphate buffer with 30% sucrose, brains were stored at -80°C .

RT-PCR and in situ hybridization

Full-length mRNA was extracted as described previously (Clarke et al., 2008), and cDNA libraries were made for each tissue by reverse transcription (Sprint Powerscript kit; Clontech). PCR amplification was performed with Taq DNA polymerase (Invitrogen, Paisley, United Kingdom) and primers specific to *PIP5K2A* (forward: 5'-AAGAGTCTGATGCCAAGAA-CCTGT-3' and reverse: 5'-TGCAGTGCAACTTAAGGATGGTAA-3'), *PIP5K2B* (forward: 5'-CATCCTCACAGAAGAACATGGC-3' and reverse: 5'-CCTGGTCATTACCGTCTCA-3'), *PIP5K2C* (forward: 5'-CATCTTCCACTGCTAATGTGTCTCC-5' and reverse: 5'-TTGAGTTATGGCTCTGACTCCTCTCT-3'), and β -actin (forward: 5'-GACGATATCGCTGCGCTGGT-3' and reverse: 5'-CCACGATGGAGGGGAATA-3'), generating PCR products of 108, 104, 95, and 100 bp, respectively. Reactions were cycled 30 times (96°C for 1 minute, 60°C for 1 minute, 72°C for 3 minutes) on a Techne Progene thermal cycler and the products analyzed by 2% (w/v) agarose gel electrophoresis.

For in situ hybridization, two oligonucleotide probes for *PIP5K2C* were 3'-tail labeled with [³⁵S]dATP (NEN, Hounslow, United Kingdom), hybridized with 20- μ m mouse tissue sections, and autoradiographed for 5 weeks, as previously described (Giudici et al., 2004). Results for the probe designed to the 3'-untranslated region (5'-GACTGGGTGGATTGAGTT-ATGGCTCTGACTCCTCT-3') were confirmed with the probe designed to the coding sequence (5'-ATAGGAGATAAGGAA-ACGGCCATCACTGCCTTCAG-3'). Both probes identified only *PIP5K2C* when BLAT searched (Kent, 2002) against the EMBL mouse database. Slides were treated with autoradiographic emulsion and counterstained with methyl blue after development (12 weeks). Control incubations contained an excess of unlabeled probe in addition to the labeled oligonucleotide.

Primary antibody characterization

PIP4K γ was detected using a custom rabbit polyclonal antibody raised against a synthetic peptide representing amino acids 333–352 from the variable region of murine PIP4K γ . This antibody specifically stains purified recombinant PIP4K γ protein and a 47-kDa molecular weight band from mouse brain by Western blotting, and this signal is abolished in blotting and immunocytochemistry experiments by preincubation with excess antigenic peptide (this study; Clarke et al., 2008). Mouse monoclonal antibody to α -tubulin (Sigma-Aldrich, Gillingham, United Kingdom; catalog No. T9026, lot No. 093K4880) was raised against purified chick brain tubulin and stains a single band of 57-kDa molecular weight by Western blot (Blöse et al., 1984). Mouse anti-calbindin D-28k monoclonal antibody [Swant, Bellinzona, Switzerland; code No. 300, lot No. 07(F)] was raised against purified chicken duodenum calbindin

D-28k (Celio et al., 1990). The antibody recognizes a single band of 28 kDa by Western blot, and no specific immunohistochemical staining is seen in the brain of a calbindin D-28k knockout mouse (manufacturer's technical information). Anti-parvalbumin mouse monoclonal antibody [Swant; code No. 235, lot No. 10-11(F)] was raised against purified carp muscle parvalbumin (Celio et al., 1988). The antibody specifically identifies 12-kDa, IEF-4.9 parvalbumin by 2D immunoblot and shows an absence of staining in the brain of a parvalbumin knockout mouse (manufacturer's technical information). Mouse monoclonal antibodies to GM130, early endosome antigen 1 (EEA1), and p115 (BD Transduction Laboratories, Oxford, United Kingdom; catalog Nos. 610822, 610456, and 612260) were raised to amino acids 869–982 of rat GM130, amino acids 3–281 of human EEA1, and amino acids 843–955 of rat p115, respectively. Each antibody stained a single band in rat brain by Western blot (130-kDa, 180-kDa, and 115-kDa molecular weights, respectively, by the manufacturer's technical information) and showed representative staining as previously reported (Allan et al., 2000; Clarke et al., 2008). The guinea pig polyclonal antibody against glial fibrillary acidic protein (GFAP; Advanced Immunochemical, Long Beach, CA; catalog No. 031223) was raised against purified human GFAP, and this antibody stained cells with the classic morphology and distribution of fibrous astrocytes in our experiments. Mouse monoclonal anti-neuronal class III β -tubulin (clone TUJ1; Stem Cell Technologies Inc., Vancouver, British Columbia, Canada; catalog No. 01409) was raised against rat brain microtubules and specifically stained neuronal and not glial cell β -tubulin (manufacturer's technical information). Mouse monoclonal anti-KDEL antibody (Stressgen, Ann Arbor, MI; catalog No. SPA-827) was raised against amino acids 649–654 of rat Grp78 (BiP) and recognized Grp78 (78 kDa) and Grp94 (94 kDa) bands in mouse brain by Western blot (manufacturer's technical information). Antibodies to golgin 160 (goat polyclonal), lysosome-associated membrane protein 1 (LAMP-1; mouse monoclonal), and LAMP-2 (rabbit polyclonal) were from Santa Cruz Biotechnology (Santa Cruz, CA; catalog Nos. sc-79966, sc-17768, and sc-5571, respectively). The antibody to golgin 160 was raised against the 14 N-terminal amino acids of human golgin 160 and stained cells with a pattern previously observed (Hicks and Machamer, 2002). Anti-LAMP antibodies were raised against amino acids 1–228 and 1–207 of human LAMP-1 and LAMP-2, respectively, and stained single bands in cell lysates by Western blot (manufacturer's technical information) and cells with a pattern previously observed (Sarafian et al., 1998). Mouse monoclonal anti-Golgi 58K protein (Sigma; clone 58K-9; catalog No. G2404) was raised against rat liver Golgi 58K protein, stained a single 58-kDa band in rat brain, and gave a characteristic Golgi staining by immunofluorescence (Bloom and Brashear, 1989). A mouse monoclonal anti-calnexin antibody and rabbit polyclonal antibodies to catalase, mannose-6-phosphate receptor, and mannosidase II were obtained from Abcam Ltd. (Cambridge, United Kingdom; catalog Nos. ab31290, ab1877, ab12894, and ab12277, respectively). Anti-calnexin was raised to calnexin of human origin and stained cells with a typical ER pattern (manufacturer's technical information). Anti-catalase antibody was raised against bovine liver catalase and gave a single band of 65 kDa by Western blot in a number of tissue lysates, as well as a characteristic peroxiso-

mal staining pattern (manufacturer's technical information). Antibodies to mannose-6-phosphate receptor (amino acids 700–800) and mannosidase II were raised against protein of human origin and showed characteristic immunofluorescent staining patterns by the manufacturer's technical information.

Western blotting

Whole mouse brains were dissected under a binocular microscope and separated into nine regions, and protein lysates were prepared in RIPA buffer (150 mM sodium chloride, 50 mM Tris pH7.4, 1 mM EDTA, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS) with 10 mM tetrasodium pyrophosphate, 10 mM sodium fluoride, 17.5 mM β -glycerophosphate, and 100 μ l/ml protease inhibitor cocktail (Sigma-Aldrich; catalog No. P8340) as previously described (Clarke et al., 2008). Protein samples were resolved by 8–10% SDS-PAGE, and Western blots were carried out using antibodies to PIP4K γ (1:1,000) or α -tubulin (1:5,000). Horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG or anti-mouse IgG, 1:5,000) and SuperSignal West Dura substrate (Pierce Protein Research Products, Rockford, IL) were used to detect positive signal via the manufacturer's protocol. Samples were dephosphorylated by treatment with 1 unit calf intestinal alkaline phosphatase (Promega, Madison, WI) per microgram protein for 60 minutes at 37°C. PIP4K γ antibody for control blots was preincubated with excess antigenic peptide for 30 minutes at room temperature. Autoradiographic images were quantified by comparing average integrated pixel intensities (Image J; NIH, Bethesda, MD) for a defined area with background subtraction. These values were normalized to the loading control.

Immunohistochemistry

Tissue was sectioned (20 μ m) on a cryostat (Leica, Wetzlar, Germany) at -20°C and either mounted onto SuperFrost Plus Gold slides (Menzel-Glaser, Braunschweig, Germany) and stored at -80°C or stored as free-floating sections (0.1% sodium azide in PBS) at 4°C . Sagittal and horizontal brain sections for immunoperoxidase staining were pretreated for 20 minutes with 5% hydrogen peroxide in 20% methanol, blocked for 2 hours (1% fish skin gelatin, 0.3% Triton X-100 in PBS), and exposed to anti-PIP4K γ antibody (1:500, 24 hours at 4°C). Detection was carried out with biotinylated anti-rabbit IgG secondary antibody (1:200), Vectastain ABC reagent, and DAB substrate (Vector Laboratories, Burlingame, CA). Free-floating stained sections were mounted onto gelatinized slides, air dried, and treated with ethanol (2 minutes in 70% solution, 2 minutes in 95% solution, 3 minutes in 100%) and then with HistoClear reagent (Sigma-Aldrich). Coverslips were permanently mounted with DPX reagent (Sigma-Aldrich).

