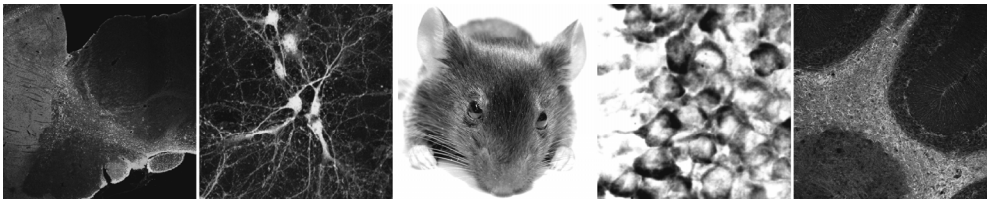
Chapter 5



Detailed analysis of mAKAP expression in the brain of young and old mice

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In preparation

Abstract

A-kinase anchoring proteins (AKAPs) form large macromolecular signaling complexes that specifically target cAMP-dependent protein kinase (PKA) to unique subcellular compartments and thus provide high specificity to PKA signaling. One important member of this family is mAKAP. Although mAKAP was reported to be present in the brain, so far knowledge on its distribution and function was lacking. In the present study we established a detailed distribution of mAKAP the mouse brain. In general, mAKAP could be found throughout the whole mouse brain. mAKAP expression was particularly abundant in the hippocampus, hypothalamus and olfactory bulb, whereas the cortex, cerebellum and striatum revealed a more moderate mAKAP immunoreactivity. The lowest expression level was detected in the brain stem. At subcellular levels, mAKAP was abundantly expressed both in perikarya (e.g: pyramidal neurons, Purkinje neurons), dendrites (e.g.: apical and basal dendrites of hippocampus pyramidal neurons) and to some extent in fibers (e.g: amygdala, cortex). Interestingly, the highest mAKAP expression was detected presynaptically in brain regions such as central amygdaloid nucleus, supraoptic nucleus, paraventricular hypothalamus nucleus, and suprachiasmatic nucleus.

In aged mice, we found a dramatic decrease in mAKAP expression in all brain regions.

Our finding that mAKAP is abundantly expressed, particularly presynaptically, suggest an important role for mAKAP in coordinating signal transduction pathways involved in proper brain functioning. Moreover, the strongly decreased expression in senescent mice suggests that mAKAP may contribute to changes in brain functioning in aged mice.

Key words: mAKAP; immunohistochemistry; brain; mouse, cAMP-dependent protein kinase, localization.

Introduction

cAMP-dependent protein kinase (PKA) mediates cellular responses to a wide range of hormones, neurotransmitters, and other signaling substances. Specificity of PKA signaling is in part achieved by binding to A-kinase anchoring protein (AKAP) molecules via interaction with the regulatory subunits (RI and RII) of PKA. Besides binding to PKA, AKAPs are capable of forming multi-protein complexes to coordinate the action of several signalling molecules all at a single location. To date, over 50 AKAPs have been described from diverse species ranging from C. elegans and rodents to humans. A prototypic example of an AKAP which carries multiple signaling enzymes is muscle specific AKAP (mAKAP, AKAP6 or AKAP100). mAKAP was for the first time identified in 1995 as AKAP100 using an interaction cloning strategy with an RIIα protein probe on a human hippocampal cDNA expression library (McCartney et al., 1995). Later AKAP100 was renamed mAKAP because of its presence in striated muscle (Kapiloff et al., 1999). A targeting domain is responsible for linkage of the complex to the nuclear envelope and sacroplasmatic reticulum (Kapiloff et al., 1999). The mAKAP gene structure was shown to be conserved in humans and rodents and two alternatively spliced forms of mAKAP were characterized: mAKAPα and mAKAPβ. mAKAPα and mAKAPβ are identical except for a 244 amino acid residue N-terminal extension in mAKAPα (Michel et al., 2005). The longer form, mAKAPα, is preferentially expressed in the brain, whereas mAKAPβ is abundant in cardiac myocytes and skeletal muscle (Michel et al., 2005).

Hitherto, the function of mAKAP in signaling events in cardiac myocytes has become increasingly clear under physiological as well as under pathological conditions. In cardiac myocytes, PKA anchored to mAKAP participates in calcium signaling via phosphorylation of the calcium-activated calcium release channel RyR (Kapiloff et al., 2001; Ruehr et al., 2003). Increased intracellular levels of calcium can activate the calcium-dependent protein phosphatase 2B (PP2B) which was also found to bind mAKAP (Pare et al., 2005). This phosphatase dephosphorylates the transcription factor NFAT, allowing for movement of NFAT into the nucleus and the transcription of genes. Interestingly, mAKAP was also found to bind a phosphodiesterase (PDE4D3). In cardiac myocytes, by tethering both PKA

and PDE4D3, mAKAP assembles a negative feedback loop that functions to regulate both PKA and PDE activity (Dodge-Kafka et al., 2005). Upon stimulation of the cell increased levels of cAMP lead to PKA activation, while activated PKA phosphorylates PDE4D3. This phosphorylation of PDE4D3 in turn increases the metabolic activity of PDE4D3 resulting in decreased cAMP levels. These effects were found to be counterbalanced by the MAP kinase extracellular-signal-regulated kinase 5 (ERK5) which phosphorylates PDE4D3 on a different serine site which results in suppressed PDE activity and increased PKA activity (Dodge-Kafka et al., 2005). Overall, by binding PKA, PP2B, RyR, PDE and a MAP kinase, mAKAP serves to integrate cAMP, calcium, and MAPK signals for the coordination of intracellular processes in the heart. The importance of proper coordination of signaling pathways is perfectly exemplified by the function of mAKAP in the heart. Defective mAKAP signaling has e.g. been linked to cardiac hypertrophy (Pare et al., 2005; Bauman et al., 2007).

