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# Differential mRNA Expression and Protein Localization of the SAP90/PSD-95–Associated Proteins (SAPAPs) in the Nervous System of the Mouse

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ABSTRACT

The supramolecular anchoring/signaling complex at the postsynaptic density of glutamatergic synapses has been proposed to play a key role in regulating synaptic function and plasticity. One class of proteins present in the complex is the SAP90/PSD-95-associated protein family (SAPAPs). The SAPAPs, identified by their direct interaction with PSD-95 family proteins, were initially proposed to function in the anchoring/signaling complex as linker proteins between glutamate receptor binding proteins and the cytoskeleton. However, recent studies have indicated that the SAPAPs also bind to signaling molecules and may thus have multiple roles at synapses. Four homologous genes encoding SAPAP proteins have been previously identified. As a first step toward understanding the physiological function of the SAPAPs, we have investigated in detail, at both the mRNA and protein levels, the localization of the individual SAPAP genes in the adult murine nervous system. We find that the SAPAP mRNAs are highly, yet differentially, expressed in many regions of the brain, including the hippocampus and cerebellum. Furthermore, SAPAP3 mRNA is targeted to dendrites, whereas SAPAP1, -2, and -4 mRNAs are detected mainly in cell bodies. The SAPAP proteins are localized at synapses in a manner consistent with mRNA expression. Surprisingly, in addition to glutamatergic synapse localization, antibody staining also reveals that the SAPAP proteins are localized at cholinergic synapses, including neuronal cholinergic synapses and the neuromuscular junction. Together, these results indicate that the SAPAPs are general components of excitatory synapses and that each of these proteins may perform a distinct function. J. Comp. Neurol. 472:24–39, 2004. 

**Indexing terms: GKAP; DAP; cholinergic synapses; neuromuscular junction; postsynaptic density; glutamatergic synapses**

Proper synaptic function depends on the precise assembly of neurotransmitter receptors and signaling molecules in the postsynaptic density. The identification of the PSD-95 family of proteins and the subsequent chain-link cloning of associated proteins by using the yeast two-hybrid system has led to a well-developed understanding of the molecular composition of the glutamatergic postsynaptic density (Scannevin and Huganir, 2000; Sheng, 2001). The wealth of known protein-protein interactions has in turn led to the proposal of a model depicting multimodular scaffolding proteins as organizers of a macromolecular signaling complex in the glutamatergic postsynaptic density (Craven and Bredt, 1998; Garner et al., 2000; Scannevin and Huganir, 2000; Sheng and Kim, 2002; Sheng and Pak, 2000).

At the center of the scaffolding model is the PSD-95 family of proteins. These proteins contain multiple protein-protein interaction domains, including three PDZ domains, an SH3 domain, and a guanylate kinase-like

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(GK) domain (Garner et al., 2000). The N-terminal PDZ domains of PSD-95 family proteins bind N-methyl-D-aspartate (NMDA) receptors (Kennedy, 2000; Sheng, 2001), whereas the C-terminal GK domain of PSD-95 family proteins interacts with many classes of intracellular proteins. One such class of intracellular proteins is the SAPAP/GKAP/DAP family of proteins [SAP90/PSD-95–associated protein/guanylate kinase domain-associated protein/discs-large associated protein, henceforth referred to as SAPAP; (Kim et al., 1997; Satoh et al., 1997; Takeuchi et al., 1997)]. SAPAP proteins have been proposed to provide a link between the PSD-95 family of proteins and the actin cytoskeleton through interactions with the Shank/ProSAP proteins, which in turn bind the actin-binding protein Cortactin (Du et al., 1998; Boeckers et al., 1999; Naisbitt et al., 1999; Sheng and Kim, 2000; Scannevin and Huganir, 2000). Additionally, Shank/ProSAP also binds to Homer, which interacts with metabotropic glutamate receptors (Tu et al., 1999). Therefore, in the current scaffolding model, PSD-95/SAPAP/Shank interactions play an important role in organizing the large postsynaptic signaling complex at glutamatergic synapses (Scannevin and Huganir, 2000; Sheng and Kim, 2000; Kennedy, 2000; McGee and Bredt, 2003).

The SAPAP family of proteins was originally cloned in yeast two-hybrid screens based on direct interactions with the GK domains of SAP90/PSD-95 and hDlg/SAP97 (Kim et al., 1997; Satoh et al., 1997; Takeuchi et al., 1997). There are four genes in the family (SAPAP1–4), each exhibiting approximately 50% identity at the amino acid level (Takeuchi et al., 1997). Although the SAPAPs were initially depicted in the scaffolding model as simple linkers acting between receptors/PSD-95 and the cytoskeleton, recent protein-protein interaction experiments have indicated that the SAPAPs may play a direct role in intracellular signaling through interactions with proteins such as neuronal nitric oxide synthase (nNOS) and nArgBP2, a protein interacting with the Abl/Arg nonreceptor tyrosine kinases (Kawabe et al., 1999; Haraguchi et al., 2000).

It has also recently been shown in cultured neurons that SAPAP protein levels in the postsynaptic density are regulated by activity, indicating a possible role for these proteins in synaptic plasticity (Ehlers, 2003). Additional experiments in cultured neurons have shown that at least one of the SAPAP proteins is among the earliest components to arrive at developing synapses (Rao et al., 1998). However, apart from a brief report that SAPAP1 expression may potentiate NMDA receptor currents in heterologously expressing oocytes (Yamada et al., 1999), there have been no direct studies of the SAPAP proteins' physiological function.

Similarly, the expression patterns of the SAPAP genes have only been minimally investigated. Northern blot analysis suggests that SAPAP1 and -2 are expressed predominantly in neural tissue (Naisbitt et al., 1997; Takeuchi et al., 1997). In situ hybridization has indicated that SAPAP1 transcription is strong in the cortex, hippocampus, and cerebellum of rat brain (Naisbitt et al., 1997). SAPAP2-4 mRNA localization has not been investigated. By using antibodies that cross-react with multiple SAPAP gene products (Allison et al., 2000), protein expression was observed in the rat hippocampus, cortex, and cerebellum (Naisbitt et al., 1997). However, the protein localizations of the individual SAPAPs have not been investigated.

As a first step in investigating the functions of the different SAPAPs, we have analyzed in detail the in vivo expression patterns of the SAPAP mRNAs and proteins in the adult murine nervous system. We report that each of the SAPAPs are expressed in the nervous system and are located predominantly at synapses. We also find differential expression of the SAPAPs in many regions of the nervous system, consistent with the possibility that the SAPAPs play distinct physiological roles from one another. Finally, we report that in addition to glutamatergic localization, SAPAP proteins are found at cholinergic synapses, including both neuronal cholinergic synapses and the neuromuscular junction, suggesting that the SAPAPs play a broader role at excitatory synapses than previously considered.

*Abbreviations*

| | |
|---|---|
| BTX | α-bungarotoxin |
| EPL | external plexiform layer |
| DG | dentate gyrus |
| GABAR | γ-aminobutyric acid A receptor |
| GC | granule cell |
| GCL | granule cell layer |
| GL | glomeruli |
| GlyR | glycine receptor |
| INL | inner nuclear layer |
| IPL | inner plexiform layer |
| LM | lacunosum moleculare |
| MC | mitral cell |
| MF | mossy fiber |
| ML | molecular layer |
| OL | oriens layer |
| ONL | outer nuclear layer |
| OPL | outer plexiform layer |
| PC | Purkinje cell |
| PG | periglomerular cell |
| PSD | postsynaptic density |
| SAP | synapse-associated protein |
| SAPAP | SAP90/PSD-95–associated protein |
| SPM | synaptic plasma membrane |
| SR | stratum radiatum |

## MATERIALS AND METHODS

### In situ hybridization

Mouse sequences encoding SAPAP1 (nucleotide #1209-2685, Genbank accession #AY243846), SAPAP2 (nucleotide #1078-2504, Genbank accession #AY243847), SAPAP3 (nucleotide #1072-2624, Genbank accession #AY243848), and SAPAP4 (nucleotide # 1658-2967, Genbank accession #AY243849) were amplified by polymerase chain reaction (PCR) and subcloned into pBluescript II SK+ vector (Stratagene, Palo Alto, CA). Plasmids were linearized with restriction enzymes that generated 5′ overhangs. Digoxigenin (DIG)-labeled RNA probes were synthesized with the MAXIscript in vitro RNA synthesis kit (Ambion, Austin, TX) by using T3 and T7 RNA polymerases. DIG-labeled probes were quantified by dot blot and probed with alkaline phosphatase-conjugated anti-DIG antibody (Roche, Indianapolis, IN).

P21-28 ICR mice were euthanized with halothane in accordance with NIH and institutional protocols. Brains were dissected, embedded in OCT compound (TissueTek, Torrance, CA), and frozen in 2-methylbutane chilled in liquid nitrogen. Sagittal sections (20 μm) were cut on a

cryostat, placed on Superfrost Plus slides (Fisher Scientific, Swanee, GA), and dried at room temperature for 1 hour.

For hybridization, sections were fixed with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) solution (150 mM NaCl, 12.1 mM $Na_2HPO_4$, 2.9 mM $KH_2PO_4$, pH 7.5) for 10 min at 4°C and washed 3× 3 minutes with PBS. Sections were then acetylated for 10 minutes at room temperature in TEA buffer (295 ml $H_2O$, 4 ml triethanolamine, 0.525 ml 12.1 M HCl; mix well and add 0.75 ml acetic anhydride just before use). After washing 3 times with PBS, sections were incubated with hybridization buffer (50% formamide, 5× SSC [750 mM NaCl, 75 mM Na-Citrate], 5× Denhardt's solution [Sigma, St. Louis, MO], 500 μg/ml salmon sperm DNA, 250 μg/ml yeast tRNA) for 2 hours at room temperature and then with DIG-labeled RNA probes (1–2 mg/ml in hybridization buffer; heat at 70°C for 10 minutes to denature, and cool on ice) overnight at 70°C in a chamber humidified with hybridization buffer.

