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Reduced expression of kinase-associated phosphatase in cortical dendrites of MAP2-deficient mice

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Abstract

We previously demonstrated that cAMP-dependent protein kinase was reduced in the dendrites of MAP2-deficient mice. In this study, we compared the expression of various protein phosphatases (PPs) between wild-type and *map2*$^{-/-}$ dendrites. Kinase-associated phosphatase (KAP) was the only PP which showed difference between the two phenotypes: (1) the expression of KAP was reduced in *map2*$^{-/-}$ cortical dendrites, and (2) the amount of KAP bound to microtubules was reduced in *map2*$^{-/-}$ brains. We also demonstrated in cultured neuroblastoma cells that KAP is not only expressed in dividing cells, but also in the neurites of differentiated cells. Our findings propose that KAP, which has been reported to function in cell-cycle control, has an as yet uncovered role in regulating dendritic functions. We also propose MAP2-deficient mice as an ideal system for identifying protein phosphatases essential for dendritic functions.

Keywords: Kinase-associated phosphatase; Dendrite; Microtubule-associated protein 2; cAMP-dependent protein kinase; Dual-specificity phosphatase; Protein phosphatase; Knockout mice

As neurons have a highly polarized morphology, the discrete subcellular location of enzymes is crucial for their specified functions. Accumulating evidence shows that many protein kinases (PKs) and PPs are anchored to the cytoskeleton or the plasma membrane of neurons, in order to locate themselves close to their substrates. PKA is the most thoroughly investigated example, and the group of proteins mediating the compartmentalization of PKA is called A-kinase anchoring proteins (AKAPs) [1]. The most abundant AKAP in neurons is MAP2 [2], a filamentous microtubule-associated protein (MAP) expressed at high levels in dendrites [3]. In our previous paper, we demonstrated a significant reduction of PKA in the mature dendrites of MAP2-deficient mouse [4]. This result led us to the presumption that the amounts of essential PPs might similarly be reduced. In this study, we therefore employed this *map2*$^{-/-}$ mouse to seek for PPs that behave similarly to PKA.

We first examined PP1, PP2A, and calcineurin (CN), the three well-characterized serine/threonine phosphatases. We expected some distributional changes of them in *map2*$^{-/-}$ neurons, because they have been reported to regulate the phosphorylation of MAP2 and to be associated with MTs [5,6]. Moreover, several scaffold proteins have been reported to anchor serine/threonine phosphatases and PKA together [1]; AKAP79 binds both CN and PKA, and yotiao binds PP1 and PKA to assemble a signaling complex with the NMDA receptor.

We next examined KAP, which has been identified as a dual-specificity phosphatase (dsPTP) interacting with cyclin-dependent kinases, and is also called Cdi1 [7,8]. Very interestingly, KAP was the only phosphatase that showed a difference in expression between the wild-type and *map2*$^{-/-}$

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mice. This was completely unexpected, because to date, there have been no reports suggesting any active roles of KAP in neurons. Our results suggest an as yet unknown crucial role of KAP in regulating the function of cortical dendrites.

Materials and methods

Generation of mutant mice. The MAP2 targeting vector was linearized with *Not*I before electroporation. Electroporation, genomic Southern blot, and blastocyst injection were performed as described previously [4].

Antibodies. Mouse monoclonal antibodies against KAP, pan-PP1, and PP2A Cα were purchased from BD Biosciences Pharmingen (San Diego, CA). The rabbit polyclonal antibody against CN/PP2B B was from Upstate Biotechnology (Lake Placid, NY). The mouse monoclonal antibody against CN (α-subunit) and FITC-conjugated DM1A antibody were from Sigma (St. Louis, MO). Alexa 488-conjugated antibodies were from Invitrogen (San Diego, CA), and alkaline phosphatase-conjugated anti-mouse IgG was from Bio-Rad (Hercules, CA). Anti-PKA catalytic subunit polyclonal sera and anti-MAP1A monoclonal antibody were obtained as described previously [4].

Immunohistochemistry. Cerebral cortices were dissected from wild-type and *map2*$^{-/-}$ mice (8-week-old) anesthetized with ether and Nembutal, and perfused with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The procedures for fixation, cryoprotection, freezing, and sectioning were as previously described [4]. For single labeling, sections were reacted with the primary antibodies, followed by Alexa 568-conjugated secondary antibodies. For double labeling, the sections were initially reacted with anti-MAP1A monoclonal antibody, followed by Alexa 568-conjugated anti-mouse IgG, and finally with the anti-KAP antibody conjugated with Alexa-488 using the Zenon Alexa Fluor 488 Mouse IgG1 Labeling kit (Invitrogen). The sections were observed using a laser scanning confocal microscope (model MRC-1024; Zeiss, Oberkochen, Germany), and the images were processed digitally using Adobe Photoshop (Adobe Systems, San Jose, CA).