Brain sections for fluorescent labeling experiments were pretreated and incubated as previously described (Clarke et al., 2008) using anti-PIP4K γ (1:750), anti-calbindin D-28k (1:1,000), anti-parvalbumin (1:1,000), anti-GM130 (1:50), anti-p115 (1:100), anti-TUJ1 (1:1,000), anti-calnexin (1:1,000), and anti-GFAP (1:2,500). Fluorescent dye-conjugated secondary antibodies (Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 546 anti-rabbit, anti-mouse, and anti-guinea pig IgG, 1:1,000; Molecular Probes, Paisley, United Kingdom) were used for fluorescence microscopy. Whole sections for brain region reference were stained with 435/455 blue fluorescent Nissl stain (NeuroTrace; 1:300; Molecular Probes) with the manufacturer's specifications. Coverslips were mounted with ProLong

Gold antifade reagent (Molecular Probes). PIP4K γ antibody for controls was preincubated with 50 μ g of recombinant protein for 30 minutes at room temperature. Brain areas were identified by using a stereotactic atlas (Lein et al., 2007).

Immunocytochemistry

Primary hippocampal cultures were isolated and maintained as a source of pyramidal neurons (Koizumi et al., 1999), and primary cerebellar granule cells were isolated and maintained as described by Giudici et al. (2004). These cells were derived from rat brain, and PIP4K γ from this source is 98.1% similar at the protein level and immunoreactive with the anti-PIP4K γ antibody. Glass coverslips seeded with neuronal cultures or transiently transfected HeLa cells expressing GFP-tagged constructs were fixed in 4% PFA for 30 minutes on ice. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 minutes and blocked with 4% fish skin gelatin (Sigma-Aldrich) in PBS for 30 minutes, then incubated for 60 minutes with anti-PIP4K γ (1:1,000), anti-GM130 (1:50), anti-p115 (1:100), anti-EEA1 (1:50), anti-mannose-6-phosphate receptor (1:500), anti-GRP78 (1:250), anti-58K (1:250), anti-mannosidase II (1:200), anti-golgin 160 (1:250), anti-catalase (1:200), anti-LAMP-1 (1:250), anti-LAMP-2 (1:250), or anti-tubulin (1:400). After incubation with secondary antibody (Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 546 anti-mouse or anti-goat IgG in 2% gelatin in PBS) or Alexa Fluor 568-conjugated phalloidin (Molecular Probes), coverslips were mounted onto slides with ProLong Gold antifade reagent (Molecular Probes) and analyzed as described below. For permeabilization experiments, cells were treated (prefixation) with 20 μ g/ml digitonin or 1% Triton X-100 for 5 minutes (in 80 mM PIPES pH 6.8, 4% PEG 8000, 1 mM MgCl₂, 1 mM EGTA).

Microscopy and figure preparation

Light microscopy was performed with an Axioskop II microscope (Carl Zeiss GmbH, Munich, Germany), and images were saved as TIF format in AxioVision software (Carl Zeiss GmbH). Confocal microscopy was carried out by sequential scanning with a Leica TCS SP5 laser scanning confocal microscope running LAS AF software (Leica Microsystems Ltd., Milton Keynes, United Kingdom). Images were tinted and output as TIF files from Image J.

Autoradiographic images developed from in situ hybridization and electrophoresis gels were scanned and output as TIF files by using a Hewlett-Packard scanjet 8200 scanner and VueScan software (Hamrick Software, Pheonix, AZ). Figures were prepared for publication in Adobe Photoshop (Adobe Systems, San Jose, CA), adjusting image brightness and contrast only.

RESULTS

PIP5K2C has an expression profile in mouse brain

Previous studies have suggested that *PIP5K2* isoforms are differentially transcribed according to tissue (Akiba et al., 2002; Clarke et al., 2008; Itoh et al., 1998), and RT-PCR with isoform-specific primers confirmed this (Fig. 1). Among the tissues tested, *PIP5K2A* mRNA levels were comparatively higher in mouse spleen, *PIP5K2B* levels were higher in muscle, and *PIP5K2C* was the predominant isoform in kidney (this study; Clarke et al., 2008). However, mRNA for each isoform

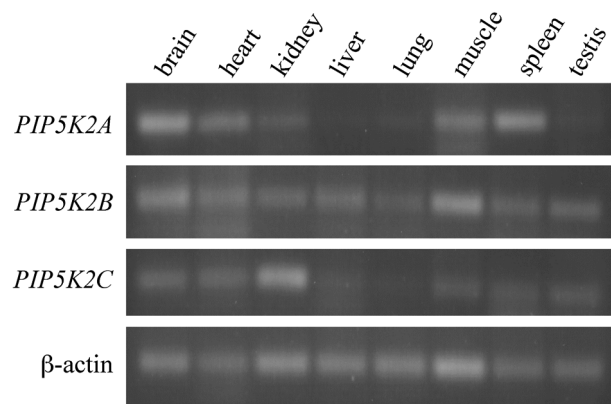


Figure 1. *PIP5K2* gene expression in adult mouse tissue cDNA libraries. Comparative levels of *PIP5K2A*, *PIP5K2B*, *PIP5K2C*, and β -actin control mRNA in different tissues as determined by RT-PCR (representative of results from cDNA libraries generated from three different animals).

was also detected, at different levels, in brain (Fig. 1). Tissue analysis by in situ hybridization with two different *PIP5K2C* probes confirmed that transcription of this isoform was up-regulated in discrete regions of the brain, compared with a control tissue (Fig. 2). Silver grain labeling of *PIP5K2C* mRNA in brain suggested that expression was confined to cells on the boundary of the molecular and granular layers of the cerebellum and in large cell bodies in the hippocampus (CA1–CA3), cerebral cortex, and olfactory bulb (Fig. 2).

Endogenous PIP4K γ is expressed in specific brain regions

With a polyclonal peptide antibody specific to the variable region of PIP4K γ (Fig. 3; Clarke et al., 2008), our results indicated that this PIP4K isoform is differentially expressed within the mouse brain. Crude dissection of mouse brain tissue and Western blotting of the protein lysates allowed direct comparison of PIP4K γ levels in these regions. Equivalent samples (by total protein loading) were standardized to the loading control (α -tubulin), and quantification indicated that PIP4K γ levels were higher in the cerebral cortex, hippocampus, spinal cord, and olfactory bulb, with significant expression also seen in the cerebellum and brainstem (Fig. 4). Tissue lysates from these regions were further investigated to resolve multiple PIP4K γ -immunoreactive bands. Western blotting of samples with comparative PIP4K γ levels indicated the presence of three distinct bands at 47, 48, and 49 kDa (Fig. 5). The two lower molecular weight bands were present in all of the six regions, whereas the 49-kDa band was equivalent in the cerebellum and hippocampus; significantly reduced in the cortex, brainstem, and spinal cord; and completely absent from the olfactory bulb (Fig. 5). Treatment of samples with phosphatase removed the 48- and 49-kDa bands, suggesting that these were phosphorylated forms of PIP4K γ (Fig. 5). Itoh et al. (1998) previously identified one phosphorylated form of PIP4K γ , and our results suggest that the presence of a third immunoreactive band could indicate a form of the protein that is specific to distinct areas of brain tissue, in contrast to other tissues studied (Clarke et al., 2008).

Immunostaining of endogenous PIP4K γ in whole-brain sections allowed the further identification of regions showing

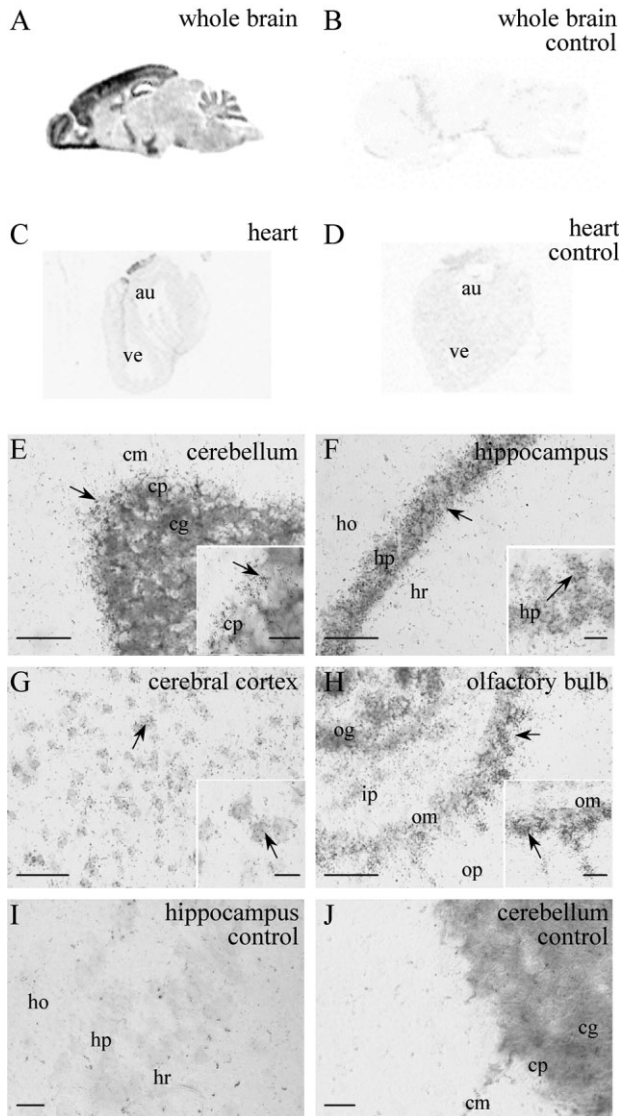


Figure 2. *PIP5K2C* gene expression in adult mouse tissues by in situ hybridization. With *PIP5K2C*-specific probes on 20- μ m mouse tissue sections, positive signal is spatially distributed in brain (A) but absent from heart tissue (C; not to scale). Control sections of brain (B) and heart (D) were incubated with excess of unlabeled probe, and identical results were obtained with three sections from two animals. Autoradiographic emulsion staining of hybridization slides (counterstained with methyl blue), shows *PIP5K2C* mRNA labeled with silver grains (E–J). Positive cells (arrows) are seen in the cerebellum (E, inset is Purkinje cell layer), hippocampal field CA1 (F, inset is stratum pyramidale), cerebral cortex (G, inset is layer V), and olfactory bulb (H, inset is mitral cell layer). Controls incubated with excess cold probe (I, J) were negative. au, Heart auricle; ve, heart ventricle; cm, molecular layer; cp, Purkinje layer; cg, granular layer (all cerebellar); ho, stratum oriens; hp, stratum pyramidale; hr, stratum radiatum (all hippocampal); op, outer plexiform layer; om, mitral cell layer; ip, inner plexiform layer; og, granule cell layer (all olfactory bulb). Scale bars = 40 μ m in E–I; 10 μ m in J and insets.