The role of mAKAP in the brain remains so far rather unexplored. It was recently reported that the unique amino terminus of the most prominent mAKAP form in the brain, mAKAPα, can spatially restrict the activity of 3-phosphoinositide-dependent kinase-1 (PDK1). Moreover, the simultaneous recruitment of PDK1 and ERK onto mAKAPα was reported to facilitate activation and release of the downstream target p90RSK (Michel et al., 2005). AKAP100 mRNA was detected in the human cerebellum, basal ganglia, hippocampus, frontal and motor cortex (McCartney et al., 1995). These data, together with the importance of the different mAKAP signaling complex members in numerous brain functions, makes its very likely that mAKAP in the brain has a similar function as in the heart i.e. the integration of several signaling pathways to regulate neuronal processes.

The present study adds to the fundamental knowledge of mAKAP in the brain by mapping its distribution in detail in young and aged mice.

Materials and methods

Animals and housing conditions

3 and 26 months old male C57Bl/6J inbred mice (Harlan, Horst, The Netherlands, n=10) were group housed in standard macrolon cages and placed in an environmentally controlled room under a 12:12 light/dark cycle. Animals had free access to water and standard food pellets. All procedures concerning animal care and treatment were in accordance with the regulation of the ethical committee for the use of experimental animals of the University of Groningen, The Netherlands (DEC 4278A).

Western blotting

CO_2/O_2 anesthetized animals were quickly decapitated and brain tissue was removed on ice. The following brain regions were excised, immediately frozen in liquid nitrogen and stored at -80 °C before further processing: hippocampus, striatum, olfactory bulb, cortex, cerebellum, hypothalamus and brain stem.

Brain, colon, and heart tissue was mechanically homogenized in 10 volumes of homogenization buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 0.2 % NP-40, 4 mM EGTA, 10 mM EDTA, 15 mM sodium pyrophosphate, 100 mM β-glycerophosphate, 50 mM sodium fluoride, 5 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM PMSF, and Complete Mini Protease Inhibitor Cocktail (Roche)). The homogenate was centrifuged at 20,000 x g for 10 min at 4 °C, and the resulting supernatant was assayed for protein concentration using the Bradford method.

Protein samples (20 µg per sample) were separated on a 6 % SDS polyacrylamide gel and transferred to PVDF membranes (Millipore, USA). The blots were blocked for 1 h in blocking buffer (0.2 % I-Block (Tropix), 0.1 % Tween20) and then incubated overnight at 4 °C either with rabbit anti-mAKAP (1:8.000; Abcam, ab24639). Mouse anti-actin antibody (1:40,000; MP Biomedicals, Irvine, CA, USA) served as control for protein loading. The blots were incubated with horse radish peroxidase conjugated secondary antibodies (HRP conjugated anti-mouse (1:5,000)) (sc-2005 Santa Cruz, CA, USA) and HRP conjugated anti-rabbit (1:5,000) (NA934V, Amersham). Western blots were developed using the

chemiluminescence method (Pierce). The immunoblots were digitized and quantified using a Leica DFC 320 image analysis system (Leica, Cambridge, UK).

Immunohistochemistry

Animals were anesthetized by intraperitoneal injection with sodium pentobarbital 6% solution and sacrificed by transcardial perfusion with saline solution containing heparin, followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). After perfusion, the brains were kept at 4 °C in 0.01 M phosphate buffered saline (PBS) containing 0.1% sodium azide for 72 h. All brains were dehydrated for 48 h in a 30% buffered sucrose solution. After dehydration, 20 µm coronal or sagittal sections were cut on a cryostat microtome. Sections were stored at 4 °C in 0.01 M PBS containing 0.1% sodium azide.

The avidin/biotin immunoperoxidase staining method was used to visualize mouse mAKAP IR. Coronal and sagittal sections were preincubated with 0.3% H_2O_2 to reduce endogenous peroxidase. Non-specific binding sites were blocked by preincubating the sections with 5% normal rabbit serum in 0.01 M PBS for 30 min. Subsequently sections were incubated with rabbit polyclonal anti mAKAP, 1:1000 dilution (Abcam, ab24639) in 0.01 M PBS containing 5% normal goat serum and 0.3% Triton X-100 for 2 h at room temperature (RT) and left overnight at 4 °C. Afterwards the sections were rinsed and incubated at RT for 2 h with biotin-conjugated goat anti-rabbit antibody (1:400) (Jackson Inc., 115-065-145) in 0.01 M PBS containing 1% normal goat serum and 3% Triton X-100. This was followed by incubation with avidin complex containing biotinylated horseradish peroxidase (1:400) (Vectastain ABC Kit, Burlingame, CA, USA) for 2 h at RT. Finally, staining was visualized with 0.03% diaminobenzidine (DAB) chromogen substrate and 0.001% H_2O_2. Immunostained sections were analyzed with an Olympus BH2 microscope (Olympus, Japan). Two independent investigators established semi-quantification of mouse mAKAP IR. For each brain region, material from 6 animals was examined to establish mAKAP immunostaining. The scoring system used for establishing the relative AKAP IR was classified as follows: absent (-), low (-/+), moderate (+), high (++) and very high (+++). In case of very high staining in which presynaptic elements were detected, the immunoreactivity was classified as ++++.

Photographs were taken with a DM1000/DFC280 Leica image analysis system (Leica, Cambridge, UK).

Antibody specificity

Since there are no blocking peptides available for any mAKAP antibodies, we ascertained the specificity of both antibodies using several different approaches. Initially, parallel staining in free-floating brain sections was performed without primary antibodies. In these sections we could not observe any staining (Fig. 1A).