Western blotting. MT pellet and supernatant fractions were prepared from wild-type and *map2*$^{-/-}$ mice (8-week-old) as previously described [4]. A high-molecular-weight MAP (HMW-MAP) fraction was isolated from purified microtubule proteins using phosphocellulose chromatography, as previously described [9]. Protein concentration was determined using the BCA Protein Assay reagent (Pierce, Rockford, IL). The MT pellet (10 μg), the supernatant (10 μg), and the HMW-MAP fraction (1.6 μg) were separated by polyacrylamide gel electrophoresis, transferred to PVDF membranes (Millipore, Billerica, MA), and immunostained with primary antibodies, followed by an alkaline phosphatase-conjugated secondary antibody.

Cell culture and immunocytochemistry. Neuro-2A neuroblastoma cells were first subcloned to obtain a highly uniform population. Cells were then plated on collagen-coated coverslips in DMEM (Invitrogen) with 10% fetal calf serum. To induce neurite outgrowth, the medium was supplemented with 2% serum and 20 μM retinoic acid (Sigma). After about five days, the cells were permeabilized (0.02% saponin, 10 μM taxol in PBS, 5 min), fixed (4% paraformaldehyde in PBS, 37 °C, 15 min), and double-stained with the anti-KAP antibody followed by Alexa 568-conjugated anti-mouse IgG, and with the FITC-conjugated DM1A antibody (anti-tubulin antibody).

Results

KAP is reduced in cortical dendrites of map 2$^{-/-}$ *mice*

We previously demonstrated a significant reduction of PKA in the dendrites of *map2*$^{-/-}$ mice [4]. Here, we stained sections of cerebral cortices from the wild-type and *map2*$^{-/-}$ mice with various antibodies against PPs.

We first examined the three major serine/threonine phosphatases, PP1 PP2A and CN, using antibodies against pan-PP1, PP2A Cα (the major catalytic subunit of PP2A), CNA (catalytic subunit of CN), and CNB (regulatory subunit of CN). As shown in Figs. 1B–E′, there was no difference in the distribution between the wild-type and *map2*$^{-/-}$ mice.

We next tested KAP, which belongs to the dsPTPs, using an anti-KAP monoclonal antibody. Contrary to our expectation, KAP was the only PP that showed differences in localization between the wild-type and *map2*$^{-/-}$ mice (Figs. 1A and A′). As demonstrated in Fig. 2, the staining pattern of KAP was remarkably similar to that

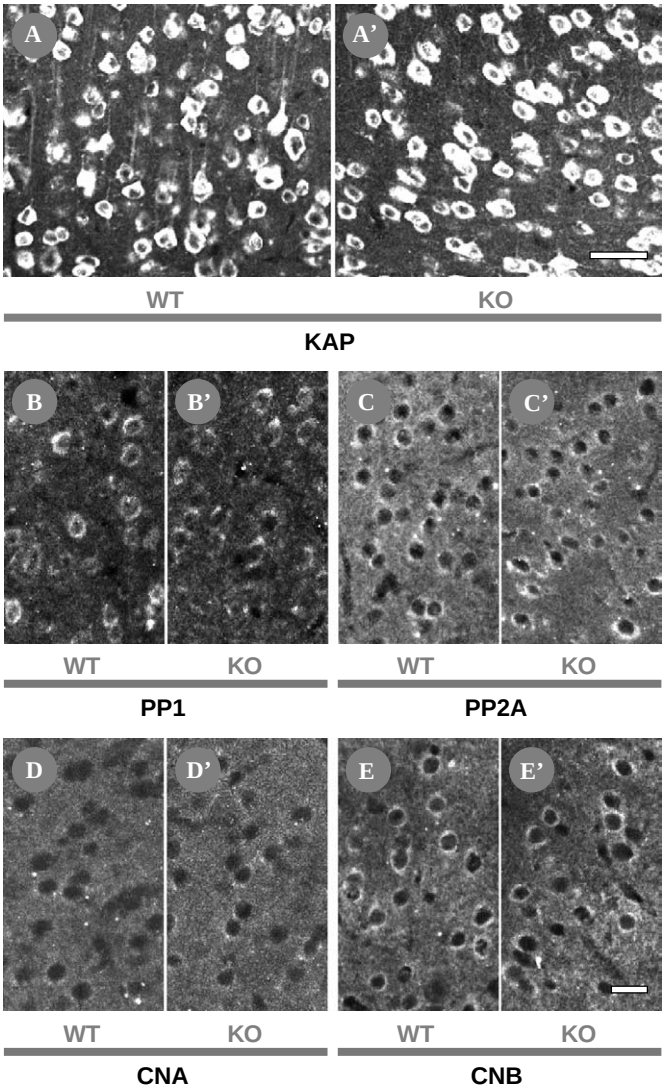

Fig. 1. Localization of various PPs in cerebral cortex of wild-type and *map2*$^{-/-}$ mice. Panels show sections of the cerebral cortex from a wild-type mouse (WT), and from a *map2*$^{-/-}$ mouse (KO), immunostained for KAP (A and A′), PP1 (B and B′), PP2A (C and C′), CNA (D and D′), and CNB (E and E′). KAP was clearly expressed in the dendrites of the wild-type mouse (A), but not in the dendrites of the *map2*$^{-/-}$ mouse (A′). The staining properties of PPA, PP2A, CNA, and CNB showed no distributional differences between the wild-type and *map2*$^{-/-}$ mice. Scale bars: (in A′) A–A′, 50 μm; (in E′) B–E′, 20 μm.