localized expression of PIP4K γ (Fig. 6). Detailed examination of sections, labeled with anti-PIP4K γ by immunohistochemical or immunofluorescent methods, indicated expression of this iso-

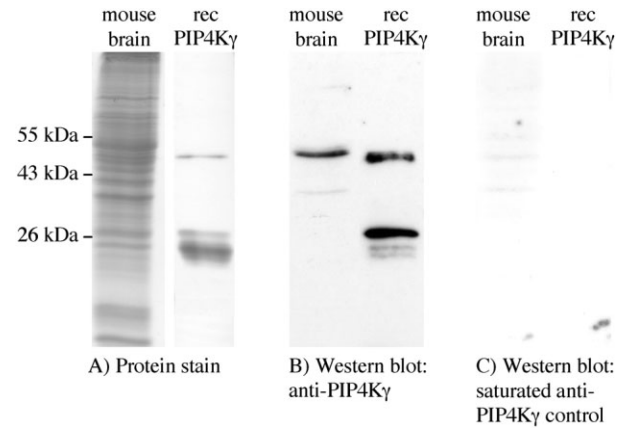


Figure 3. Specificity of antiphosphatidylinositol 5-phosphate 4-kinase γ (PIP4K γ) antibody to PIP4K γ in mouse brain. A: SDS-PAGE of total proteins from 50 μ g whole mouse brain lysate (Coomassie stained) and 50 ng of purified recombinant (rec) PIP4K γ (silver stained) showing mature protein of 47 kDa and degradation products at 26 kDa. B: Western blot of these proteins using anti-PIP4K γ antibody, showing single band specificity in brain lysate. C: Control Western blot using anti-PIP4K γ antibody preincubated with antigenic peptide. Results are representative of at least three experiments.

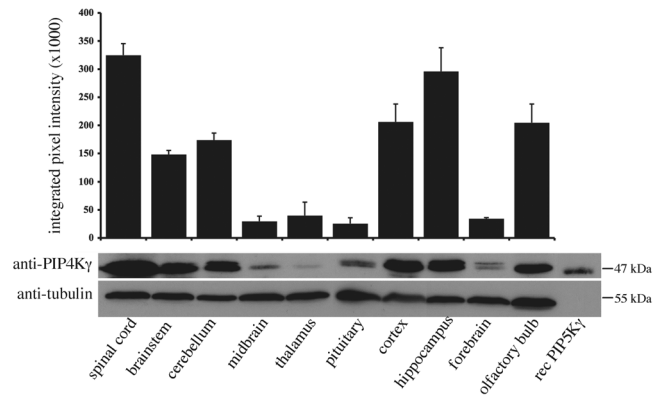


Figure 4. Endogenous PIP4K γ expression in adult mouse brain. Quantification of PIP4K γ levels in lysates (30 μ g) from 10 different adult mouse CNS regions probed with PIP4K γ -specific antibody (n = 3). Integrated pixel intensities for each band are shown (\pm SEM), normalized to the loading control (α -tubulin). Recombinant (rec) PIP4K γ was included as a positive size control.

form in isolated cells (including pyramidal and basket cells) throughout the cerebral cortical layers (Fig. 7A–C), and this expression did not extend into the corpus callosum or cingulum region or into the caudate-putamen. High levels of expression were seen in the hippocampal formation, primarily in the stratum pyramidale and extending into the stratum radiatum of CA1–CA3, and were excluded from the dentate gyrus (Fig. 7E–G). Within the cerebellar cortex, PIP4K γ was observed in large cell bodies of the Purkinje layer, extending into the dendritic trees of these cells, but was absent from the granular layer (Fig. 7M–O). PIP4K γ expression was excluded from the majority of the medulla (Fig. 8E–G) but was present in the dorsal horn of the spinal cord (Fig. 8A–C) and the spinal

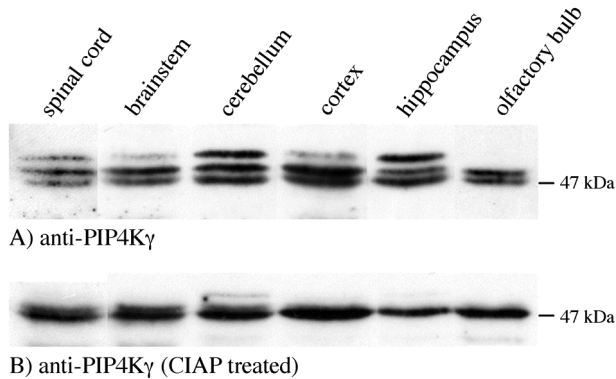


Figure 5. Multiple forms of PIP4K γ are differentially expressed in brain regions. **A:** PIP4K γ was detected as three different bands (47, 48, and 49 kDa) by 8% SDS-PAGE and Western blotting. The 49-kDa band was differentially expressed in cerebral cortex, hippocampus, spinal cord, cerebellum, and brainstem and was not present in the olfactory bulb. **B:** Only the 47-kDa band remained after lysates were pretreated with calf intestinal alkaline phosphatase (CIAP) and Western blotted with anti-PIP4K γ antibody. Results are representative of at least three experiments.

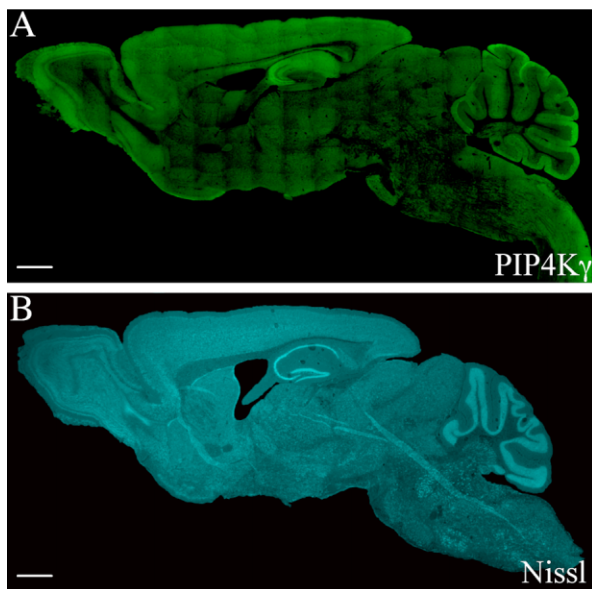


Figure 6. PIP4K γ expression in different regions of the adult mouse brain. PIP4K γ was detected in whole sagittal brain sections (approximately lateral 0.48 mm) by immunofluorescence with anti-PIP4K γ antibody (**A**). Staining indicated differential expression levels of PIP4K γ in different brain regions. A similar, fluorescent Nissl-stained section is included for morphological reference (**B**). Images are representative of a minimum of six stained sections from three different mice. Scale bars = 1 mm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

trigeminal nucleus and tract (data not shown). Positive signal was also observed throughout the cervical, thoracic, and lumbar spinal cord regions (data not shown) and large neuronal cell bodies in the dorsal root ganglia (see Fig. 12C). Lower level expression was observed in the pontine gray, and the

remainder of the pons, midbrain, thalamus, and hypothalamus were negative (Figs. 7I–K, 8I–K). In the olfactory bulb, PIP4K γ expression was seen in cells extending from the mitral cell layer to the olfactory nerve layer and into the anterior olfactory nucleus but excluded from the granular cell layer (Fig. 8M–O).

Expression of PIP4K γ is restricted to specific neurons

Further characterization of PIP4K γ -positive cells in the adult mouse brain indicated that this phosphoinositide kinase is preferentially expressed in cells that are costained with neuronal cell markers (Figs. 9–11; Supp. Info. Figs. 1, 2). Use of a fluorescent counterstain to identify neurons by abundance of Nissl substance (Quinn et al., 1995) colocalized PIP4K γ to these cells in each region studied (Figs. 9C,F,O,R, 10C,L, 11C), and this was confirmed by colocalization with the neuronal class III β -tubulin marker TUJ1 (Supp. Info. Fig. 1). PIP4K γ was not expressed in granule cells in the cerebellum (Fig. 10C) or in the dentate gyrus (Fig. 9O). Costaining with an antibody to the glial cell marker GFAP (Figs. 9I,U, 10F,O) suggested that the PIP4K γ signal was excluded from these cells. Furthermore, staining of pyramidal cell cultures (derived from hippocampus) for endogenous PIP4K γ indicated that these neurons were PIP4K γ positive (Fig. 11I), whereas primary cell cultures of granule cells (derived from cerebellum) showed little endogenous signal (Fig. 11L). Within the cerebral cortex, hippocampus, and olfactory bulb, the subset of neurons expressing PIP4K γ was excluded from the subpopulations expressing the calcium-binding protein calbindin D-28k (Figs. 9L,X, 10R), and parvalbumin (Supp. Info. Fig. 2C,F,L), a calcium-binding marker of GABAergic interneurons, which is predominantly expressed by chandelier and basket cells in the cortex (Hendry et al., 1989). In the cerebellum, both of these markers were present in PIP4K γ -positive Purkinje cells (Fig. 10I; Supp. Info. Fig. 2I), and, within the spinal cord, the population of PIP4K γ -positive neurons included subpopulations positive for both calbindin D-28k (Fig. 11F) and parvalbumin (Supp. Info. Fig. 2O).