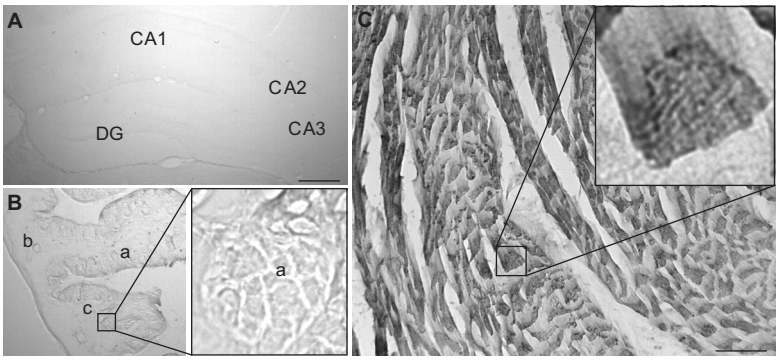

Fig. 1. mAKAP antibody specificity. (A) Control staining without mAKAP primary antibody in the hippocampus. CA1, CA2, CA3, cornu ammonis 1, 2, 3. DG, dentate gyrus, (B) mAKAP IR in the colon. a: lamina mucosa, b: lamina submucosae, c: lamina muscularis. (C) Positive mAKAP staining in mouse heart tissue. Enlarged: transversal section of muscular cells in the right ventricular wall of the mouse heart. Scale bar: A = 620 µm, B = 350 µm, C = 250 µm.

Furthermore, preincubation steps were performed to increase the specificity of mAKAP antibodies in the mouse brain. It was previously reported the mAKAP is not expressed in the colon (Kapiloff et al., 1999). Therefore, we tested the antibody specificity using mouse colon as a negative control and mouse heart and brain tissue as positive control. Colon, heart and brain tissue from perfused mice were dehydrated for 48 h in a 30% buffered sucrose solution. After dehydration, 20 µm coronal or sagittal sections were directly collected on precoated microscope glasses. mAKAP staining was performed on glass as described for immunostaining of free-floating sections. Controls without primary antibodies were also included. mAKAP immunoreactivity could not be detected in the colon (Fig. 1B)

while brain and heart tissue showed high mAKAP staining (Fig. 1C). In addition, no staining was detected in the control sections without primary or secondary antibody. Also in Western blot experiments mAKAP was not found in colon tissue. Overall we conclude that the rabbit polyclonal anty mAKAP antibody is specific for mAKAP in mouse tissue.

Results

Western blot analysis of mAKAP expression levels in various brain compartments

We detected mAKAP in homogenates of heart and cortex (brain) tissue but not in the colon of mice. Western blot analysis showed that our antibody can specifically detect both mAKAPα in the brain and mAKAPβ in the heart (Fig. 2A).

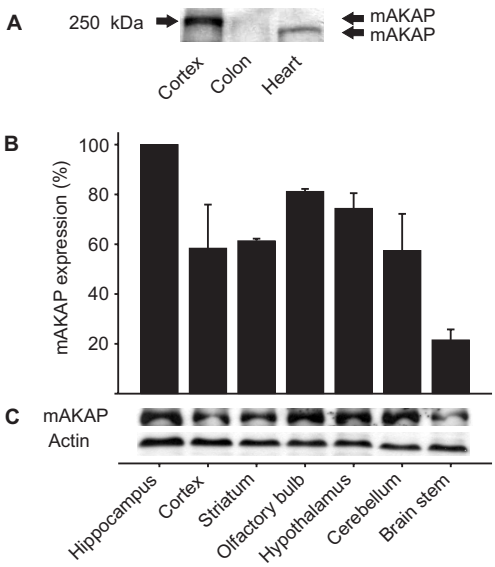

Fig. 2. Western blot analysis of mAKAP expression levels in mouse brain. (A) mAKAP antibody detects both mAKAPα and mAKAPβ. Tissue source is indicated below each lane. Colon was used as negative control (B) Bar graph representing mAKAP expression levels in different compartments of the mouse brain. Values represent mean percentages of integrated optical density (I.O.D) ± S.E.M. Hippocampus was set to 100% (C) Representative Western blot with protein extracts from different brain regions. Actin served as control for protein load. Blots are representative of three independent experiments.

Western blot analysis on protein extracts from different mouse brain regions revealed the highest expression level of mAKAP in hippocampus, hypothalamus, and olfactory bulb (Fig. 2B, C). Relatively high expression levels were also detected in the cortex, striatum, and cerebellum, while the brain stem showed low levels of mAKAP (Fig. 2B, C).

General overview of mAKAP IR in mouse brain

Immunohistochemical staining on coronal brain slices was performed to investigate the distribution of mAKAP in more detail. Antibody specificity was ascertained as described in materials and methods (section "Antibody specificity", Fig. 1). Overall, mAKAP was found to be widely distributed throughout the mouse brain. The highest mAKAP expression was detected presynaptically. For example, areas such as periventricular nucleus, supraoptic nucleus, suprachiasmatic nucleus and central amygdala were highly immunoreactive for mAKAP (Table 1, Fig. 3). High mAKAP immunoreactivity (IR) was also observed in various other regions like olfactory bulb, cortex, cerebellum, and hippocampus (Fig. 3). At the cellular level mAKAP was mostly expressed in cell bodies (e.g. the Purkinje cell layer of the cerebellum, pyramidal neurons in the cortex and hippocampus), but it was also found in fibers (e.g. stratum radiatum of the hippocampus, mossy fibers of the dentate gyrus). Some brain regions did not show any IR, such as the corpus callosum and the fornix (Fig. 3). Immunopositive endothelial cells or glia could not be detected.

Detailed description of mAKAP expression in mouse brain

Olfactory system

mAKAP is strongly expressed in the olfactory system. The accessory olfactory bulb (one of the strongest mAKAP immunoreactive regions in the mouse brain) was very densely stained and revealed mAKAP staining in fibers. The external plexiform layer and mitral cell layer also showed very high levels of IR of mAKAP. In these areas clear perikarya staining could be detected. In contrast, the glomerular layer, the granule layer, and internal plexiorm layer showed only moderate levels of mAKAP IR. Very high mAKAP IR was also found in the olfactory tubercle, in tenia tecta layers 1-3 and anterior olfactory nucleus, where perikarya staining was pronounced. (Fig. 3A, B, Table 1).