After hybridization, sections were first rinsed 2× 5 minutes with 5× SSC followed by 4× 1 hour with 0.2× SSC at 70°C. Sections were then cooled to room temperature and incubated with blocking buffer (10% normal sheep serum, 0.2% blocking reagent; Roche, Indianapolis, IN) for 1 hour followed by incubation with alkaline phosphatase-conjugated anti-DIG antibody (1:2000; Roche) overnight at 4°C. Sections were washed (4× 10 minutes) with TBS (150 mM NaCl, 10 mM Tris-HCl, pH 7.5) and incubated with color detection buffer (100 mM NaCl, 50 mM $MgCl_2$, 0.24 mg/ml levamisole, 100 mM Tris-HCl, pH 9.5) for 5 minutes. Color reactions were developed in the presence of nitroblue tetrazolium (NBT; 0.35 mg/ml) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP; 0.175 mg/ml) in color detection buffer. Color reactions were terminated by incubating sections in TE buffer (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). Sections were mounted with 0.2 μm filtered 90% glycerol/$H_2O$ for imaging.

## Antibody production and purification

The sequences of the rat SAPAP 1, -3, and -4 genes (Takeuchi et al., 1997) were used to design PCR primer sets amplifying a region of each gene whose encoded amino acid sequence was unique. The amplified regions corresponded to the following mouse amino acid sequences: SAPAP1, 584-662 (accession #AY243846); SAPAP3, 560-641 (accession #AY243848); and SAPAP4, 612-689 (accession #AY243849). The primers were then used to amplify corresponding SAPAP regions from mouse brain cDNA by PCR using Platinum Hi-fidelity Taq polymerase (Invitrogen, Carlsbad, CA).

The amplified products were cloned in frame into the pET-23b(+) vector in order to produce histidine-tagged fusion proteins by using the pET protein overexpression system (Novagen, Madison, WI). Soluble fusion proteins from isopropylthio-β-galactoside (IPTG)-induced bacterial lysates were purified on HisBind Quick Columns (Novagen) per the manufacturer's instruction. Insoluble fusion proteins (inclusion bodies) were solubilized with 6 M guanidine and purified on a HisBind Quick Column in the presence of 6 M guanidine. Purified soluble fusion proteins were dialyzed against 2 L of PBS at 4°C (6× 12 hours) and collected. Purified insoluble fusion proteins were dialyzed against 2 L of the following solutions (12 hours each at 4°C): 3 M Guanidine/PBS, 1.5 M guanidine/PBS, 0.75 M guanidine/PBS, PBS (3×). At the end of the third PBS dialysis, the fusion proteins were collected.

All HisBind column-purified fusion proteins were further purified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The fusion protein band was carefully cut from the gel, and the strips of gel containing the fusion proteins were sent to Washington Biotechnology (Baltimore, MD) for use as antigens in immunizing rabbits.

Affinity columns for purifying SAPAP antibodies were prepared as follows. Antigenic fusion protein was first generated and purified as above. Strips of gel containing fusion protein were masticated, and the fusion protein was electroeluted (model 422 Electro-elutor, Bio-Rad, Hercules, CA). The fusion protein was then dialyzed (4× 12 hours) against coupling buffer (CB) solution (0.1 M $NaHCO_3$, 0.1 M NaCl, pH 8.4) to rid the fusion protein of Tris-containing buffer. Following dialysis, the fusion protein was collected, concentrated (Centricon columns, Millipore, Bedford, MA), and dissolved in 4 ml CB containing 1% SDS. Cyanogen bromide-activated sepharose 4B beads (Sigma; 0.15 mg) were washed in 50 ml 1 mM HCl on ice for 1hour and poured into an empty column. The column was washed 5 times with 10 ml of cold $H_2O$ and once with 5 ml CB. Immediately after the CB wash, fusion protein was added. The fusion protein was allowed to couple with the cyanogen bromide-activated beads for 2 hours at room temperature. Following coupling, the column was washed 5 times alternately with CB and acetate buffer (0.1 M acetic acid, 0.1 M NaCl, pH 4.0), and then with 20 ml of PBS. The column was stored at 4°C with PBS/0.2% sodium azide until antibody purification. A few beads were pipetted out of the column and immunostained with serum to confirm that the coupling reaction was successful for each fusion protein.

Serum from each immunized rabbit was partially purified by ammonium sulfate precipitation (Harlow and Lane, 1988). Affinity purification of the antibody on the column was carried out according to standard protocols (Harlow and Lane, 1988). The affinity-purified antibodies were dialyzed against PBS (4× 12 hours) and collected. Antibody concentrations were determined by BCA protein assay (Pierce, Rockford, IL), and each antibody was diluted to 250 μg/ml in 0.2% bovine serum albumin (BSA)-PBS.

## Determination of antibody specificity

PCR primer sets were designed to clone the full-length sequences of the mouse SAPAP genes from mouse brain cDNA. Each of the mouse SAPAPs was subcloned into a vector with the CMV promoter (BD Clontech, Palo Alto, CA), and quail fibroblast QT6 cells were transfected (Lipofectamine 2000, Invitrogen, Carlsbad, CA) with each of the SAPAPs. The transfected QT6 cells were lysed by using SDS-PAGE loading buffer, and each of the three affinity-purified antibodies was tested on QT6 cell lysates by Western blot (antibody concentration 1 μg/ml); the affinity-purified antibodies were determined not to cross-react with the other SAPAPs (see Fig. 5).

## Postsynaptic density (PSD) preparation

The PSD fraction of whole mouse brain was prepared as described (Carlin et al., 1980; Cho et al., 1992; Ehlers, 2003). Mouse brain was homogenized in HEPES-buffered sucrose (HBS) solution (0.32 M sucrose, 4 mM HEPES, pH 7.4)

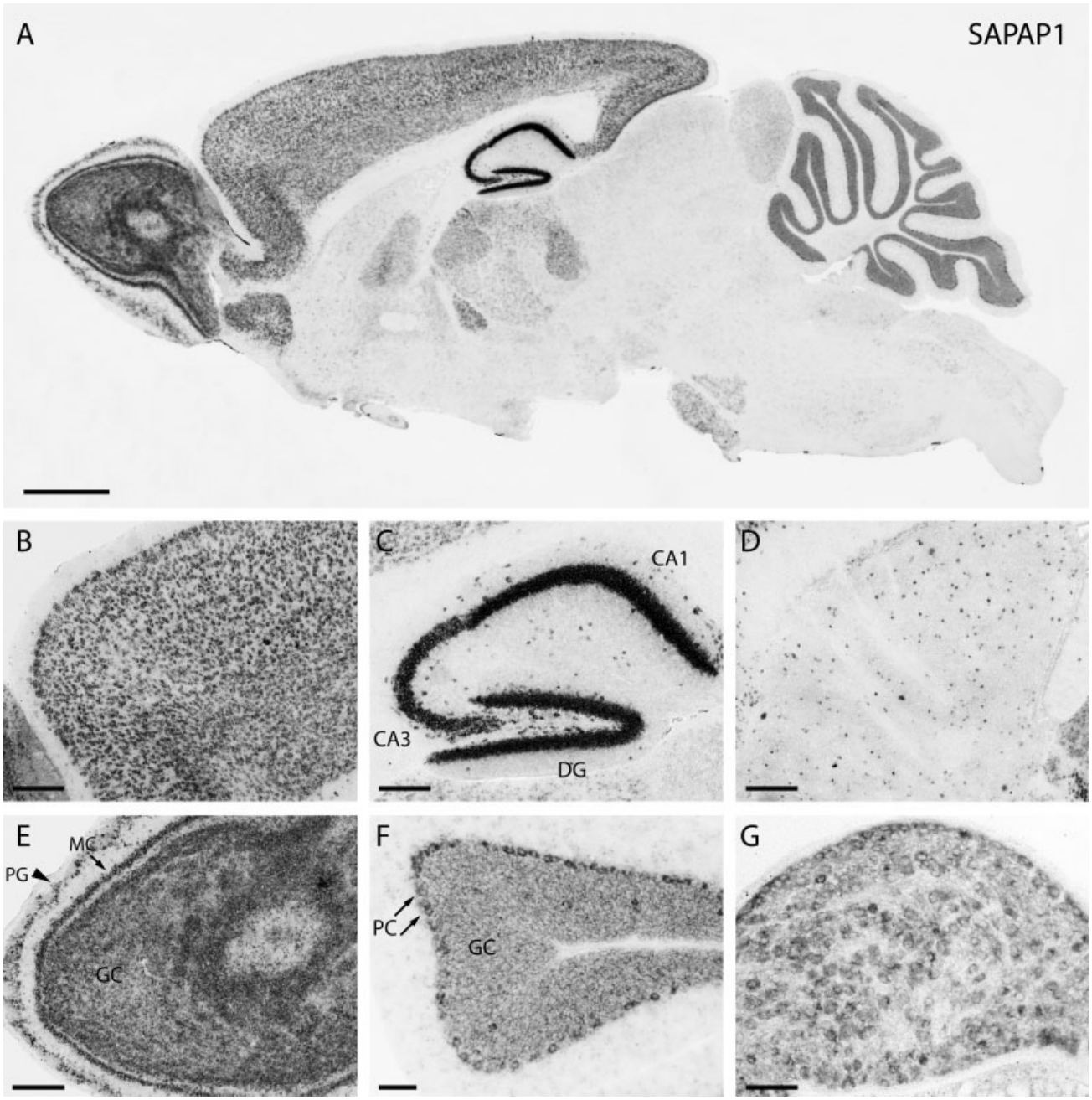


Fig. 1. Expression of SAPAP1 mRNA in the nervous system. **A:** A whole mouse brain section was probed with DIG-labeled antisense RNA of SAPAP1. SAPAP1 mRNA is highly expressed in the olfactory bulb, neocortex, hippocampus, and cerebellum. Moderate expression is also seen in many other parts of the brain (see Table 1). **B–F:** Higher magnification views of neocortex (B), hippocampus (C), striatum (D), olfactory bulb (E), and cerebellum (F). **G:** SAPAP1 mRNA is also expressed in neurons of the superior cervical ganglion. For abbreviations, see list. Scale bars = 1 mm in A; 300 μm in B–E; 100 μm in F,G.

containing protease and phosphatase inhibitors (0.1 mM phenylmethylsulfonyl fluoride [PMSF], 1.5 μg/ml aprotinin, 10 μg/ml antipain, 10 μg/ml leupeptin, 10 μg/ml chymostatin, 10 μg/ml pepstatin, 0.1 mg/ml benzamidine, 10 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 1 mM pap-nitrophenylphosphate, 1 mM sodium orthovanadate, and 0.1 mM ammonium molybdate). Homogenates were centrifuged (1,000*g*) to remove nuclei and tissue debris (brain lysate fraction). The supernatant was centrifuged at 10,000*g* to yield the crude brain membrane fraction (P2 pellet) and the soluble fraction. The P2 pellet was washed with HBS containing protease and phosphatase inhibitors and re-entrifuged to obtain the washed crude synaptosomal pellet (P2′). The P2′ fraction was subjected to hypo-osmotic shock (4 mM HEPES, pH 7.4) and centrifuged at 25,000*g* to yield the lysed synaptosomal membrane fraction (P3). The synap-