PIP4K γ has a distinct subcellular compartmentalization

Confocal microscopy at high resolution and costaining with a range of markers for different subcellular compartments allowed the identification of a distinct vesicular location for PIP4K γ . In all of the neurons observed expressing PIP4K γ , endogenous signal was present in the cell body and dendritic projections but was mostly excluded from the nucleus (Fig. 12). Subcellular localization was cytoplasmic and partially perinuclear, with clear, punctate staining (Fig. 12A–C), similar to that observed in kidney cells (Clarke et al., 2008). Costaining of endogenous PIP4K γ in brain tissue with markers for the endoplasmic reticulum and Golgi apparatus suggested that the vesicular PIP4K γ compartment might partially colocalize with one of these structures (Fig. 12F,I,L).

To investigate this potential association in more detail, HeLa cells were transiently transfected with constructs expressing PIP4K γ fused to the GFP reporter protein, because very low endogenous levels of PIP4K γ were observed in neuronal cell lines such as SH-SY5Y (data not shown). Cells were permeabilized to reduce the levels of free cytosolic protein produced by overexpression, as judged by the reduction of overexpressed control GFP levels (Supp. Info. Fig. 3). Treat-

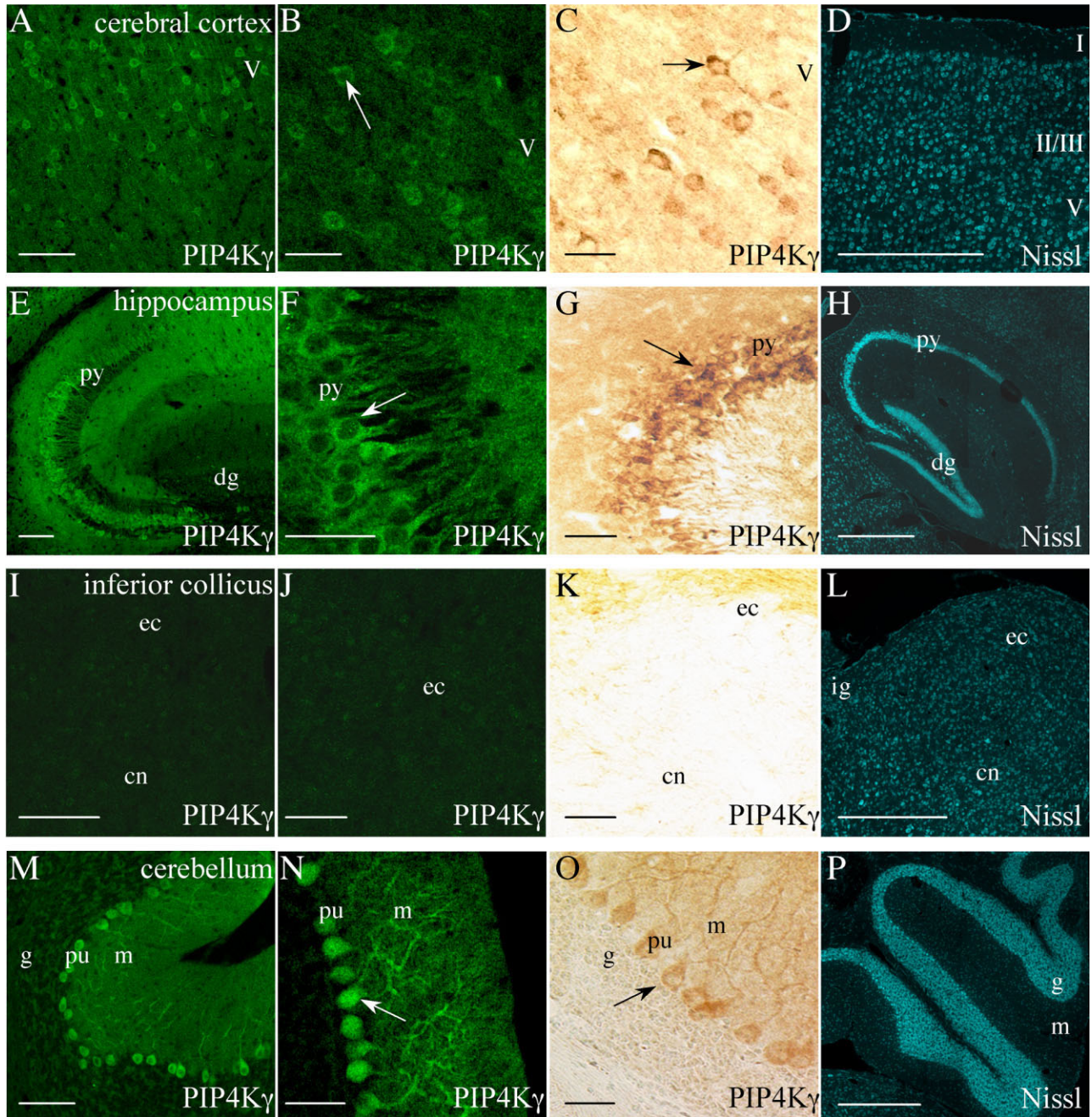


Figure 7. A–P: Localization of PIP4K γ in adult mouse brain regions. Positive PIP4K γ signal (arrows) was observed in the cerebral cortex (A–C), CA1–CA3 of the hippocampus (E–G) and cerebellum (M–O). No significant signal was observed in the inferior colliculus (I–K). Sagittal brain sections were stained by immunofluorescent (A,B,E,F,I,J,M,N) and also immunohistochemical (C,G,K,O) methods. Fluorescent Nissl-stained images are included for reference (D,H,L,P). Images are representative of a minimum of six stained sections from three different mice. I, II/III, V, cerebral cortex layers; dg, dentate gyrus; py, hippocampal pyramidal cells; ec, external cortex of the inferior colliculus; cn, central nucleus of the inferior colliculus; ig, intermediate gray layer of the superior colliculus; pu, cerebellar Purkinje cells; g, cerebellar granular layer; m, cerebellar molecular layer. Scale bars = 100 μ m in A,E,I,M; 50 μ m in B,F,J,N; 20 μ m in C,G,K,O; 500 μ m in D,H,L,P. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

ment with the glycoside digitonin (Lorenz et al., 2006) significantly reduced cytosolic levels of GFP and all three PIP4K isoforms, and stronger detergent extraction completely removed GFP signal, but nuclear PIP4K β and vesicular PIP4K γ

remained resistant to this procedure, suggesting a stronger association with a cellular compartment (Supp. Info. Fig. 3). A proportion of PIP4K γ remains localized to the nucleus during extraction, which may also be significant. Permeabilization