Cerebral cortex

mAKAP protein was found to be expressed throughout all layers of the cortex (Fig. 4A) The highest mAKAP IR in the mouse cortex was observed in cortical layer II (external granular layer), cortical layer III (external pyramidal layer) and cortical layer V (internal

pyramidal layer). Cortical layer II was highly immunoreactive for mAKAP protein in the perikarya of its granule cells (Fig. 4A). In layers III and V, which represent the principal output system of the neocortex, pyramidal neurons were strongly stained for mAKAP (Fig. 4A). Layer IV (internal granular layer) and the deepest cortical layer, layer VI (polymorphic layer), were immunoreactive for mAKAP to a somewhat lower extent (Fig. 4A). The lowest cortical IR was found in cerebral cortex layer I (molecular layer).

In addition, the entorhinal cortex, the major source of afferents to the hippocampal formation, was intensely stained (Table 1). At subcellular level, besides perikarya, dendrites were immunoreactive for mAKAP while occasionally immunopositive fibers with varicosities were detected (data not shown).

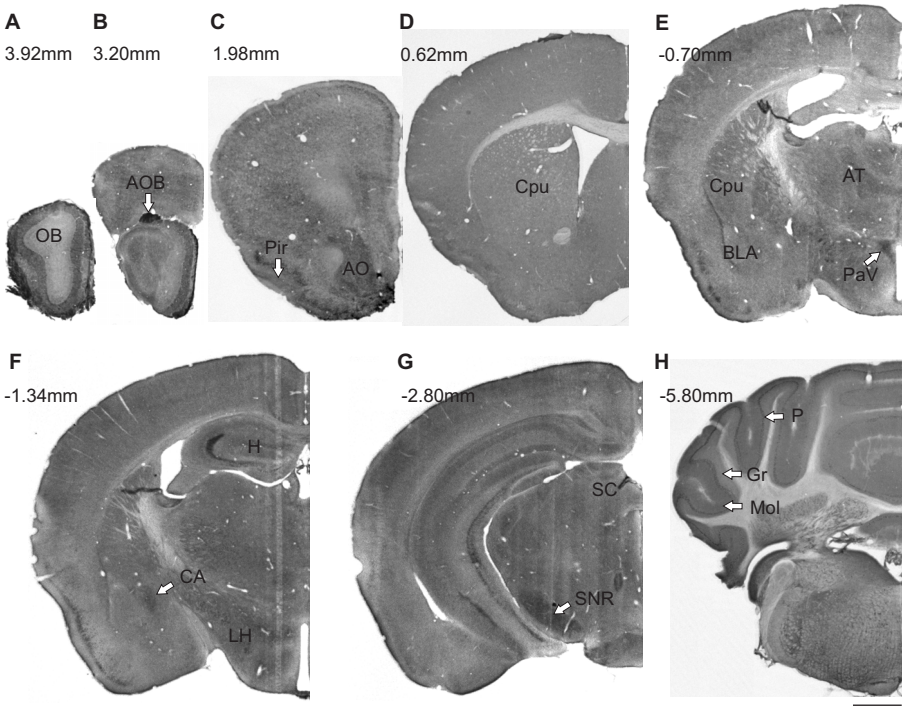

Fig. 3. Overview of mAKAP expression in coronal sections of the brain. Distance from bregma is indicated directly on the photographs (Franklin & Paxinos, 1997). (A) OB, olfactory bulb, (B) AOB, accessory olfactory bulb, (C) Pir, piriform cortex, AO, anterior olfactory nucleus, (D) Cpu, caudate putamen (striatum), (E) AT, anterior thalamus, BLA, basolateral amygdala, PaV, paraventricular hypothalamic nucleus ventral, Cpu, caudate putamen, (F) LH, lateral hypothalamic area, H, hippocampus, CA, central amygdala, (G) SC, superior colliculus, SNR, substantia nigra pars reticulata, (H) Mol, cerebellar molecular layer, Gr, cerebellar granular layer, P, Purkinje cell layer. Scale bar = 1300 µm

Hippocampus

Overall within this laminar brain structure, mAKAP displayed high IR (Fig. 4B; Table 1). Especially in the CA1-3 region of the hippocampus, mAKAP showed a very high expression particularly in the pyramidal layer where very high IR was detected in the perikarya of the pyramidal neurons (Fig. 4B, D, E).