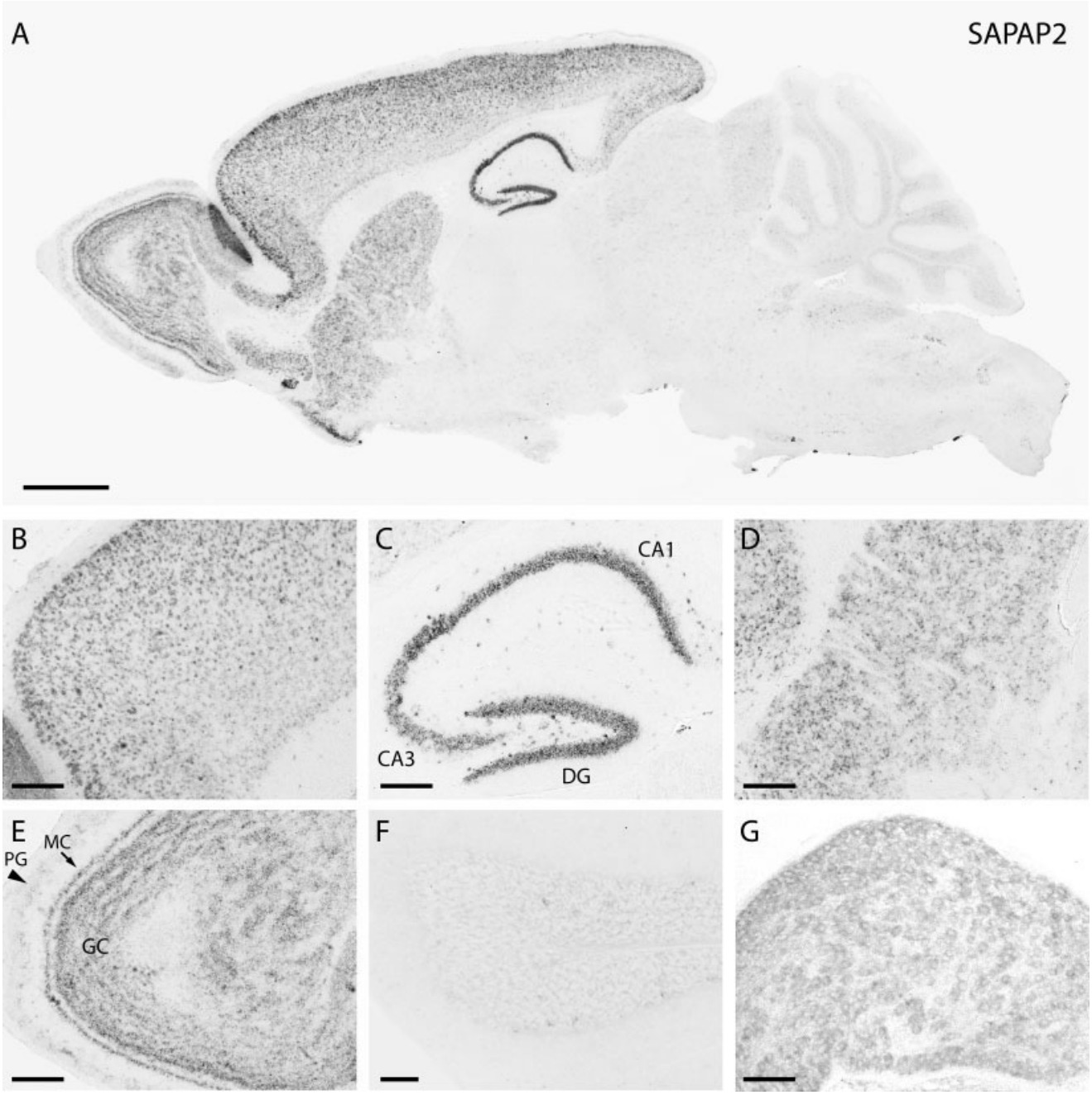


Fig. 2. Expression of SAPAP2 mRNA in the nervous system. **A:** In situ hybridization showing expression of SAPAP2 mRNA in the brain. Expression is observed in olfactory bulb, neocortex, hippocampus, and striatum. **B–F:** Higher magnification views of neocortex (B), hippocampus (C), striatum (D), olfactory bulb (E), and cerebellum (F). **G:** Weak SAPAP2 hybridization signal is also seen in neurons of the superior cervical ganglion. For abbreviations, see list. Scale bars = 1 mm in A; 300 μm in B–E; 100 μm in F,G.

tic plasma membrane (SPM) fraction was obtained by sucrose gradient centrifugation of resuspended P3 (Ehlers, 2003). SPM was washed once (PSDI fraction) or twice (PSDII) with 0.5% Triton X-100 (Sigma) in HEPES/EDTA (HE) buffer (50 mM HEPES, 2 mM EDTA, pH 7.4) and pelleted. To obtain the PSDIII fraction, SPM was washed once with 0.5% Triton X-100 in HE buffer and once with 3% Sarcosyl (Sigma) in HE buffer.

## Fluorescent immunohistochemistry

P21–P28 mice were deeply anesthetized with halothane and perfused transcardially with lactated Ringer's solution. Brains were dissected, embedded in OCT compound (TissueTek), and quick-frozen in ethanol-dry ice slush. Brains were stored at −80°C until sectioning.

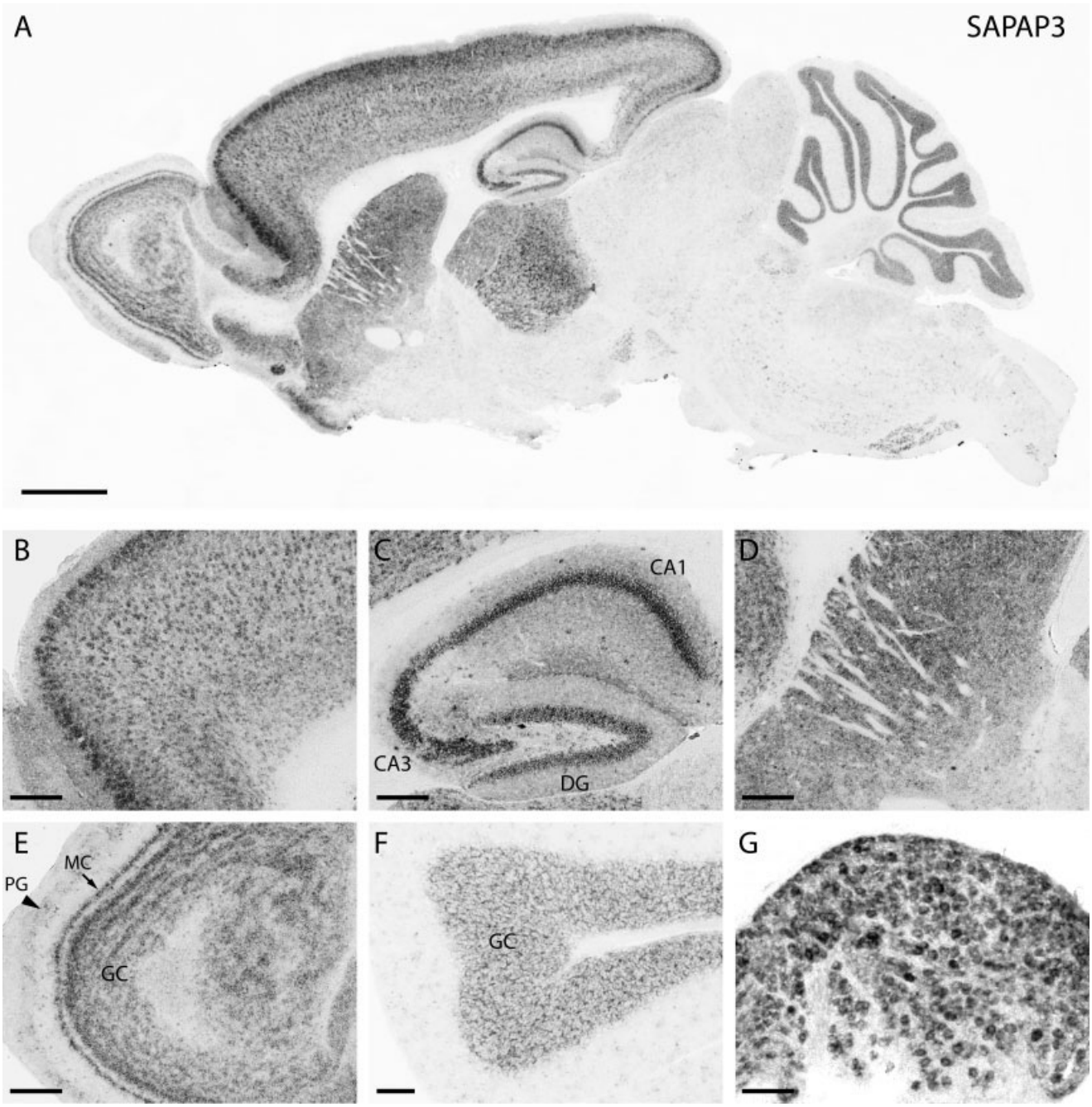


Fig. 3. Expression of SAPAP3 mRNA in the nervous system. **A:** Many regions of the brain show high levels of expression of SAPAP3 mRNA, including neocortex, hippocampus, striatum, olfactory bulb, cerebellum, and thalamus. **B–F:** Higher magnification views of neocortex (B), hippocampus (C), striatum (D), olfactory bulb (E), and cerebellum (F). **G:** SAPAP3 mRNA is also expressed in neurons of the superior cervical ganglion. For abbreviations, see list. Scale bars = 1 mm in A; 300 μm in B–E; 100 μm in F,G.