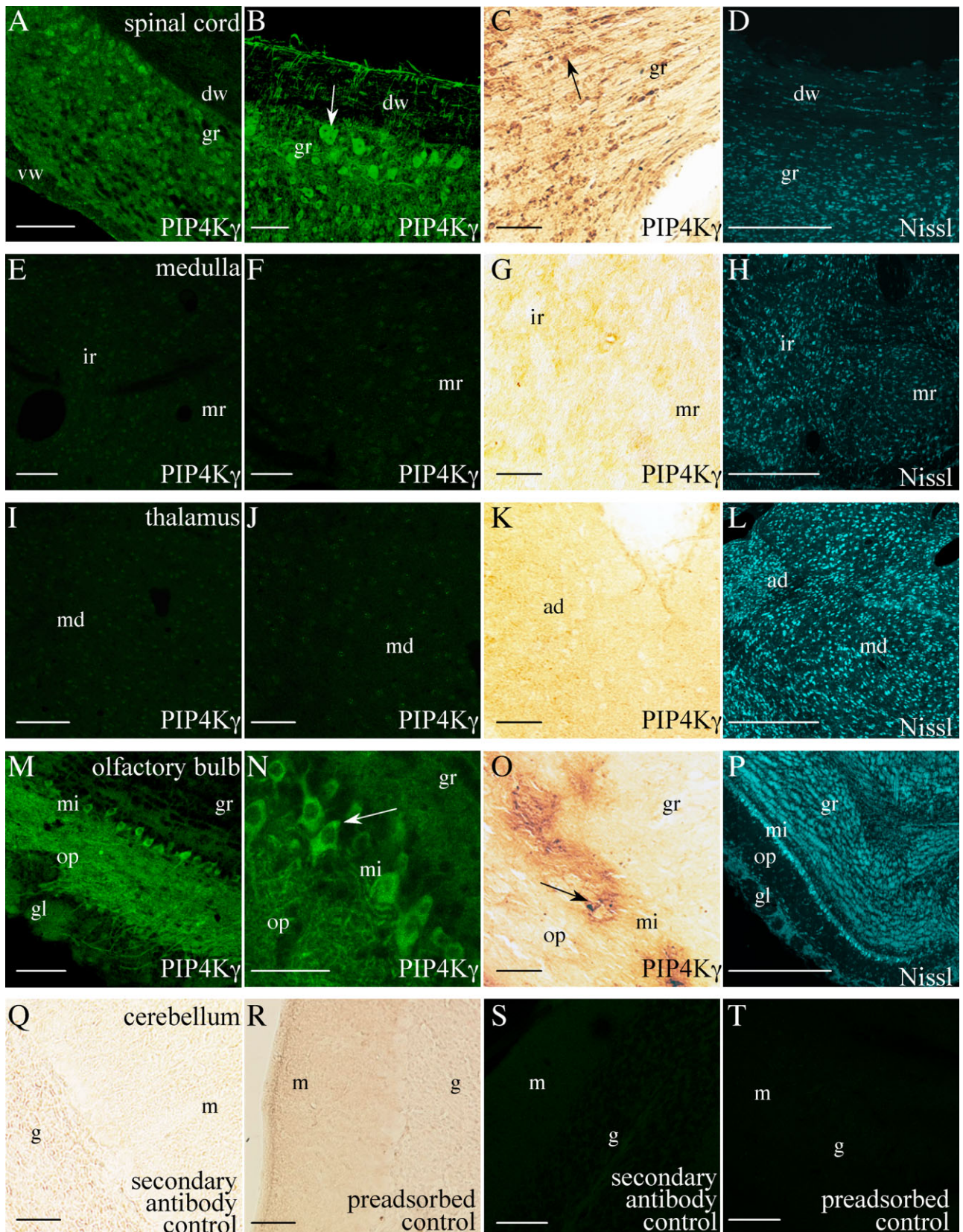


Figure 8. A–T: Localization of PIP4K γ in adult mouse brain regions. Positive PIP4K γ signal (arrows) was observed in the cervical spinal cord (longitudinal section, A–C) and olfactory bulb (M–O). No significant signal was observed in other areas such as the medulla (E–G) or thalamus (I–K). Sagittal brain sections were stained by immunofluorescent (A, B, E, F, I, J, M, N, S, T) and also immunochemical (C, G, K, O, Q, R) methods. Fluorescent Nissl-stained images are included for reference (D, H, L, P). Controls for immunochemical and immunofluorescent labeling (incubated with secondary antibody only or using preadsorbed anti-PIP4K γ) are also included (Q–T). Images are representative of a minimum of six stained sections from three different mice. dw, Dorsal white matter of the spine; vw, ventral white matter of the spine; gr, spinal gray matter; ir, intermediate reticular nucleus of the medulla; mr, medullary reticular nucleus; md, mediodorsal/paracentral thalamic nuclei; ad, anterodorsal thalamic nucleus; gl, olfactory glomerular layer; op, olfactory outer plexiform layer; gr, olfactory granule cell layer; mi, olfactory mitral cells; g, cerebellar granular layer; m, cerebellar molecular layer. Scale bars = 100 μ m in A, E, I, M; 50 μ m in B, F, J, N; 20 μ m in C, G, K, O, Q–T; 500 μ m in D, H, L, P. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

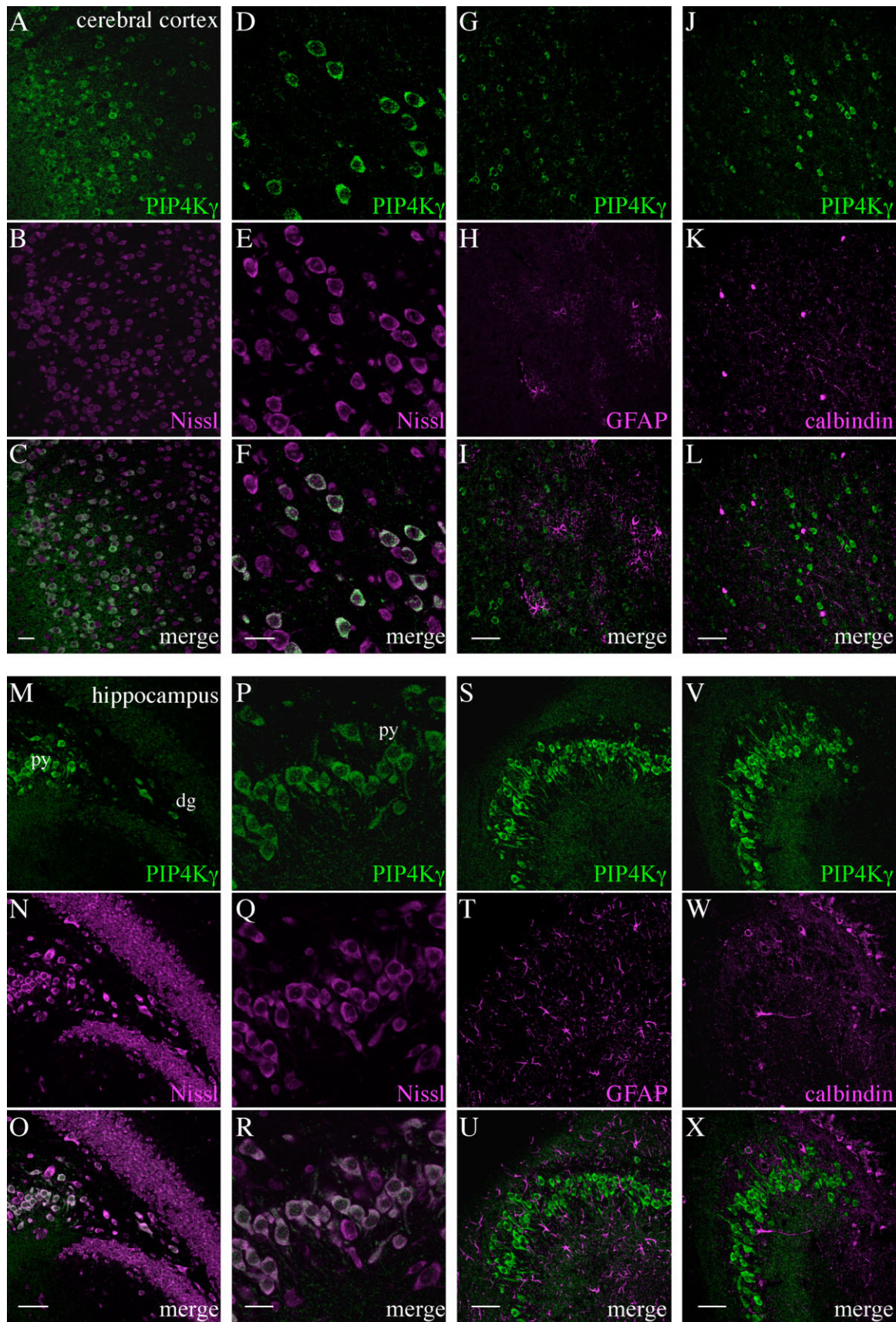


Figure 9.

Identification of cell types expressing PIP4K γ in the adult mouse cerebral cortex and hippocampus. Endogenous PIP4K γ was detected in cerebral cortex (A–L) and hippocampus (M–X) by using anti-PIP4K γ antibody (green). Counterstaining for Nissl substance (B,E,N,Q) indicated that PIP4K γ was expressed in neuronal cells (C,F,O,R). PIP4K γ was excluded from glial cells stained with anti-glial fibrillary acidic protein (GFAP; G–I,S–U). PIP4K γ was not expressed in cells that were positive for the neuronal cell marker calbindin D-28k in these regions (J–L,V–X). Images are representative of three stained sections. py, Stratum pyramidale of hippocampus; dg, dentate gyrus. Scale bars = 50 μ m in C (applies to A–C); 20 μ m in F (applies to D–F); 50 μ m in I (applies to G–I); 50 μ m in L (applies to J–L); 50 μ m in O (applies to M–O); 20 μ m in R (applies to P–R); 50 μ m in U (applies to S–U); 50 μ m in X (applies to V–X).