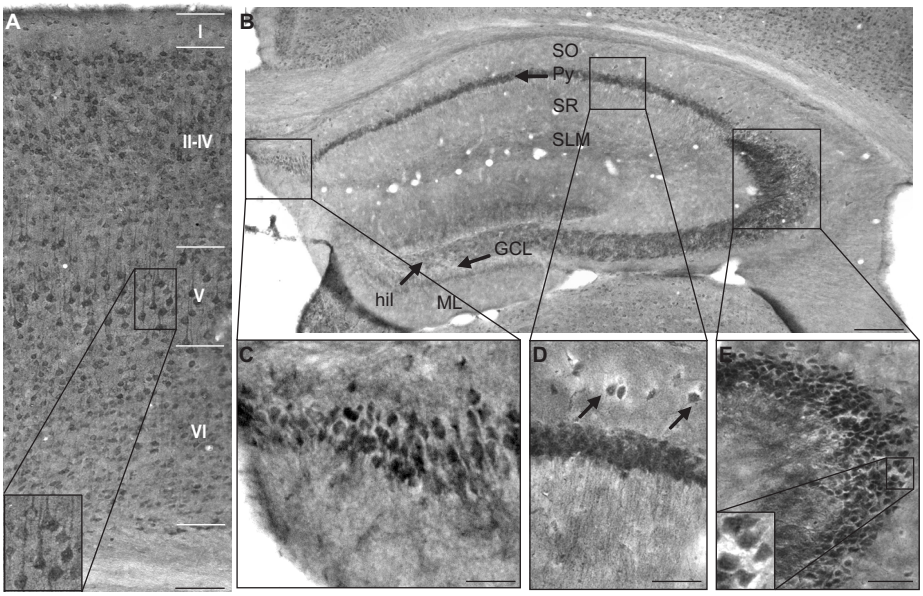

Fig. 4. Distribution of mAKAP protein in the cerebral cortex and hippocampus. (A) Overview of mAKAP protein expression in the cerebral cortex somatosensory 1, trunk region: I-VI cortical layers: I - molecular layer, II - external granular layer, III - external pyramidal layer, IV - internal granular layer, V - internal pyramidal layer, VI polymorphic layer. Enlarged: densely stained pyramidal neurons in cortical layer V, (B) mAKAP IR in the hippocampus: SO, stratum oriens, SR, stratum radiatum, SLM, stratum lacunosum moleculare, Py, stratum pyramidale, ML, dentate gyrus molecular layer, GCL, dentate granular cell layer and hil, hilus , (C) mAKAP staining in subiculum, (D) mAKAP IR in CA1 area of the hippocampus. Non-principal cells are also stained for mAKAP (arrows), (E) mAKAP IR in the CA3 area of the hippocampus. Enlarged: CA3 pyramidal neurons densely immunoreactive for mAKAP. Scale bar: A = 175 µm, B = 400 µm, C, D and E = 100 µm.

In stratum oriens and stratum lacunosum moleculare moderate to high mAKAP IR was observed. A divergent staining pattern was observed in the stratum radiatum (the apical dendrites of pyramidal neurons): Whereas in the CA1 area the apical dendrites were moderately stained (Fig. 4D), in the CA3 area extremely dense staining could be observed

(Fig. 4E). Moreover, the densely stained stratum lucidum revealed a mixture of mAKAP IR in mossy fibers and dendrites of CA3 pyramidal neurons. The subiculum also showed very high mAKAP IR in perikarya (Fig. 4C). Interestingly, scattered non-principal cells in the hippocampus were strongly stained for mAKAP protein (Fig. 4D).

In the dentate gyrus, mAKAP IR was characterized by moderate staining in the granule cells, while the granule cell layer showed a somewhat higher mAKAP IR (Fig. 4B). The staining made it difficult to distinguish subcellular compartments within the granule cell layer. The molecular layer of the dentate gyrus showed only moderate staining, whereas the hilus was slightly more immunoreactive (Fig. 4B).

Amygdala

The distribution of mAKAP was clear and strong throughout all amygdaloid nuclei (Table 1). The most prominent staining was detected in the central amygdaloid nucleus (Fig. 5), the basomedial amygdaloid nucleus (anterior and posterior) and intercalated nuclei (Table 1). In spite of the dense staining, perikarya staining could still be observed in these nuclei.

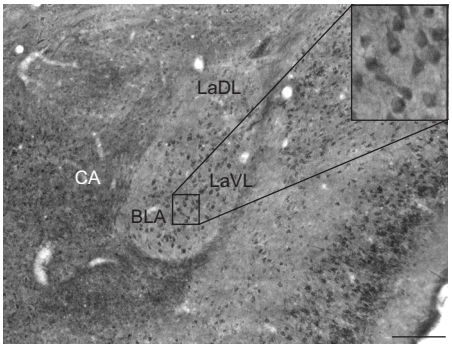

Fig. 5. mAKAP IR in the amygdala. CA, central amygdaloid nucleus, LaDL, lateral amygdaloid nucleus dorsolateral, LaVL, lateral amygdaloid nucleus ventrolateral, BLA, basolateral amygdaloid nucleus. Enlarged: cluster of perikarya in the basolateral amygdala. Scale bar = 200 μm.

The basolateral complex revealed a strong IR in the ventral nucleus and a more moderate staining in the anterior nucleus in which both neuronal perikarya and fibers were detected (Figs. 3E, F, 5, Table 1). Moderate mAKAP IR was also observed in the medial amygdaloid nucleus, lateral amygdaloid nucleus, amygdalohippocampal area and amygdalopiriform transition area (Table 1).

Septal nuclei and striatopallidal system

Overall, the distribution of mAKAP in the septal nuclei and the striatopallidal system was strong with only slight differences in the density of staining (Table 1). In the septum, the lateral nucleus was strongly stained, whereas the medial nucleus showed a somewhat lower expression (Fig. 3D; Table 1). In the caudate putamen all transected fiber bundles are mAKAP immunoreactive and also perikarya staining is present. Claustrum and ventral pallidum were also highly IR for mAKAP.

Epithalamus and thalamus

In the epithalamus mAKAP showed a moderate to high expression. In the medial habenula we observed high IR with visible perikarya staining (Table 1), whereas the lateral habenula was only moderately stained. Moreover, in the habenula, occasionally mAKAP positive could be detected.

At the level of the thalamus the overall distribution of mAKAP revealed a very high expression (Fig. 3E, Table 1). Detailed analysis of the stained thalamus displayed strong staining in the posterior nuclear group, the parafascicular thalamic nuclei and the subgeniculate nucleus. The ventrolateral thalamic nucleus was also highly immunoreactive for mAKAP. In contrast to the other areas, stained perikarya could be observed in this region. The remainder of the thalamus (i.e.: paratenial nucleus, rhomboid nucleus, ethmoid nucleus, mediodorsal nucleus) showed only moderate levels of mAKAP (Table 1). The lowest mAKAP IR was observed in the rhomboid nucleus and reuniens.

Hypothalamus

In general, the expression level of mAKAP in the hypothalamus was high. Accordingly, paraventricular and suprachiasmatic nucleus displayed the highest mAKAP staining whereas the median eminence, arcuate nucleus, mammilary nuclei and periventricular nucleus showed a reduced but still strong pattern of staining (Fig. 3F, Table 1), (Table 1).