Sections of 8 μm were cut on a cryostat, placed on gelatin-coated slides, and dried at room temperature for at least 1 hour before storage at −20°C. For staining, neural tissue sections were first lightly fixed (0.5% PFA/PBS, 2 minutes) and then blocked for 1 hour with 5% normal goat serum (Sigma) and 2% BSA (Sigma) in PBS. Muscle sections were blocked in the same fashion but were left unfixed. Following blocking, sections were incubated with affinity-purified SAPAP antibodies (1 μg/ml) for 2–3 hours, washed 3× 5 minutes in PBS, and incubated for 2 hours with Cy3 goat anti-rabbit secondary antibodies diluted 1:1,500 (Jackson ImmunoResearch, West Grove, PA). Following secondary antibody incubation, sections were washed 3× 5 minutes in PBS and mounted for fluorescent imaging with anti-fade PPD solution (1 mg/ml para-phenylenediamine [ICN, Aurora, OH] in 90% glycerol/PBS).

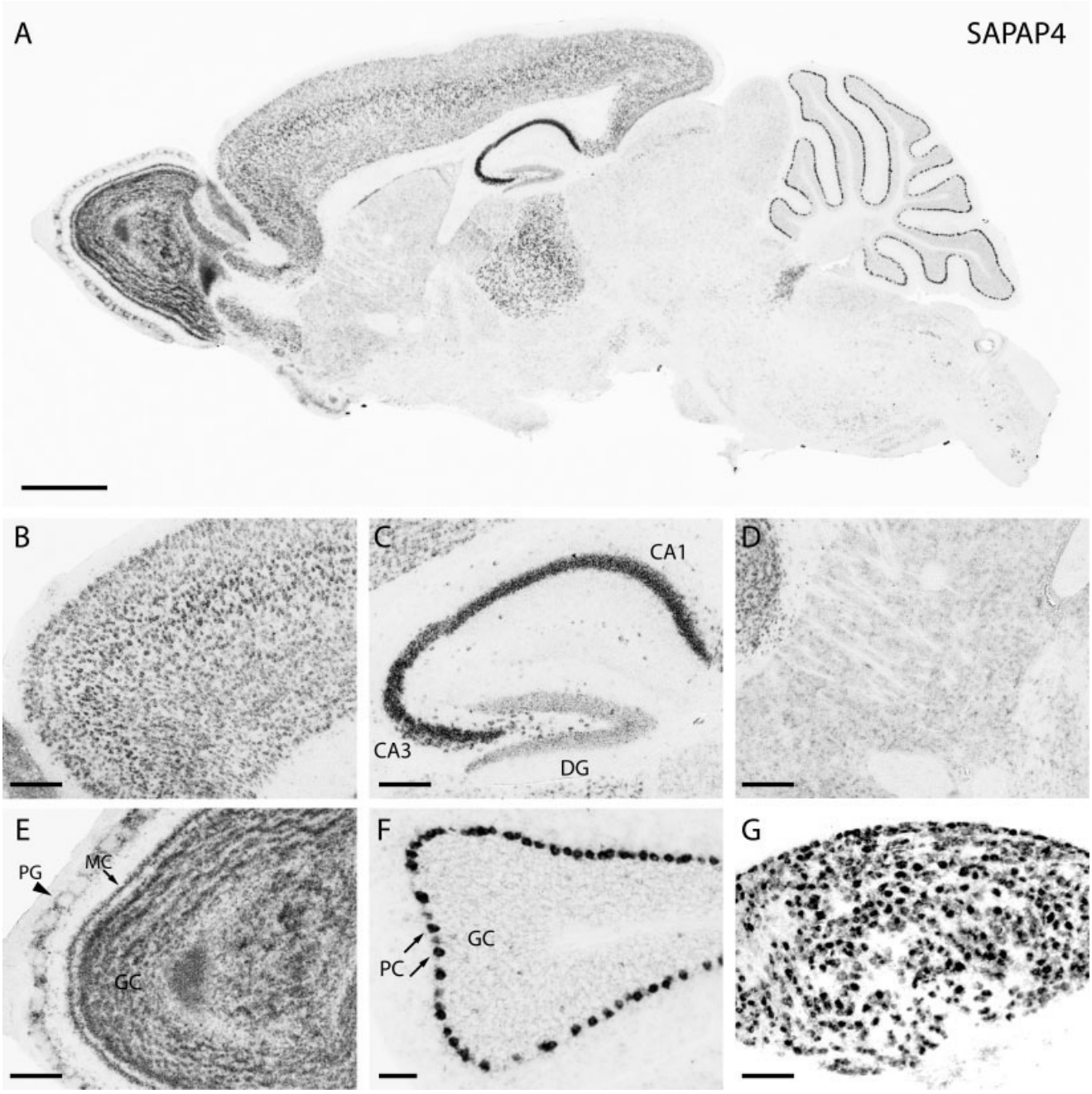


Fig. 4. Expression of SAPAP4 mRNA in the nervous system. **A:** SAPAP4 mRNA is expressed in the neocortex, hippocampus, olfactory bulb, cerebellum, and thalamus. **B–F:** Higher magnification views of neocortex (B), hippocampus (C), striatum (D), olfactory bulb (E), and cerebellum (F). **G:** SAPAP4 mRNA is highly expressed in neurons of the superior cervical ganglion. For abbreviations, see list. Scale bars = 1 mm in A; 300 μm in B–E; 100 μm in F,G.

Various mouse monoclonal antibodies were applied with the SAPAP affinity-purified antibodies for use as synaptic markers. These antibodies were used at the following dilutions from the original manufacturer's stock: anti-synaptophysin mouse monoclonal IgG1 (Synaptic Systems, Götingen, Germany), 1:500; anti-glycine receptor α1 subunit (mAb 2A, generous gift of Dr. Heinrich Betz, Max Planck Institute of Brain Research, Frankfurt, Germany), 1:100; anti-$GABA_A$ receptor β2/3 chains (clone BD17, mAb 341 Chemicon, Temecula, CA), 1:100; and anti-PSD-95 mouse monoclonal IgG2a (Affinity BioReagents, Golden, CO), 1:100. These synaptic marker antibodies were recognized by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG1 secondary antibody (Roche) or FITC-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch). Alexa-488–conjugated α-bungarotoxin

TABLE 1. SAPAP mRNA Expression in the Central and Peripheral Nervous Systems[1]

| | SAPAP1 | SAPAP2 | SAPAP3 | SAPAP4 |
|---|---|---|---|---|
| Central nervous system | | | | |
| Neocortex | | | | |
| Layer I | +/− | +/− | ++ | +/− |
| Layer II/III | +++ | +++ | ++++ | ++ |
| Layer IV | +++ | ++ | ++ | ++ |
| Layer V | +++ | ++ | +++ | +++ |
| Layer VI | +++ | + | ++ | ++ |
| Hippocampus | | | | |
| CA1 | ++++ | ++++ | ++++ | ++++ |
| CA3 | +++ | +++ | ++++ | ++++ |
| Dentate gyrus | ++++ | ++++ | ++++ | + |
| Cerebellum | | | | |
| Granule cell layer | +++ | +/− | +++ | + |
| Molecular layer | +/− | +/− | +/− | +/− |
| Purkinje cell layer | +++ | +/− | +/− | ++++ |
| Olfactory bulb | | | | |
| Granule cell layer | +++ | +++ | +++ | ++++ |
| Mitral cell layer | +++ | +++ | +++ | ++++ |
| External plexiform layer | +/− | +/− | +/− | +/− |
| Periglomerular cells | +++ | + | ++ | +++ |
| Anterior olfactory nucleus | +++ | +++ | +++ | +++ |
| Hypothalamus | +/− | +/− | +/− | +/− |
| Thalamus | ++ | +/− | +++ | +++ |
| Striatum | + | +++ | +++ | + |
| Substantia nigra | +/− | +/− | ++ | + |
| Superior colliculus | +/− | +/− | +/− | + |
| Inferior colliculus | + | +/− | +/− | +/− |
| Peripheral nervous system | | | | |
| Superior cervical ganglion | ++ | + | ++++ | ++++ |

[1]Relative SAPAP mRNA expression levels in different areas of the central and peripheral nervous systems of the adult mouse (++++, highest expression within each section; +/−, negligible expression). See also Figure 1–4.

TABLE 2. Localization of SAPAP1, -3, and -4 Proteins in the Central and Peripheral Nervous Systems[1]

| | SAPAP1 | SAPAP3 | SAPAP4 |
|---|---|---|---|
| Central nervous system | | | |
| Neocortex | | | |
| Layer I | ++++ | ++++ | ++++ |
| Layer II/III | ++++ | ++++ | +++ |
| Layer IV | ++++ | +++ | ++++ |
| Layer V | ++++ | ++ | ++++ |
| Layer VI | ++++ | ++ | +++ |
| Hippocampus | | | |
| CA1 oriens layer | ++++ | ++++ | ++++ |
| CA1 stratum radiatum | ++++ | ++++ | ++++ |
| CA3 oriens layer | ++ | +++ | +++ |
| CA3 stratum radiatum | ++ | +++ | +++ |
| Lacunosum moleculare | + | ++ | +++ |
| Dentate gyrus molecular layer | ++++ | +++ | + |
| Cerebellum | | | |
| Granule cell layer | ++++ | ++++ | +++ |
| Molecular layer | ++ | + | ++++ |
| Mossy fiber layer | +/− | +/− | +/− |
| Olfactory bulb | | | |
| Glomerular layer | +++ | ++++ | +++ |
| External plexiform layer | + | ++ | ++ |
| Granule cell layer | ++ | + | +++ |
| Hypothalamus | + | +/− | + |
| Thalamus | ++ | +++ | +++ |
| Striatum | + | ++++ | ++ |
| Superior colliculus | + | + | ++ |
| Inferior colliculus | ++ | ++ | +++ |
| Retina | | | |
| Ganglion cell layer | + | +++ | +/− |
| Inner plexiform layer | ++++ | ++++ | ++++ |
| Inner nuclear layer | +/− | + | ++ |
| Outer plexiform layer | + | +++ | ++++ |
| Outer nuclear layer | +/− | +/− | ++ |
| Spinal cord | | | |
| Ventral horn | +++ | +++ | +++ |
| Dorsal horn | ++++ | ++++ | ++++ |
| White matter | +/− | +/− | +/− |
| Peripheral nervous system | | | |
| Neuromuscular junction | ++++ | ++++ | ++ |
| Submandibular ganglion | ++++ | ++++ | ++++ |

[1]Relative expression levels of SAPAP1, -3, and -4 proteins in different areas of the central and peripheral nervous systems of the adult mouse (++++, highest expression within each section; +/−, negligible expression). See also Figures 6–11.