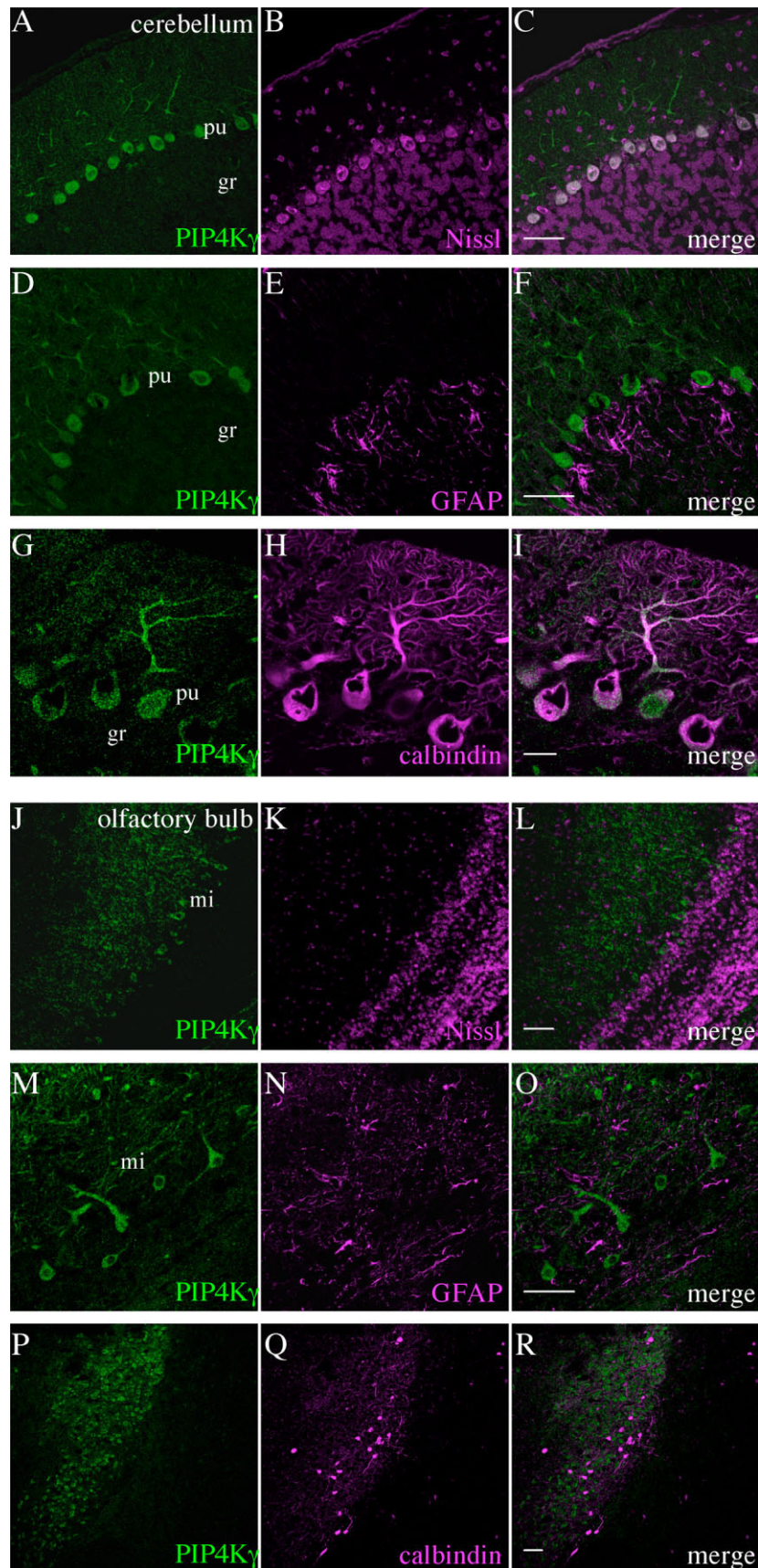


Figure 10.

Identification of cell types expressing PIP4K γ in the adult mouse cerebellum and olfactory bulb. PIP4K γ (green) was detected in the cerebellum (A–I) and olfactory bulb (J–R). Signal was coincident with fluorescent Nissl stain (A–C, J–L) and was excluded from glial cells stained with anti-GFAP (D–F, M–O), indicating that PIP4K γ was expressed in neuronal cells. PIP4K γ was expressed in cells that were positive for the neuronal cell marker calbindin D-28k in the cerebellum (G–I) but not in the olfactory bulb (P–R). Images are representative of three stained sections. gr, Cerebellar granular layer; pu, cerebellar Purkinje layer; mi, mitral cell layer of olfactory bulb. Scale bars = 50 μ m in C (applies to A–C); 50 μ m in F (applies to D–F); 20 μ m in I (applies to G–I); 50 μ m in L (applies to J–L); 20 μ m in O (applies to M–O); 50 μ m in R (applies to P–R).

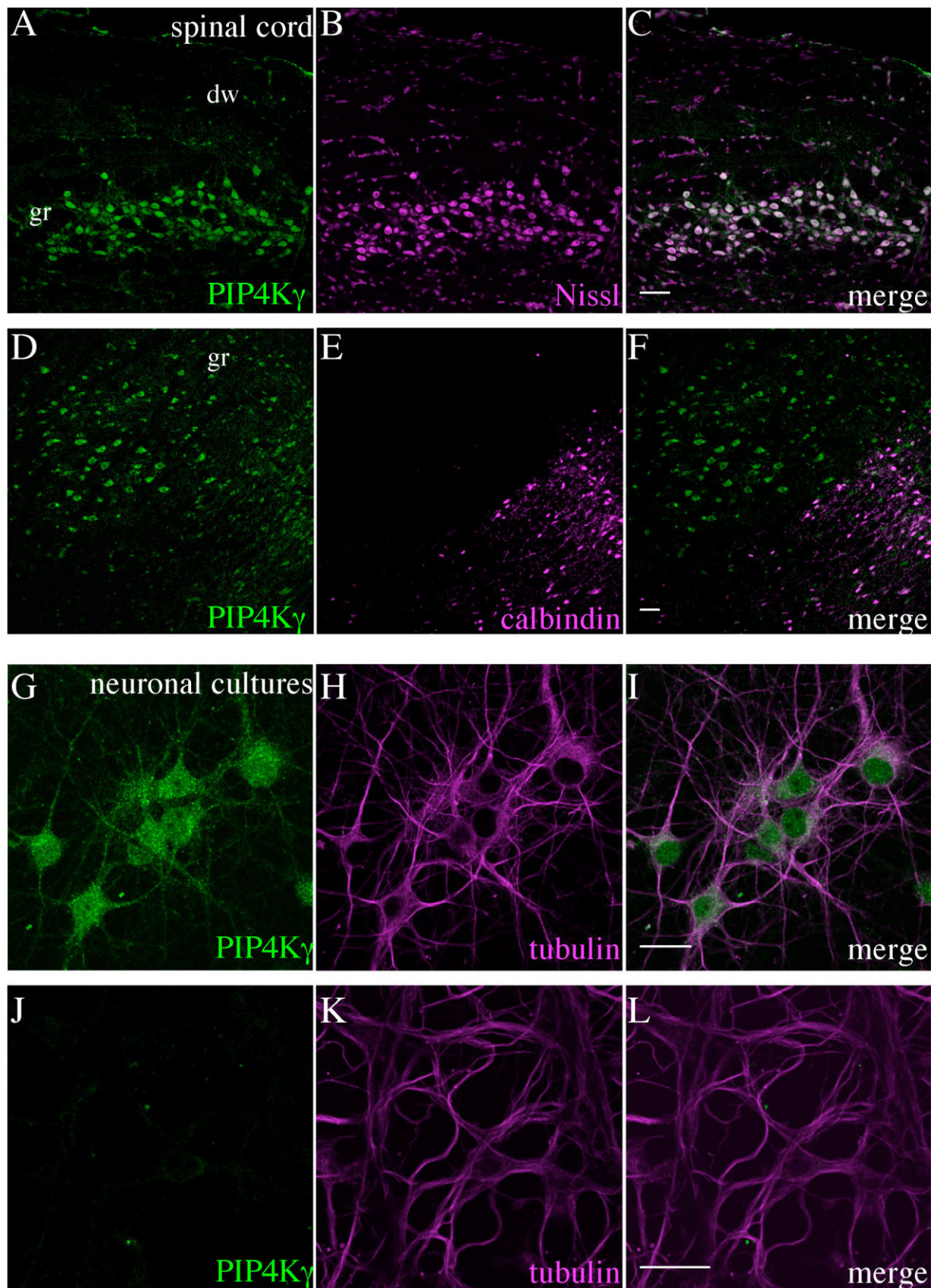


Figure 11. Identification of cell types expressing PIP4K γ in the adult mouse spinal cord and in primary neuronal cultures. Endogenous PIP4K γ (green) was detected in neurons in the spinal cord (A-F) by fluorescent costaining for Nissl substance (A-C) and was also seen in the subpopulation of neurons positive for calbindin D-28k (D-F). Hippocampal primary cell cultures enriched for pyramidal cells expressed PIP4K γ (G-I), whereas cultures enriched for cerebellar granule cells did not (J-L). Images for (A-F) are representative of three stained sections. dw, Dorsal white matter of the spine; gr, spinal gray matter. Scale bars = 50 μ m in C (applies to A-C); 50 μ m in F (applies to D-F); 20 μ m in I (applies to G-I); 20 μ m in L (applies to J-L).