Midbrain

mAKAP protein was moderately to very strongly expressed in the midbrain region (Table 1). The highest staining was observed in the substantia nigra pars reticulata, interpeduncular

and pointine nuclei (Fig. 3G). Moderate levels of mAKAP IR were detectable in inferior and superior colliculus. Low levels were found in the periaqueductal gray and brachium pontis (Table 1).

Cerebellum

A high homogeneous staining was seen throughout the entire cerebellar cortex with no apparent expression difference between the molecular and granule cerebellar layer (Fig. 6A). The Purkinje cell bodies and their fibers were very clearly stained covering the complete cerebellar cortex (Fig 6, Table 1).

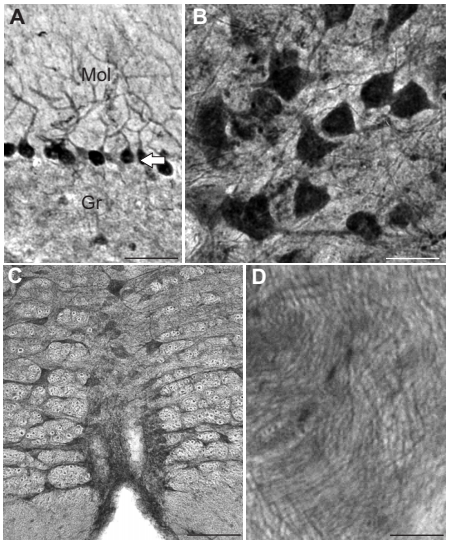

Fig. 6. mAKAP expression in the cerebellum. (A) Mol, molecular layer, Gr, granular layer. White arrow shows purkinje neurons with staining in both cell body and dendrites, (B) facial nucleus, (C) raphne magnus nucleus, (D) spinal trigeminal tract. Scale bar: A, C and D = 15 µm, B = 30 µm.

Brain stem

High levels of mAKAP were also detected in the raphne nucleus, facial nucleus, lateral (dentate) cerebellar nucleus and the dorsal cochlear nucleus (Fig. 3H, Tabel 1). In these nuclei, at higher magnification, the staining was detected in both cell bodies and fibers. In the spinal trigeminal tract, moderate staining was detected in fibers. Lower mAKAP IR was observed in the inferior cerebellar peduncle (Tabel 1).

mAKAP expression in aged mice

Finally, we compared mAKAP protein levels of young (4-6 months) and old (24-26 months) mice. Immunohistochemical analysis revealed a different expression level for mAKAP in young and old mice. Accordingly, mAKAP IR was dramatically decreased throughout the entire brain of old mice. Brain regions in which mAKAP was very highly expressed in young mice (e.g.: paraventricular hypothalamic nucleus, central amygdala, etc.) exhibit only low mAKAP expression in old mice (Fig. 7).

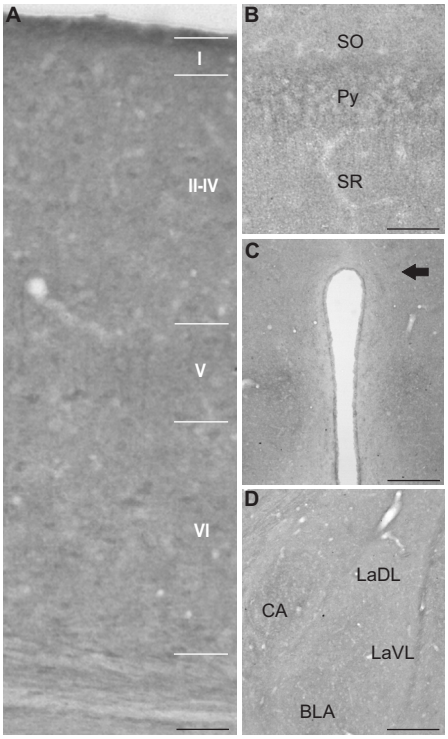

Fig. 7. mAKAP expression in several brain regions of 26 months old mouse brain: (A) mAKAP expression in the cortex. I-VI cortical layers: I - molecular layer, II - external granular layer, III - external pyramidal layer, IV - internal granular layer, V - internal pyramidal layer, VI polymorphic layer, (B) mAKAP expression in the CA1 area of the hippocampus: SO, stratum oriens, Py, stratum pyramidale, SR, stratum radiatum, (C) Arrow indicates low mAKAP IR in paraventricular hypothalamus nucleus, (D) mAKAP IR in the amyglada: CA, central amygdaloid nucleus, LaDL, lateral amygdaloid nucleus dorsolateral, LaVL, lateral amygdaloid nucleus ventrolateral, BLA, basolateral amygdaloid nucleus. Scale bar: A = 180 µm, B = 100 µm, C and D = 250 µm

To confirm these results, we compared mAKAP protein expression in the hippocampus and cortex from young and old mice using Western blot analysis. Both in the cortex and the hippocampus of old mice, the levels of mAKAP were significantly decreased when compared to young controls (cortex 61% reduction, hippocampus 47 % reduction; Fig. 8).