(Molecular Probes, Eugene, OR) was used as a marker for muscle nicotinic acetylcholine receptors at the neuromuscular junction.

## Image processing

All images were collected on a Zeiss Axioskop 2+ (Carl Zeiss, New York, NY) with Zeiss Achromat and Zeiss Neofluar objectives using a Zeiss Axiocam digital camera (Carl Zeiss). Images were subsequently processed in Adobe Photoshop version 6.0 (Adobe Systems, San Jose, CA) to optimize brightness and contrast.

# RESULTS

## Expression of SAPAP mRNA in the brain

Probes specific for SAPAP1, -2, -3 or -4 were generated and used to determine the mRNA expression patterns of each of the SAPAP genes in the brain (Figs. 1–4, Table 1). In situ hybridization of adult mouse whole brain sections showed that SAPAP1, -3, and -4 were highly expressed in many regions of the brain, whereas SAPAP2 mRNA was expressed in a more limited fashion (Figs. 1A, 2A, 3A and 4A). Additionally, in many regions of the brain, the SAPAP mRNAs showed interesting differential expression patterns.

One of the brain regions showing differential expression of the SAPAPs was the hippocampus. Figure 1A and C shows that expression of SAPAP1 mRNA was very high in the pyramidal cells of CA1 and CA3 and the granule cells of the dentate gyrus. In contrast, SAPAP4 mRNA, although strongly expressed in pyramidal cells of CA1 and CA3, showed only minimal expression in granule cells of the dentate gyrus (Fig. 4A,C). SAPAP2 and -3 mRNAs were expressed in all areas of the hippocampus (Figs. 2A,C, 3A,C). Interestingly, SAPAP3 mRNA was highly localized in dendritic layers of the hippocampus (molecular layer of the dentate gyrus, the lacunosum moleculare, the stratum radiatum, and the oriens layer), whereas SAPAP1, -2, and -4 mRNA localizations were generally restricted to cell bodies (Figs. 1C, 2C, 3C, 4C). Additionally, all the SAPAP mRNAs appeared to be expressed by inhibitory interneurons in the hippocampus, particularly those in the hilus.

In the cerebellum, SAPAP1 mRNA was highly expressed in both granule cells and Purkinje cells (arrows in Fig. 1F). By comparison, there was virtually no expression of SAPAP2 mRNA in the cerebellum (Fig. 2A,F). SAPAP3 and -4 mRNAs showed differential expression patterns in the cerebellum. SAPAP3 was strongly expressed in cerebellar granule cells but not in Purkinje cells (Fig. 3A,F). Conversely, SAPAP4 was strongly expressed in Purkinje cells, with low expression in granule cells (Fig. 4A,F). These cerebellar mRNA expression patterns lead to the prediction that SAPAP3 and -4 proteins present at dendritic postsynaptic densities should be found in different layers of the cerebellum (see below).

SAPAP1, -2, -3, and -4 mRNAs were all expressed in the cerebral cortex (Figs. 1A,B, 2A,B, 3A,B, 4A,B). SAPAP1 mRNA showed uniformly high expression in layers 2–6 of the cerebral cortex, whereas SAPAP2 and -3 mRNAs showed relatively strong expression in layers 2/3 (Figs. 1B, 2B, 3B), and SAPAP4 mRNA showed relatively strong expression in layer 5 (Fig. 4A,B). Additionally, SAPAP3

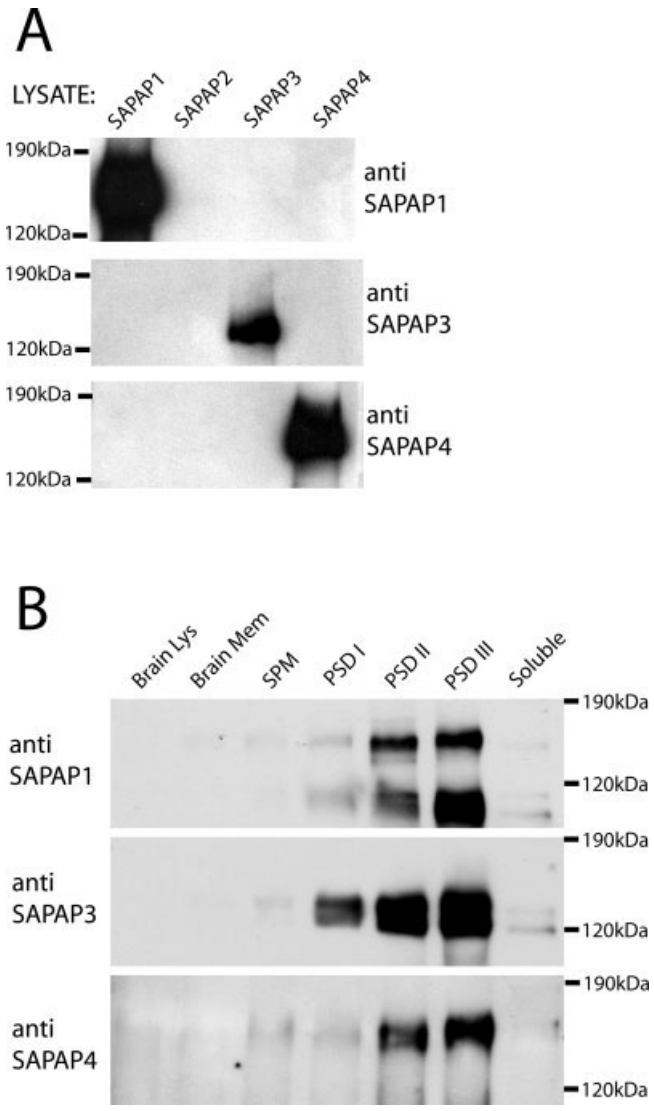


Fig. 5. SAPAP1, -3, and -4 are all enriched in PSD fractions. **A:** Rabbit polyclonal antibodies were raised against SAPAP1, -3, and -4 and affinity purified (see Materials and Methods). QT6 cells were transiently transfected with SAPAP1, -2, -3, or -4 and lysed. The lysates were immunoblotted with the indicated affinity purified antibody. No cross-reactivity was observed among the affinity-purified antibodies to SAPAP1, -2, -3, and -4. SAPAP expression in each of the lysates was also checked with an antibody recognizing all four of the SAPAPs (data not shown). **B:** PSD fractions (5 μg per lane), prepared from whole mouse brain (see Materials and Methods), were probed with the affinity-purified antibodies. The SAPAP1 antibody recognizes two bands of approximately 100 and 140 kDa. The SAPAP3 antibody recognizes two major bands of approximately 110 and 130 kDa. The SAPAP4 antibody recognizes only a single band of approximately 145 kDa. For abbreviations, see list.

mRNA was localized in layer 1 of cerebral cortex, again suggesting dendritic localization (Fig. 3B).

SAPAP mRNAs were also differentially expressed in the striatum (Figs. 1A,D, 2A,D, 3A,D, 4A,D). SAPAP2, -3, and -4 mRNA was expressed in many cells in the striatum, with the in situ signal for SAPAP3 being particularly strong (Figs. 2D, 3D, 4D). In contrast, SAPAP1 was only expressed in a small subset of cells in the striatum (Fig. 1D).

SAPAP mRNAs were all highly expressed in the olfactory bulb. Cell types expressing SAPAPs in the olfactory bulb included periglomerular cells, tufted cells, mitral cells, and granule cells (Figs. 1E, 2E, 3E, 4E).

## Expression of SAPAP mRNA in superior cervical ganglion

Up to now, studies of SAPAPs have been focused only in the central nervous system. To determine whether SAPAPs are also expressed in the peripheral nervous system, we investigated SAPAP mRNA expression in one of the autonomic ganglia, the superior cervical ganglion (SCG) of the sympathetic chain. SAPAP1, -3, and -4 mRNAs were highly expressed in SCG (Figs. 1G, 3G, 4G), whereas expression of SAPAP2 mRNA in SCG was minimal (Fig. 2G). Because SCG neurons received cholinergic inputs, expression of SAPAP mRNAs in these neurons implies that SAPAP proteins may be localized to cholinergic synapses (see below).

## Antibody generation and characterization

Based on their high levels of mRNA expression in the brain and SCG, we developed polyclonal antibodies specific for SAPAP1, -3, and -4 by using immunogenic fusion proteins (see Materials and Methods). To test the specificity of the antibodies, we transiently transfected quail fibroblast QT6 cells with mammalian expression constructs encoding each of the full-length SAPAPs. We prepared lysates from the transfected cells and probed the lysates with each of the antibodies on Western blots. As shown in Figure 5A, no cross-reactivity was observed, demonstrating that the antibodies are specific for SAPAP1, -3, and -4.

SAPAP1 was originally identified as a binding partner of the prototypical postsynaptic density (PSD) protein, PSD-95, and is known to be present in PSD fractions prepared from brain (Kim et al., 1997; Takeuchi et al., 1997). To test whether SAPAP3 and -4 were also present in the PSD, we prepared PSD fractions from adult mouse brain (see Materials and Methods) and probed them with the antibodies to SAPAP1, -3, and -4 using Western blots (Fig. 5B). We found that SAPAP1, -3, and -4 were all highly enriched in PSD fractions. The SAPAP1 antibody recognized two bands of approximately 100 and 140 kDa in PSD fractions, consistent with two previously identified alternative splicing forms (Kim et al., 1997; Takeuchi et al., 1997); the SAPAP3 antibody recognized two bands of approximately 110 and 130 kDa, suggesting that this gene may also be alternatively spliced; and the SAPAP4 antibody recognized a single band of approximately 145 kDa.