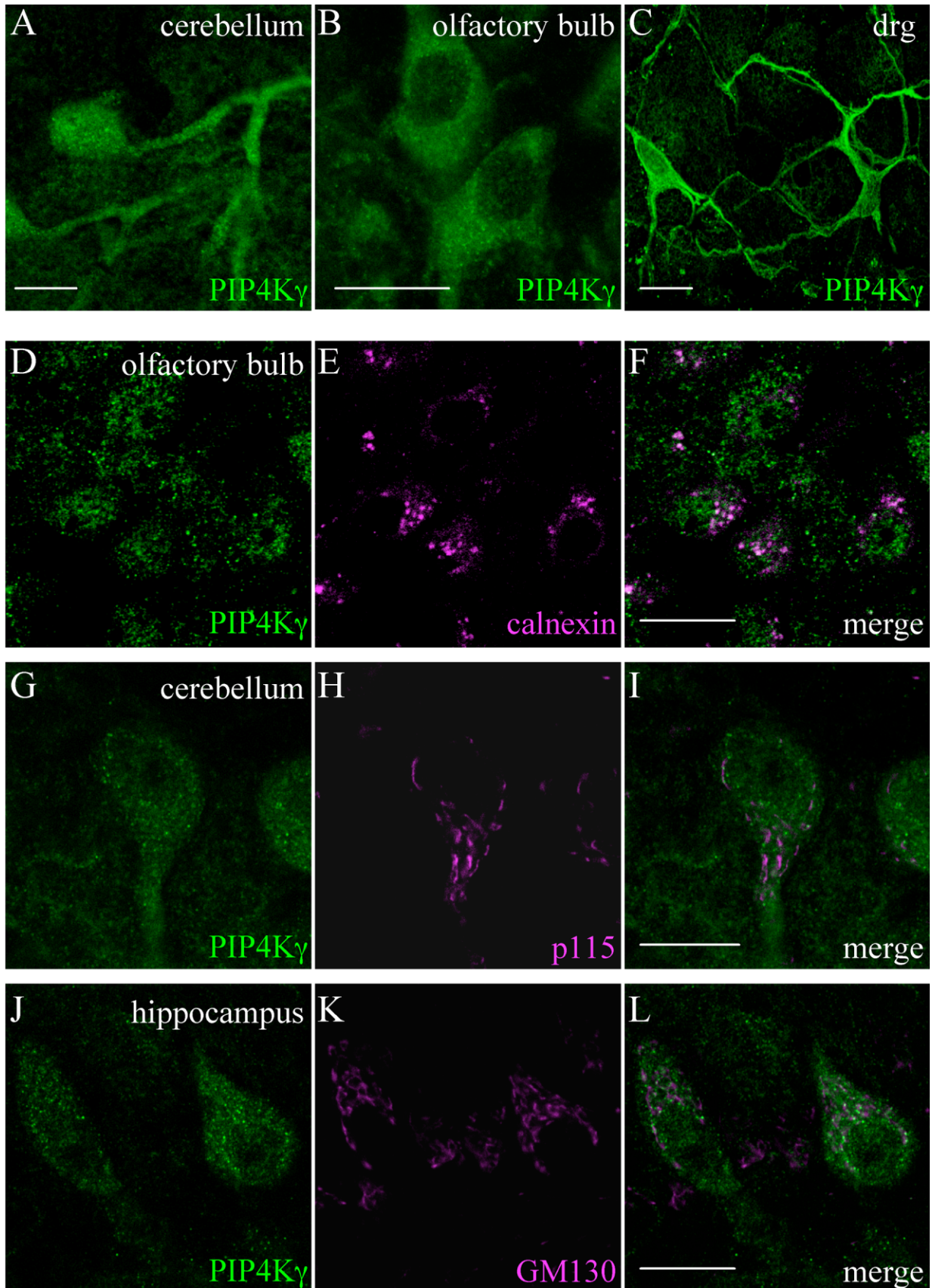


Figure 12.

Subcellular localization of PIP4K γ in neurons. Endogenous PIP4K γ was detected in the cell body and dendritic trees of cerebellar Purkinje cells (A), olfactory bulb mitral cells (B), and large neurons in dorsal root ganglion (drg) preparations (C). PIP4K γ was mostly excluded from the nucleus and was present in a vesicular compartment in the cytoplasm. This compartment seemed to have a stronger, but partial, colocalization with the *cis*-Golgi structural marker GM130 (J-L) than with the ER resident calnexin (D-F) or the -ergic marker p115 (G-I), although this could not be fully characterized in brain tissue. Images are representative of results from at least three stained sections. Scale bars = 20 μ m in A-C; 20 μ m in F (applies to D-F); 20 μ m in I (applies to G-I); 20 μ m in L (applies to J-L).

did not affect the staining of other cellular compartment markers. PIP4K γ was not seen to colocalize with the endoplasmic reticulum resident Grp78 (Supp. Info. Fig. 5C), in accordance with previous observations (Clarke et al., 2008), or the -ergic Golgi vesicle docking protein p115 (Fig. 13C). Partial colocalization after digitonin treatment was observed with the Golgi markers GM130 (Fig. 13F), 58K, mannosidase II, and golgin 160 (Supp. Info. Fig. 4) and the endosomal markers EEA1 and mannose-6-phosphate receptor (Fig. 13I,L), but not the lysosomal markers LAMP-1 and LAMP-2 or the peroxisomal marker catalase (Supp. Info. Fig. 5F,I,L). However, the PIP4K γ protein remaining after stronger detergent treatment did not completely localize to any of these compartments (Fig. 13O,R,U,X; Supp. Info. Fig. 6).

PIP4K γ expression during postnatal development

Examination of similar brain sections in a murine postnatal developmental series indicated that the onset of PIP4K γ expression from neurons was between postnatal days 7 and 14 (Fig. 14). Establishment of Purkinje cells and defined dendritic trees in the Purkinje layer of the cerebellum was apparent at P14, and staining for endogenous PIP4K γ indicated that this enzyme was also expressed by Purkinje cells at this time. Spaces in the Purkinje cell layer may be due to naturally occurring neuronal cell death (Madalosso et al., 2005). Similar results were observed for PIP4K γ expression in hippocampal pyramidal cells and the mitral cell layer of the olfactory bulb (Fig. 14).

DISCUSSION

Here we have investigated the expression of the PIP4K isoforms in the murine brain, with specific reference to PIP4K γ . We have further characterized the restricted localization of this PIP4K in different regions of the brain, identified the neuronal cells expressing the protein, and suggested a subcellular compartmentalization that has implications for the physiological function of this isoform.

PIP4K expression and neuronal localization

Gene expression profiles for the *PIP5K2s*, collated as MIAME-compliant microarray data on the EMBL ArrayExpress Warehouse database (Parkinson et al., 2007), support the observations that there are tissue-specific differences in isoform abundance (Clarke et al., 2008). PIP4K α is the most abundant isoform in spleen and blood components, such as platelets (Hincliffe et al., 1998; Morris et al., 2000), whereas PIP4K β levels are enhanced in skeletal muscle (Castellino et al., 1997), and PIP4K γ is most abundant in kidney (Clarke et al., 2008; Itoh et al., 1998). However, all three PIP4K isoforms are also transcribed in specific areas of the mammalian brain but have different spatial distributions (Akiba et al., 2002), and our *in situ* hybridization studies conform to the *PIP5K2C* gene expression pattern reported in the Allen Brain Atlas (Lein et al., 2007). Our detection of endogenous PIP4K γ protein in brain is consistent with these transcription levels, and the results from Western blotting of nine different brain regions concur with the results obtained from immunohistochemistry on brain sections, with the majority of protein expression observed in the cerebral cortex, hippocampus, cerebellum, and olfactory bulb. We also note a significant level of expression in the spinal cord, and specifically in the large neuronal bodies

found in the dorsal root ganglia. Within the brain regions, PIP4K γ was expressed in specific neuronal cell populations, and expression in granule cells and neuroglia was negligible. These neurons could be identified as Purkinje cells in the cerebellum, pyramidal cells in the hippocampus, and cells in the mitral cell layer of the olfactory bulb. The expression of PIP4K γ was also seen to be homogeneous in these areas and not restricted to neuronal populations within specific hippocampal subfields or cerebellar compartments. The population of PIP4K γ -positive neuronal cells in the cerebral cortex and spinal cord was a defined subset, based on our marker studies, and requires further characterization.

PIP4K γ and neuronal development

Gene ontology (GO) terms (Ashburner et al., 2000) for neuron-enriched gene expression in the CNS suggest that there are categories involved with synaptic function, neuronal cell signalling and metabolism, neurogenesis, and neuronal transport, including vesicle-mediated trafficking (Lein et al., 2007). Recent reports have also implicated PtdIns5P (Xie et al., 2005) and PI-metabolizing enzymes (Coulson et al., 2008; Polleux et al., 2002) in neurogenesis and neuronal development and migration. Our results indicate that, in the developing postnatal mouse brain, PIP4K γ expression is first observed after P7 and is established at P14, a developmental stage at which neurogenesis and neuronal migration and differentiation of Purkinje cells in the cerebellum, pyramidal cells in the hippocampus, and mitral cells in the olfactory bulb should be completed (Finlay and Darlington, 1995; Hatten, 1999; Madalosso et al., 2005). The lack of observed PIP4K γ expression before postnatal stage P7 would suggest that this PIP4K does not participate in phosphoinositide-regulated events prior to this, but this has yet to be determined for embryonic developmental stages.

PIP4K γ subcellular distribution and neuronal function

The expression of PIP4K γ in a neuronal subpopulation from P14 to adult would suggest that this enzyme might have a specific involvement with neuronal function. Our observation that PIP4K γ is present in large neuronal cell bodies, as well as throughout their dendritic projections, would suggest a central rather than a peripheral role. Our results also suggest that, as with specialized cells in the mammalian nephron (Clarke et al., 2008), PIP4K γ is localized to a vesicular cytoplasmic compartment that is partially coincident with components of the endomembrane system. The absence of a recognized signal peptide within the PIP4K γ sequence suggests that this protein would not be directly targeted to the ER or plasma membrane, in accordance with our colocalization studies, and hence may be associated with the external surface of a trafficking compartment (Alvarez et al., 2001). Our evidence of partial colocalization of PIP4K γ with Golgi complex and endosomal markers may suggest that this protein is associated with specific transport vesicles that shuttle between different endosomal organelles. The presence of PtdIns5P in trafficking vesicles is suggested by the substrate preference of a Golgi-localized lipid phosphatase, PLIP (Merlot et al., 2003), and the involvement of this phosphoinositide with vesicle translocation to the plasma membrane (Sbrissa et al., 2004). Furthermore, a recent comparative genomics study suggests that PtdIns5P is regulated by protein complexes, involving a ki-