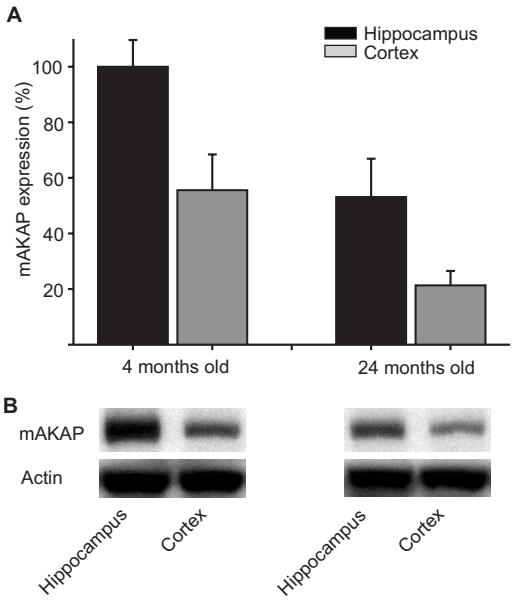

Fig. 8. Western blot analysis of mAKAP expression levels in young and old mouse brain (A) mAKAP expression levels of hippocampus and cortex in brain tissues from 3 and 26 months old mice. Values represent mean percentages of integrated optical density (I.O.D). ± S.E.M. Hippocampus was set to 100%, (B) Representative Western blot for mAKAP. Actin was used as control for protein load.

Table 1 - Expression of mAKAP protein in various compartments of the mouse central nervous system.

Brain region	mAKAP
Telencephalon	
Olfactory system	
Glomerular layer	++
External plexiform layer	+++
Mitral cell layer	+++
Internal plexiform layer	+++
Granule layer	++
Anterior olfactory nucleus	++
Olfactory tubercle	+++
Tenia tecta, layers 1-3	++/+++
Anterior commisure, anterior	-/+
Accessory olfactory bulb	+++
Cerebral cortex	
Layer I	+/++
Layer II	+++
Layer III	+++
Layer IV	++
Layer V	++/+++
Layer VI	++
Piriform cortex	++
Hippocampal formation	
Dentate gyrus	
Granule cell	+
Granule cell layer	++
Molecular layer	++/+++
Hilus	++
CA1 region	
Stratum oriens	++
Stratum radiatum	++
Stratum lacunosum moleculare	++/+++
Stratum pyramidale	
CA3 region	
Stratum oriens	++
Stratum radiatum	+++
Stratum pyramidale	+++
Stratum lucidum	+++
Subiculum	+++
Entorhinal cortex	++/+++
Amygdala	
Central amygdaloid nucleus	++++
Medial amygdaloid nucleus	+/++
Lateral amygdaloid nucleus	++
Basolateral amygdaloid nucleus, anterior	++
Basolateral amygdaloid nucleus, ventral	++/+++
Basomedial amygdaloid nucleus	++/+++
Amygdalohippocampal area	+++
Amygdalopiriform transition area	++
Intercalated nuclei	+++
Posteromedial cortical amygdaloid nucleus	+++
Septal and basal magnocellular nuclei	
Bed nucleus stria terminalis	++++
Nucleus accumbens core	++/+++
Nucleus accumbens shell	++/+++

Tabel 1 (Continued)

Brain region	mAKAP
Septal and basal magnocellular nuclei	
Substantia innominata	+++
Septum lateral nucleus	+++
Septum medial nucleus	++
Striatopallidal system	
Caudate putamen	++
Globus pallidus	++
Lateral globus pallidus	++
Ventral pallidum	++
Claustrum	++
Islands of Calleja	+/++
Diencephalon	
Thalamus	
Ventrolateral nucleus	++/+++
Ventral posterolateral	++/+++
Ventral posteromedial	++/+++
Mediodorsal nucleus	++
Central nuclei	++
Paracentral nucleus	++
Parafascicular thalamic nuclei	+++
Paraventricular thalamic nuclei	++
Subgeniculate nucleus	++/+++
Reticular thalamic nucleus	+/++
Posterior nuclear group	+++
Paratenial nucleus	++
Rhomboid nucleus	+/++
Ethmoid nucleus	++
Reuniens	+/++
Subthalamic nucleus	++/+++
Zona incerta	++
Epithalamus	
Habenula medial	+++
Habenula lateral	++
Hypothalamus	
Median eminence	++++
Arcuate nucleus	+++
Supraoptic nucleus	+++
Paraventricular nucleus	++++
Periventricular nucleus	+++
Suprachiasmatic nucleus	++++
Mammillary nuclei	+++
Fornix	++
Mesencephalon	
Midbrain	
Superior colliculus	++
Inferior colliculus	++
Interpeduncular nuclei	+++
Pontine nuclei	+++
Substantia nigra	
pars reticulata	++/+++
pars compacta	++
Periaqueductal gray	+
Brachium pontis	+
Rhombencephalon	
Cerebellum	
Molecular layer	++
Purkinje cell layer	+++
Granule cell layer	++
Raphne nucleus	++/+++
Facial nucleus	+++
Lateral cerebellar nucleus	++/+++
Dorsal cochlear nucleus	++
Spinal trigeminal tract	++
Inferior cerebellar peduncle	+

Scoring was classified as absent (-), low (-/+), moderate (+), high (++), very high (+++), very high including presynaptic staining (++++).

Discussion

Previous studies reported that mAKAP is predominantly expressed in cardiac and skeletal muscle, but it was also observed to some extent in the brain (Kapiloff et al., 1999). Nevertheless, detailed knowledge on the localization and the function of mAKAP in the brain remained so far rather limited. In this study we investigated the distribution of mAKAP in the brain of young and old mice in detail. Our results show that overall, mAKAP is abundantly expressed in the mouse brain and its distribution is thus not

restricted to a particular compartment. Very high mAKAP IR was detected in the cortex, cerebellum, thalamus, hypothalamus and hippocampus. These results are in good agreement with the reported mRNA expression of AKAP100/mAKAP in several human brain regions (Kapiloff et. al., 1999; McCartney et al., 1995). AKAP100 mRNA was reported to be highly expressed in the human motor and frontal cortex, hippocampus, basal ganglia and cerebellum (McCartney et al., 1995). Moreover, our results from immunohistochemistry were confirmed by Western blot analysis in several compartments of the mouse brain. So far two mAKAP variants have been identified: mAKAPα (preferentially expressed in the brain) and mAKAPβ (preferentially expressed in the heart) (Michel et al., 2005). mAKAPβ is a shorter form of the anchoring protein that lacks the first 244 amino acids. The unique amino terminus of mAKAPα can spatially restrict the activity of 3-phosphoinositide-dependent kinase-1 (PDK1) (Michel et al., 2005). Indeed, we detected on Western blot proteins with a lower motility in the mouse brain corresponding to mAKAPα whereas in the mouse heart the shorter mAKAPβ was observed (Kapiloff et al., 1999).