## SAPAP protein localization in hippocampus

Differential expression observed in the hippocampus by using in situ hybridization was further evidenced with fluorescent immunohistochemistry (Fig. 6). In the hippocampus, SAPAP1, -3, and -4 proteins were mainly localized in the dendritic layers of the hippocampus, consistent with a postsynaptic localization suggested by our biochemical data (Fig. 5B) and previous studies (Kim et al., 1997; Naisbitt et al. 1997). The highest expression level of SAPAP1 protein was seen in the CA1 (oriens layer and stratum radiatum, but not lacunosum moleculare) and dentate gyrus (molecular layer; Fig. 6A). A lower level of

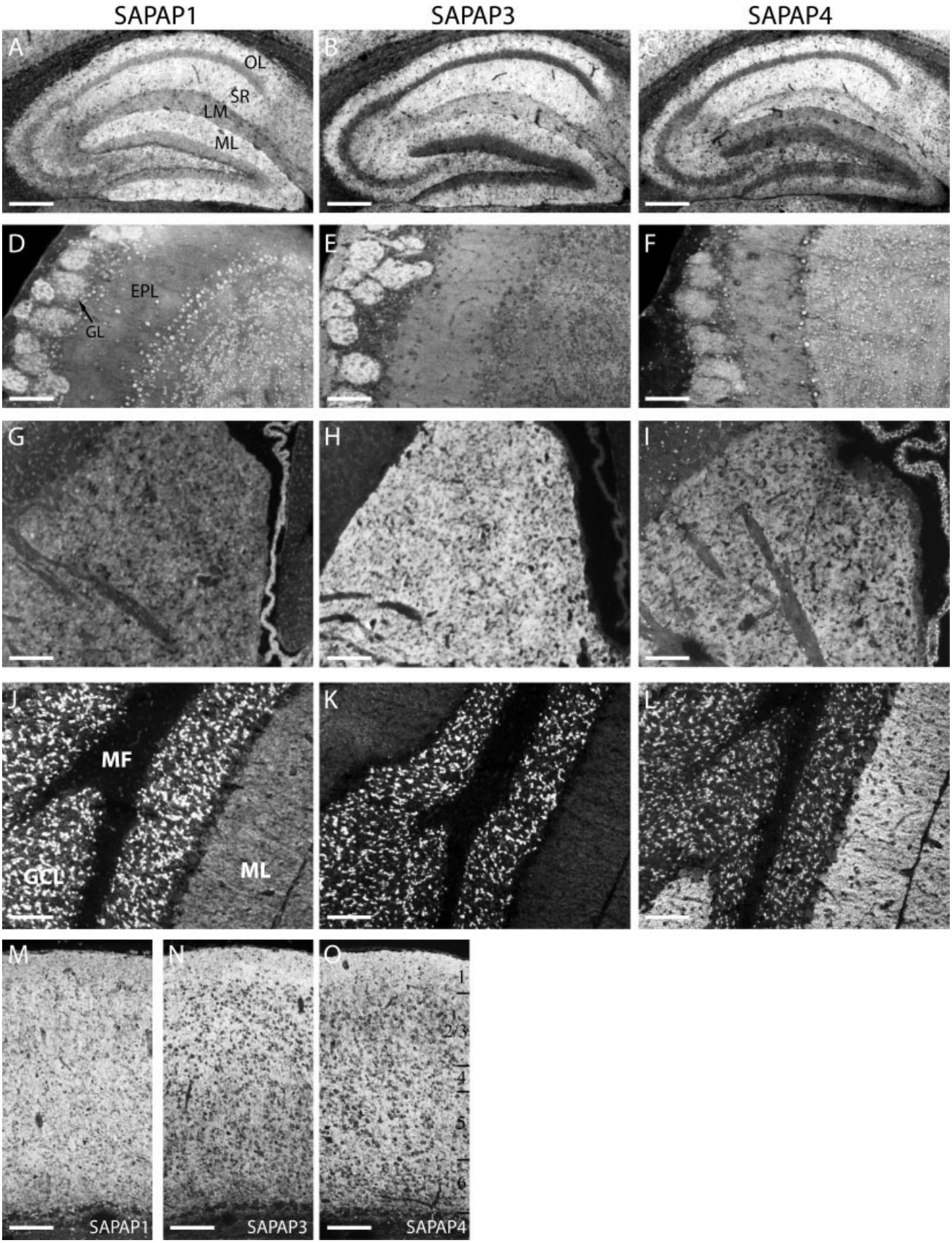


Fig. 6. SAPAP1, -3, and -4 proteins are differentially expressed in the brain as revealed by fluorescent immunohistochemistry. SAPAP1, -3, and -4 proteins are differentially expressed hippocampus (**A–C**), olfactory bulb (**D–F**), striatum (**G–I**), cerebellum (**J–L**), and neocortex (**M–O**). For abbreviations, see list. Scale bars = 300 μm in A–C, G–I; 200 μm in M–O; 100 μm in D–F, J–L.

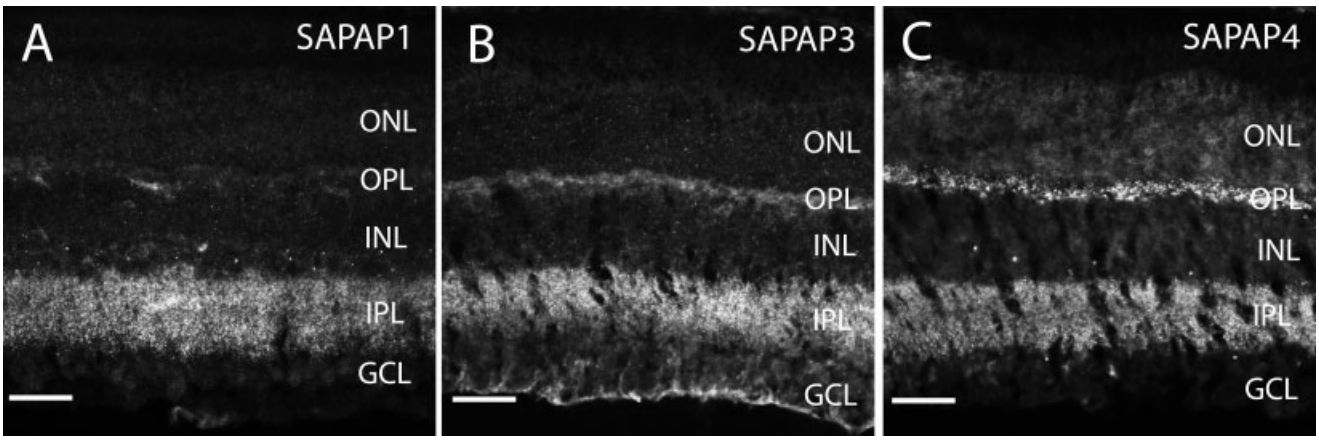


Fig. 7. SAPAP1, -3, and -4 proteins are differentially expressed in the retina. **A–C:** Fluorescent immunohistochemical staining of retinal sections reveals that SAPAP1, -3, and -4 proteins are all highly expressed in the inner plexiform layer (IPL). SAPAP3 and -4 proteins are also expressed in the outer plexiform layer (OPL). In addition, SAPAP3 expression is observed in the ganglion cell layer (GCL), and SAPAP4 expression is observed in the inner and outer nuclear layers (INL and ONL). Scale bars = 50 μm.

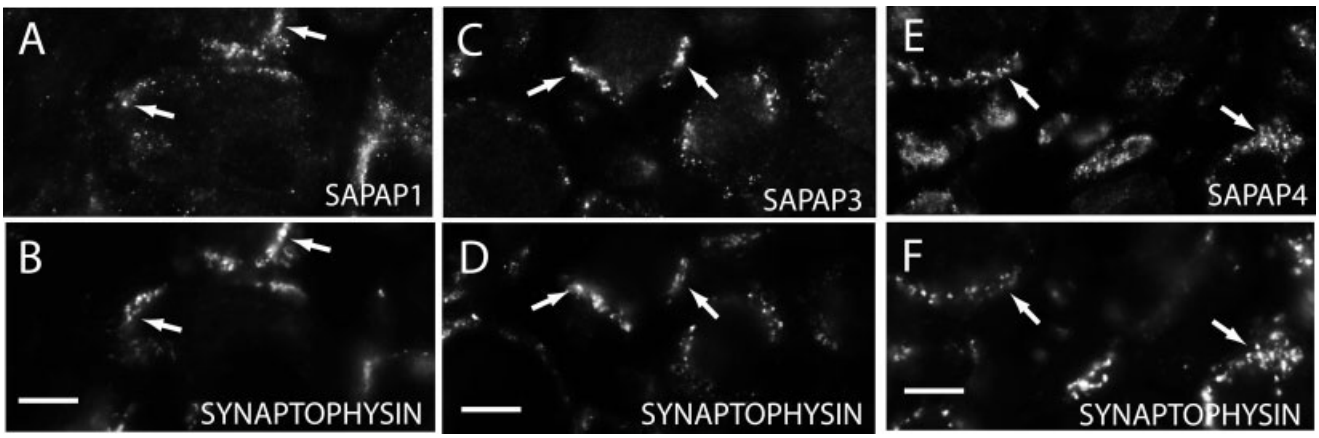


Fig. 8. SAPAP1, -3, and -4 are expressed at neuronal cholinergic synapses. **A,C,E:** Fluorescent immunostaining of submandibular ganglion sections using SAPAP1, -3, and -4 antibodies. Staining for the SAPAP proteins is predominantly synaptic (compare with presynaptic synaptophysin staining in **B,D,F**). Arrows indicate examples of synaptic regions. For abbreviations, see list. Scale bars = 10 μm.

expression of SAPAP1 protein was also seen in CA3. SAPAP3 protein was highly expressed in the oriens layer and stratum radiatum of the CA1 region, with a medium level of expression in the lacunosum moleculare, CA3, and the molecular layer of the dentate gyrus (Fig. 6B). Consistent with in situ hybridization results (Fig. 4C), there was little expression of SAPAP4 protein in the molecular layer of the dentate gyrus (Fig. 6C). SAPAP4 protein expression was high in CA1 (oriens layer, stratum radiatum, and lacunosum moleculare) and medium in CA3 (Fig. 6C).

## SAPAP protein localization in cerebellum

Fluorescent immunohistochemical staining in cerebellum revealed high SAPAP1 protein localization in both the granule cell layer and the molecular layer (Fig. 6J). This was consistent with SAPAP1 mRNA expression in both granule cells and Purkinje cells (Fig. 1F). SAPAP3 protein was highly expressed in the granule cell layer, with weak expression in the molecular layer (Fig. 6K). The weak molecular layer staining was probably caused by a lack of SAPAP3 mRNA expression in Purkinje cells (Fig. 3F). When co-staining with synaptophysin, SAPAP1 and -3 expression in the granule cell layer can be seen as highly localized at the mossy fiber synapses using higher power objectives (data not shown).