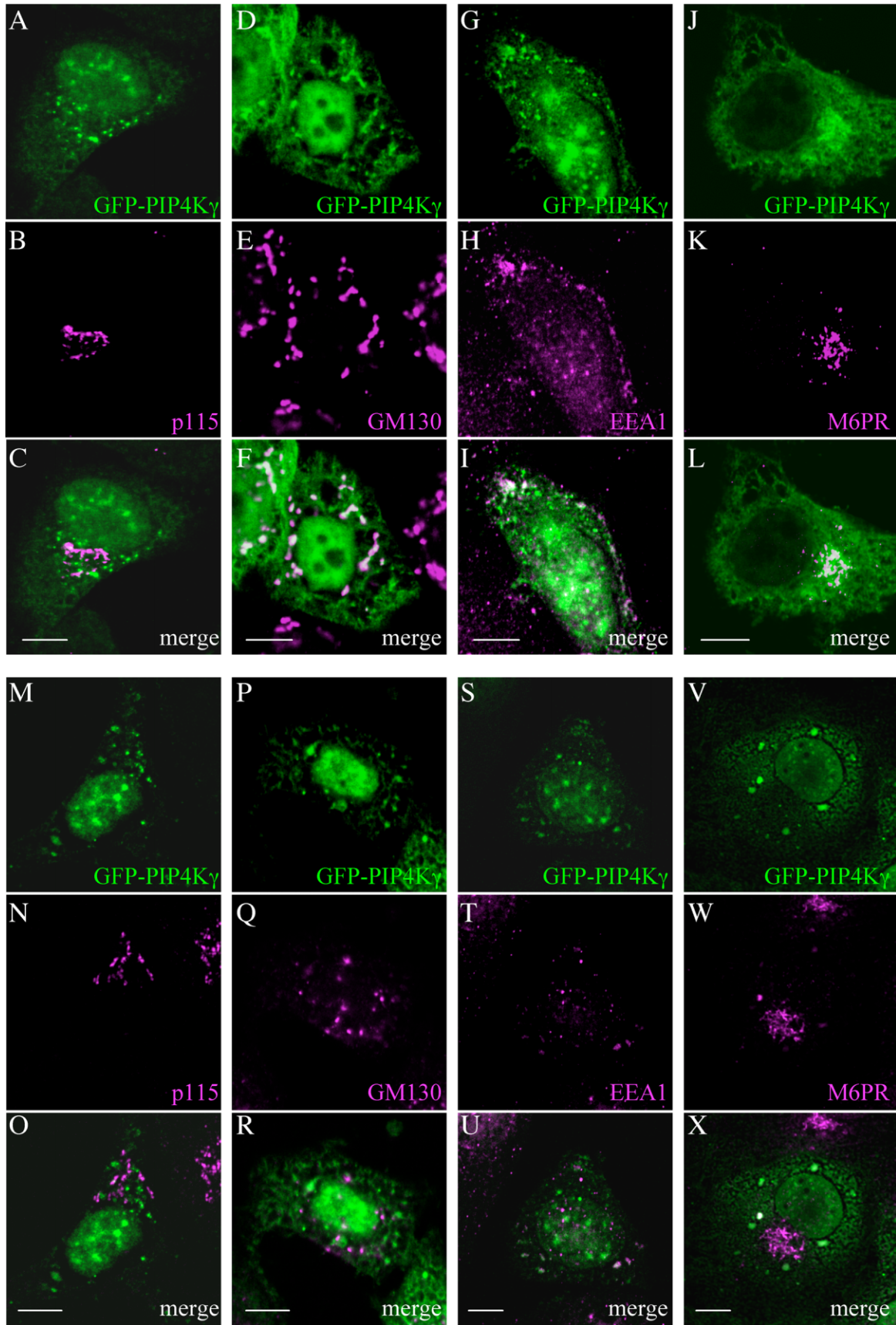


Figure 13. PIP4K γ associates with a vesicular compartment that partially colocalizes with components of the endomembrane system. Expression of GFP-tagged PIP4K γ in HeLa cells and mild permeabilization with digitonin (A–L) indicated a vesicular cytoplasmic pool of PIP4K γ that partially colocalized with the Golgi marker GM130 (D–F) and the endosomal markers EEA1 (G–I) and mannose-6-phosphate receptor (M6PR; J–L) but not with the -ergic marker p115 (A–C). Results observed after permeabilization with Triton X-100 (M–X) suggested that the main pool of PIP4K γ did not remain localized to any of these markers (O,R,U,X). Scale bars = 10 μ m in C (applies to A–C); 10 μ m in F (applies to D–F); 10 μ m in I (applies to G–I); 10 μ m in L (applies to J–L); 10 μ m in O (applies to M–O); 10 μ m in R (applies to P–R); 10 μ m in U (applies to S–U); 10 μ m in X (applies to V–X).

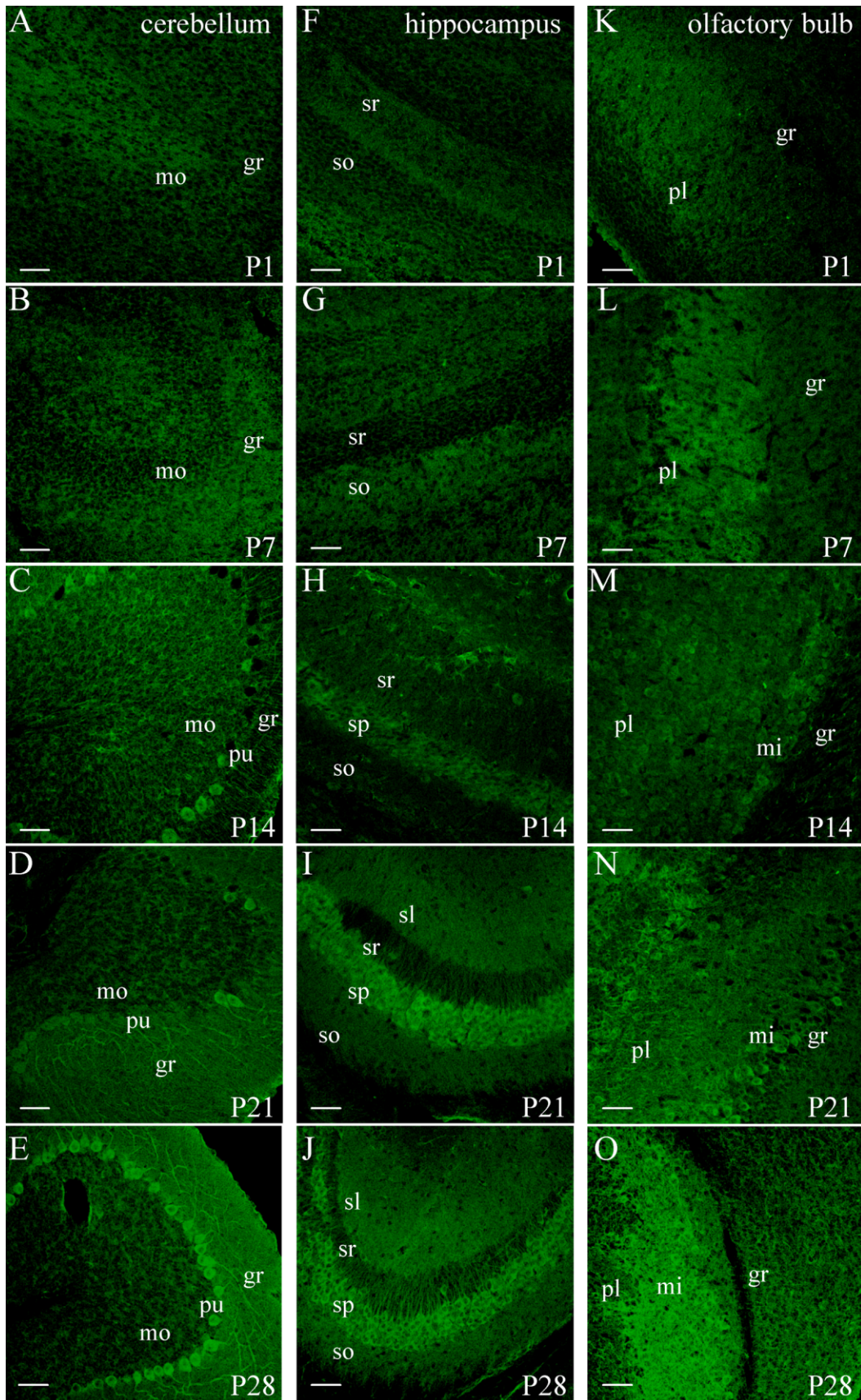


Figure 14.

Expression of PIP4K γ in the developing mouse brain. Immunohistochemical staining of mouse brain sections at different postnatal development stages (P1–P28 days after birth) indicated the levels of PIP4K γ (green) detected in three different regions; cerebellum (A–E), hippocampal field CA3 (F–J), and olfactory bulb (K–O). Brain regions are pictured in the same orientation, and images are representative of two animals at each developmental stage. mo, Molecular layer; pu, Purkinje layer; gr, granular layer (cerebellum); so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum; sl, stratum lacunosum-moleculare (hippocampal formation); pl, plexiform layer; mi, mitral cell layer; gr, granule layer (olfactory bulb). Scale bars = 50 μ m. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

nase such as PIP4K, and has a role in membrane trafficking (Lecompte et al., 2008). PIP4K γ has also been associated with actin remodelling during endocytic transport (Pelkmans et al., 2005), suggesting that modification of the PtdIns5P signal or synthesis of PtdIns(4,5)P₂ in a specific compartment might have a role in this process. Recombinant PIP4K γ has no detectable intrinsic kinase activity, although enzyme recovered from a mammalian expression system is seen to be active (Itoh et al., 1998). Our previous study suggested that this activity could also be attributable to the ability of PIP4K γ to associate with other, more active PIP4K isoforms (Clarke et al., 2008) and that PIP4K γ might function to recruit this activity to a specific cellular compartment. It should also be noted that there have been several recent reports of the regulation of various membrane channels by phosphoinositides (for reviews see Gamper and Shapiro, 2007; Suh and Hille, 2005), which could be coincident with delivery of the channel complexes to the plasma membrane. Although there is as yet no direct evidence for the involvement of PIP4K γ in these processes, it remains an intriguing possibility that the PIP4Ks are involved in neuronal function via one of these mechanisms.

The restricted expression of PIP4K γ in the CNS may be significant within the context of the specialized function of different neurons. It remains to be seen whether the role of phosphoinositide signalling involving this kinase is a common feature to the population of neurons that express it or whether there are different, neuron-specific functions of PtdIns5P, or localized pools of PtdIns(4,5)P₂, in these cells.

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