Michel and colleagues generated mice that have mAKAPα exchanged for mAKAPβ. mAKAPα null mice had reduced viability and a smaller body size as compared to wild-type and heterozygous littermates and showed craniofacial defects (Michel et al., 2005). These data suggest that switching the tissue-specific expression pattern of mAKAP forms has critical effects on the formation of brain signaling complexes which are maintained by this anchoring protein.

Although our results showed mAKAP IR throughout the entire mouse brain, detailed analysis revealed some striking differences between particular brain regions. For example, the highest mAKAP IR was detected in discrete regions of the limbic system such as paraventricular hypothalamic nucleus, supraoptic nucleus, suprachiasmatic nucleus, central amygdala, and bed nucleus stria terminalis. Interestingly, this presynaptic expression pattern overlaps remarkably well with the expression pattern for corticotrophin releasing factor (CRF) (Pilcher & Joseph, 1984). CRF and the related urocortin peptides mediate behavioral, cognitive, autonomic, neuroendocrine and immunologic responses to aversive stimuli by activating CRF receptor 1 or CRF receptor 2 in the central nervous system and

anterior pituitary. The paralleled distribution of mAKAP with the CRF system suggests that mAKAP may also be involved in a variety of functions such as emotion, behaviour, memory, and autonomic responses. For example, it has been shown that cardiac function is critically regulated by the autonomic nervous system to adapt to the physical activity and emotional stress and mAKAP was reported to play a very important role in heart physiology. However, it remains to be determined whether there is a crosstalk between CRF and mAKAP in the brain and in the heart.

Besides its localization in the limbic system at similar sites as CRF, there are several additional lines of evidence indicating that mAKAP could be involved in cognition. Long-term changes in synaptic strength are thought to be the cellular basis underlying learning and memory processes. These changes are mediated through alterations in gene expression via the activation of transcription factors such as NFAT and cAMP responsive element binding protein (CREB) (Wang et al., 2006). Like in the heart, calcineurin, which is anchored to mAKAP, mediates NFAT translocation to the nucleus and this is critically dependent on increased intracellular calcium levels in neurons (Graeff et al., 1999). Furthermore, several effectors of the mAKAP complex including intracellular calcium release and the ERK pathway are known to affect PKA-induced CREB phosphorylation and CREB-dependent transcription (Zanassi et al., 2001). Thus one could speculate that mAKAP is important in coordinating gene expression at the nuclear membrane which is essential for e.g. long-term memory storage. We observed reduced levels of mAKAP in aged mice. In this respect it is interesting to note that cAMP levels are also reduced over the course of aging leading to improper regulation of CREB activation (Sugawa & May, 1994).

Our immunohistochemical analysis revealed that mAKAP is expressed both in cell bodies (e.g.: pyramidal neurons in the cortex and hippocampus, neurons in the striatum and amygdala) and in dendrites (e.g.: apical and basal dendrites of hippocampal pyramidal neurons). It was previously reported that mAKAP is targeted to the nuclear membrane of differentiated myocytes (Kapiloff et al., 1999). Moreover, immunohistochemical analysis revealed that AKAP100 (mAKAP) was localized to the sarcoplasmic reticulum in both cardiac and skeletal cell lines (McCartney et al., 1995; Yang et al., 1998). Smooth

endoplasmic reticulum is the best characterized Ca^{2+} signaling organelle and represents a heterogeneous endomembrane system that extends from the neuronal soma to most cell compartments, including axons and dendritic spines (Meldolesi, 2001). In the mouse brain mAKAP is also detected in dendrites. This could be due to the fact that mAKAP is attached to the endoplasmic reticulum in this subcellular compartment.

In the heart, mAKAP recruits PKA to the cardiac ryanodine receptor (RyR) via an interaction with a leucine zipper-like motif (Marx et al., 2001). This channel is found on both the sarcoplasmatic reticulum and the nuclear envelope in cardiac and skeletal muscle cells and mediates calcium-induced calcium release from intracellular stores (Bootman et al., 2000). In cardiomyocytes, mAKAP facilitates the PKA-induced increase in channel conductance by linking this kinase to the RyR (Kapiloff et al., 2001, Ruehr et al., 2003). Moreover, localization of PKA by mAKAP at RyR1 increases both PKA-dependent RyR phosphorylation as well as efflux of Ca^{2+} through the RyR (Ruehr et al., 2003). RyR are also present in dendritic spines in the brain as indicated by immunohistochemical localization studies (Martone et al., 1997; Sharp et al., 1993) and by Ca^{2+} imaging (Emptage et al., 1999; Raymond & Redman, 2006). Although is difficult to speculate on the role of mAKAP in the brain on the basis of its distribution, it may very well be that mAKAP is involved in Ca^{2+} store-mediated signaling in the brain similar to its function in the heart. For example, a target of the Ca^{2+} store-mediated events could be CaMKII, which is known to associate with PSD following LTP induction (Shen et al., 2000).
Overall, the widespread distribution of mAKAP suggests an important role for this protein in multiple brain functions. This hypothesis is further strengthened by the fact that all the molecules so far identified as members of the mAKAP macromolecular complex such as PKA, PDE, Epac, calcineurin and MAPK play a crucial role in multiple brain processes and mAKAP may act as an important coordinator of these signaling pathways. Moreover, the age-dependent decrease in mAKAP expression may influence the proper coordination of signal transduction pathways and thereby contribute to changes in brain function during ageing.

Acknowledgments

We thank Martijn Clausen and Marcia Peters for valuable technical support.

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