In contrast to SAPAP3, SAPAP4 protein was present at high levels in the cerebellar molecular layer (Fig. 6L). The strong molecular layer staining for SAPAP4 was presumably due to localization of SAPAP4 protein in Purkinje cell dendrites, consistent with high SAPAP4 mRNA expression in Purkinje cells (Fig. 4F). In comparison with the molecular layer, weaker expression of SAPAP4 was observed in the granule cell layer (Fig. 6L).

## SAPAP protein localization in the cerebral cortex and striatum

SAPAP1, -3, and -4 proteins were all highly expressed in the cerebral cortex. SAPAP1 protein was uniformally distributed throughout layers 1–6, whereas SAPAP3 showed higher expression in layers 1–3, and SAPAP4 showed a slightly lower expression in layers 2/3 (Fig. 6M–O). In the striatum, consistent with in situ hybridization results (Figs. 1D, 3D, 4D), SAPAP3 was the most abundantly expressed of the SAPAP proteins (Fig. 6G–I).

## SAPAP protein localization in olfactory bulb

In the olfactory bulb, SAPAP1, -3, and -4 proteins were all highly expressed in the glomeruli, which contains the dendrites of the mitral cells and tufted cells (Fig. 6D–F). Lower levels of SAPAP protein expression were also observed in the external plexiform layer and the granule cell layer. Additionally, a significant amount of SAPAP1 and -4 proteins could be seen in cell bodies of tufted cells, mitral cells, and granule cells, whereas very little SAPAP3 protein was localized to cell bodies.

## Localization of SAPAP proteins in the retina

In addition to observations of differential expression in the brain, differential regulation of SAPAP1, -3, and -4 expression was also observed in the retina (Fig. 7). SAPAP1, -3, and -4 were all highly localized in the inner plexiform layer (IPL) which contains bipolar and amacrine cell synapses onto ganglion cells. In the outer plexiform layer (OPL), which contains synaptic connections among photoreceptors, horizontal cells, and bipolar cells, only SAPAP4 was highly expressed. SAPAP3 showed moderate OPL expression, with SAPAP1 showing little expression in this layer. Additionally, SAPAP3 showed significant expression in the ganglion cell layer, whereas SAPAP1 and -4 showed little expression in this layer. Finally, SAPAP4 showed significant expression in the inner and outer nuclear layers (INL and ONL), whereas SAPAP1 and 3 were minimally expressed in these layers.

## SAPAP protein localization in the autonomic nervous system

Although the SAPAPs have generally been investigated only at glutamatergic synapses due to their interaction with the NMDA receptor-binding protein, PSD-95, our results from in situ hybridization studies of the SCG indicate that the SAPAPs may also be present at neuronal cholinergic synapses. To explore the possible localizations of SAPAP1, -3, and -4 at neuronal cholinergic synapses, we performed fluorescent immunohistochemical stainings on sections of the parasympathetic submandibular ganglion. We chose this ganglion because the preganglionic neurons make cholinergic axosomatic connections and the synapses are easily visualized, as shown in Figure 8 (Snell, 1958; Yamakado and Yohro, 1977; Purves et al., 1987). SAPAP1, -3, and -4 were highly enriched at synapses in the submandibular ganglion, as demonstrated by simultaneously staining the sections for the presynaptic marker protein synaptophysin (Fig. 8B,D,F). This result strongly suggests that SAPAPs are part of the postsynaptic components of neuronal cholinergic synapses.

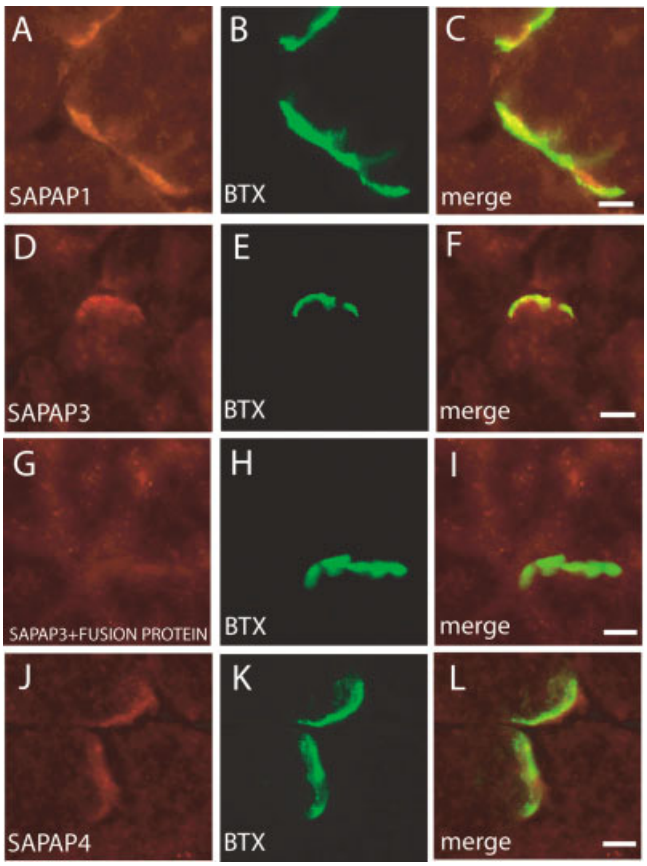


Fig. 9. SAPAP1, -3, and -4 are expressed at the neuromuscular junction. **A,D,J:** Fluorescent immunostaining of muscle sections using the SAPAP1, -3, and -4 antibodies. Staining for SAPAP1, -3, and -4 is predominantly found at the neuromuscular junction and matches with BTX staining (**B,E,K**), as shown by overlays of both stainings in **C**, **F**, and **L**. **G:** Staining of a muscle section using SAPAP3 antibody that was preincubated with the antigenic fusion protein used to make the SAPAP3 antibody. In this case, staining of the neuromuscular junctions is largely blocked (BTX staining and overlay are shown in **H** and **I**). For abbreviations, see list. Scale bars = 50 μm.

## Localization of the SAPAP proteins at the neuromuscular junction

Following the observation that SAPAP1, -3, and -4 were localized at neuronal cholinergic synapses, we decided to examine whether SAPAPs were also expressed at the neuromuscular junction (NMJ), another excitatory cholinergic synapse. In muscle, we found SAPAP1 and -3 to be highly localized at the NMJ (Fig. 9). SAPAP4 was also observed at the NMJ, but the expression was weaker in comparison with SAPAP1 and -3 (Fig. 9). Because many antibodies can stain the NMJ in a nonspecific fashion (G.F, personal observations), we tested the specificity of the SAPAP3 NMJ staining by preincubating the SAPAP3 antibody with the soluble antigenic fusion protein used to produce the SAPAP3 antibody. In this case, the staining of the NMJs was largely blocked, implying that the staining for SAPAP3 at the NMJ was specific (Fig. 9G–I).

The heavily synaptic localization of SAPAP1, -3, and -4 in the peripheral nervous system demonstrated that the SAPAP proteins are localized to cholinergic synapses as

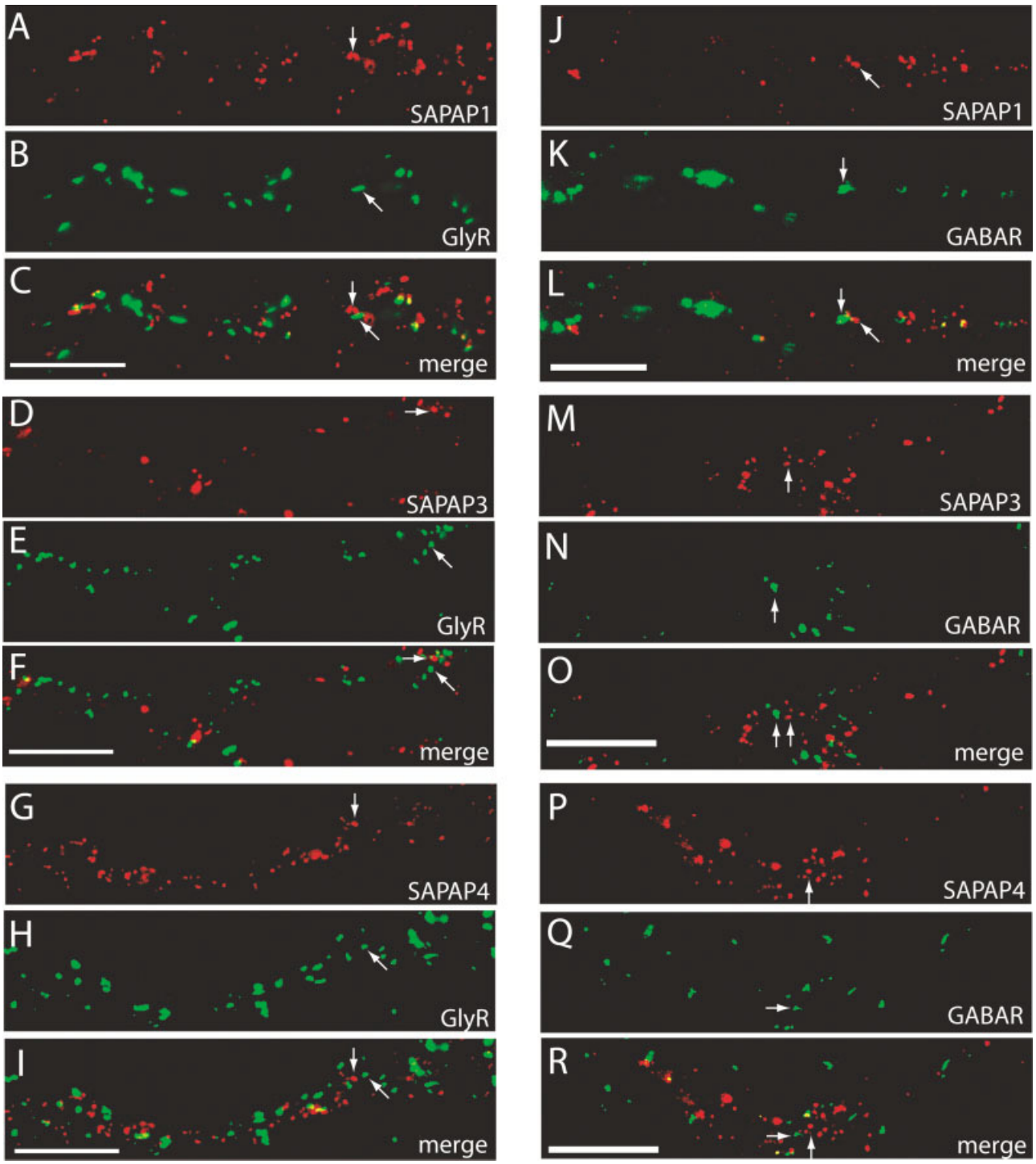


Fig. 10. SAPAP proteins do not colocalize with glycine or $GABA_A$ receptors. High-magnification pictures of dendrites in the spinal cord double-stained for SAPAP proteins and inhibitory receptors. **A,D,G:** SAPAP staining is highly punctate, consistent with synaptic localization. **B,E,H:** The same dendrites imaged in A, D, and G showing glycine receptor staining. The overlays of the SAPAP and GlyR stainings, shown in **C**, **F**, and **I**, indicate that neither SAPAP1, -3, nor -4 are localized at glycinergic inhibitory synapses. Arrows show examples of SAPAP and glycine receptor puncta that do not colocalize. **J,M,P:** SAPAP staining. **K,N,Q:** The same dendrites imaged in J, M, and P showing $GABA_A$ receptor staining. Overlays shown in **L**, **O**, and **R** indicate that neither SAPAP1, -3, nor -4 are localized at GABAergic inhibitory synapses. Arrows show examples of SAPAP and $GABA_A$ receptor puncta that do not colocalize. For abbreviations, see list. Scale bars = 10 μm.

well as to glutamatergic synapses. This novel observation supports the idea that the SAPAPs are important core components of several types of synapses and may therefore play general roles in regulating the formation and/or maintenance of postsynaptic specializations. The lower level of SAPAP4 protein at the NMJ implies that SAPAP1, -3, and -4 expression is differentially regulated in the peripheral nervous system as well as in the central nervous system.

### SAPAPs are not localized at inhibitory synapses

Following the observation that SAPAP1, -3, and -4 were found at cholinergic synapses, we investigated whether or not inhibitory synapses might also contain these proteins. Previous studies had indicated that guanylate kinase-associated protein (GKAP)/SAPAP1 was not located at γ-aminobutyric acid (GABA)ergic synapses (Naisbitt et al., 1997). Using spinal cord sections, we investigated whether or not SAPAP1, -3, or -4 were localized at glycinergic or GABAergic synapses in vivo. Figure 10 shows that SAPAP1, -3, and -4 were not localized at glycinergic or GABAergic synapses in the spinal cord. These experiments were performed by double labeling spinal cord sections for a SAPAP protein and either glycine or $GABA_A$ receptors. High-power images of spinal cord dendrites were then taken and the SAPAP labeling and inhibitory receptor labeling were overlaid. The overlaid images reveal no colocalization between the SAPAP proteins and the inhibitory receptors (Fig. 10).

As a positive control, the same experiments were performed using double labeling with the NMDA receptor binding protein, PSD-95. SAPAP1, -3, and -4 all showed extensive colocalization with PSD-95 (Fig. 11), implying, as expected, that SAPAP1, -3, and -4 were located at glutamatergic synapses in the spinal cord. The extensive colocalization of individual SAPAPs with PSD-95 also suggests that most glutamatergic synapses in the spinal cord express multiple SAPAPs.

## DISCUSSION

We have presented a detailed study of the expression of SAPAP genes in the adult murine nervous system. Our observations are threefold: 1) expression of the SAPAP genes is differentially regulated in many areas of the central and peripheral nervous systems, 2) the SAPAPs are localized to cholinergic synapses in addition to glutamatergic synapses, and 3) SAPAP3 mRNA is localized in dendrites.

The first observation is based on differential expression of the SAPAP mRNAs and differential localization of the SAPAP proteins within the various tissues of the nervous system. Differential regulation of SAPAP expression is consistent with the possibility that the SAPAPs play separate molecular roles from one another in vivo. Individual SAPAPs might endow a synaptic scaffold with particular stability and/or conformational properties required by different synapses, or it may be that individual SAPAPs have different binding affinities for other scaffold proteins, such as Shank, in order to build different scaffolding complexes. Either of these explanations would be consistent with previous models in which the SAPAPs are assumed to play a role in linking receptors to a scaffold that stabi-

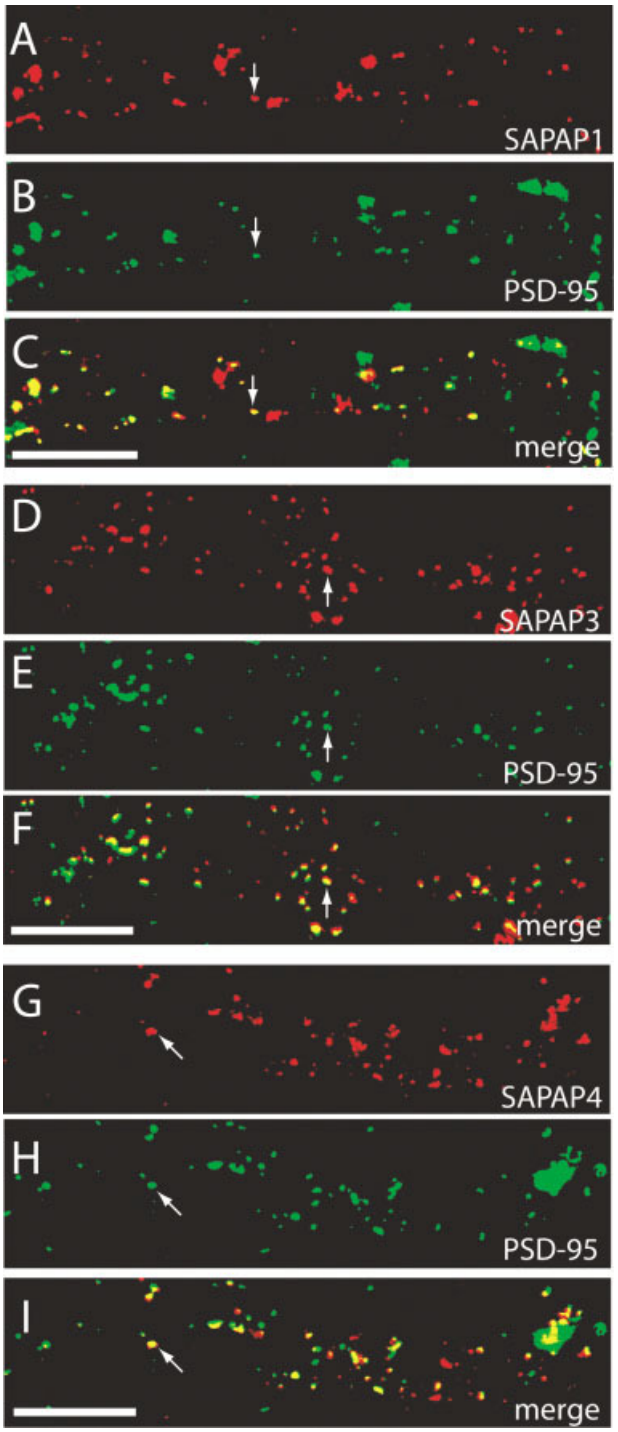


Fig. 11. SAPAP proteins colocalize with PSD-95. **A,D,G:** SAPAP-stained dendrites in the spinal cord as in Figure 10. **B,E,H:** The same dendrites imaged in A, D, and G showing co-staining for PSD-95, a postsynaptic protein that binds NMDA receptors. **C,F,I:** Overlays of SAPAP staining and PSD-95 staining indicate that SAPAP1, -3, and -4 are all localized at glutamatergic excitatory synapses. Arrows show examples of SAPAP and PSD-95 colocalizing puncta. For abbreviations, see list. Scale bars = 10 μm.

lizes synapses by connection to the cytoskeleton (Sheng and Kim, 2000; Sheng, 2001).

However, in addition to playing a structural role, it may be that SAPAP proteins are involved in recruiting signaling molecules such as nNOS and nArgBP2 to the postsynaptic scaffold (Kawabe et al., 1999; Haraguchi et al., 2000). Different types of synapses probably require different downstream signaling molecules. This might explain the differential expression patterns of the SAPAPs if these proteins recruit different signaling molecules to particular synapses. Although the SAPAPs are highly homologous, there are several regions of the proteins that show widely divergent amino acid sequences (Takeuchi et al., 1997), possibly representing binding sites for different signaling molecules. In this type of model, the SAPAPs might play a more complex role than previously envisaged.

A second important finding of this study is that the SAPAP1, -3, and -4 proteins are expressed at both neuronal cholinergic synapses and the neuromuscular junction. The SAPAPs have previously been investigated only at glutamatergic synapses based on their interaction with the NMDA receptor binding protein, PSD-95. The demonstration that the SAPAPs are present at several types of excitatory synapses is consistent with the possibility that the SAPAP proteins are a general "core" component of all excitatory synapses. Moreover, PSD-95 family proteins have been found at neuronal cholinergic synapses (Conroy et al., 2003; Parker et al., 2003), and there have also been reports that PSD-95 and SAP97 are located at the neuromuscular junction (Rafael et al., 1998; Huang et al., 2002). Together, these observations suggest that there may be an entire "core" postsynaptic complex that is generally required for the structure and/or function of all excitatory synapses.

We have also demonstrated that SAPAP proteins are not located at glycinergic nor GABAergic synapses in the spinal cord in vivo, implying that the SAPAP proteins are components only of excitatory synapses. This observation further advances the growing evidence that the molecular underpinnings of inhibitory postsynaptic specializations are fundamentally different from those of their excitatory counterparts (Feng et al., 1998; Kirsch, 1999; Kneussel and Betz, 2000).

A final interesting observation is that SAPAP3 mRNA, but not SAPAP1, -2, and -4 mRNA, is localized in dendrites. Dendritic protein synthesis plays an important role in synaptic plasticity (Steward and Schuman, 2003; Kiebler and DesGroseillers, 2000). Additionally, a recent study shows that the molecular composition of the PSD is dynamically regulated by activity through local protein turnover, possibly suggesting that dendritic protein synthesis is involved in modulating the composition of the PSD (Ehlers, 2003). The dendritic localization of SAPAP3 suggests that SAPAP3 protein can be locally synthesized and may thus play a unique role in plasticity requiring local synthesis/degradation. This finding adds another layer of complexity to our understanding of the in vivo function of the individual SAPAPs.

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