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Yotiao protein, a ligand for the NMDA receptor, binds and targets cAMP-dependent protein kinase II[1]

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Abstract A yeast two-hybrid screen revealed that regulatory subunits (RII) of PKAII bind the Yotiao protein. Yotiao interacts with the NR1 subunit of the NMDA receptor. A purified C-terminal fragment of Yotiao binds PKAII, via an RII binding site constituted by amino acid residues 1452–1469, with a dissociation constant (K_d) between 50 and 90 nM in vitro. A stable complex composed of Yotiao, RII and NR1 was immunoprecipitated from whole rat brain extracts. Immunostaining analysis disclosed that Yotiao, RIIβ and NR1 colocalize in striatal and cerebellar neurons. Co-assembly of Yotiao/PKAII complexes with NR1 subunits may promote cAMP-dependent modulation of NMDA receptor activity at synapses, thereby influencing brain development and synaptic plasticity.

Key words: Yotiao protein; NMDA receptor; Protein kinase A

1. Introduction

Phosphorylation of intracellular substrates by protein kinases conveys signals generated at the plasma membrane to regulatory molecules that control multiple cell functions [1]. Serine/threonine protein kinases and phosphatases are often targeted to specific subcellular locations by anchor proteins [2–4]. Anchored PKAII is implicated in the regulation of certain ion channel activities, hormone secretion, sperm motility and cAMP-regulated gene transcription [2–8]. Furthermore, expression of a mitochondrion-associated AKAP (AKAP121) is markedly induced by cAMP [9]. This suggests that hormones may differentially control the distribution of cAMP signals to specific intracellular sites by altering the concentration of an AKAP. Some AKAPs (e.g. AKAP79) are thought to be multifunctional proteins that bind several signaling molecules (protein kinase C, calcineurin, PKAII) thereby integrating information from multiple regulatory inputs [3].

N-methyl-D-aspartate (NMDA) receptors are expressed throughout the mammalian central nervous system. They mediate excitatory synaptic transmission and may be involved in brain development, excitotoxicity, learning and memory [10]. NMDA receptors are pentameric proteins composed of one or two NR1 subunits and a subset of NR2 A-D subunit isoforms [10]. Molecular diversity of NMDA receptors is further increased by the existence of numerous NR1 splice variants [10]. Critical physiological and functional properties of the receptor are contributed principally by NR1 subunits [10]. Cyclic AMP stimulates NMDA receptor-mediated currents and Ser^{897} in NR1 is a good target site for PKA-catalyzed phosphorylation [11–13].

Yotiao is a coiled coil protein that is expressed in mammalian brain and accumulates near neuromuscular junctions [14]. It interacts with the C1-exon specific splice variant of NR1 and forms a protein complex with the NMDA receptor in vivo.

We now identify Yotiao as an AKAP that binds and targets PKAII to a microenvironment that contains the NR1 subunit of the NMDA receptor. Enrichment of PKAII in proximity with clustered NR1 (and NMDA receptors) would increase the sensitivity of a critical target/effector to regulation by locally generated cAMP.

2. Materials and methods

2.1. Yeast two-hybrid screening

Mouse full-length RIIα cDNA was cloned in the yeast bait plasmid pBD-Gal4 (Stratagene). The corresponding cDNA encodes a fusion protein with the DNA binding domain of Gal4 fused to the NH_2-terminus of RIIα. Approximately 1×10^6 clones were screened via the two-hybrid interaction assay, using a rat thyroid cDNA library cloned in the yeast expression vector pBD-Gal4 (Stratagene) [9]. Auxotrophic yeast clones growing on selective medium lacking Leu, Trp and His were isolated and tested for β-galactosidase activity.

2.2. Expression of recombinant proteins in Escherichia coli and RII binding analysis

Amino-terminal truncations of the protein encoded by RBP64 cDNA were generated by polymerase chain reaction using specific oligonucleotide primers. Recombinant proteins were expressed in *E. coli* BL-21(DE3). The hexahistidine (H6)-tagged fusion proteins were purified as previously described [4]. A peptide derived from the α-helical wheel of Yotiao protein was synthesized:

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[1] Data deposition: the sequence of RBP64 clone has been deposited in the GenBank/EBI Data Bank with the accession number AF133906.

Abbreviations: PKA, protein kinase A (cAMP-dependent protein kinase); R, regulatory subunit of PKA; C-PKA, catalytic subunit of PKA; AKAP, PKA anchor protein; NMDA, *N*-methyl-D-aspartate

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$_{1450}$ERLEEEIAKVIVSMSIAFAQQTE$_{1472}$-amide. A mutated peptide with Ala^{1457} > Pro and Val^{1459} > Pro was also synthesized (Primm, Italy). RII binding assays were carried out as previously described [4].

2.3. Antibodies, immunoblotting and immunoprecipitation

Rabbits were immunized with a His-tagged fusion protein of RBP64. Immune serum (anti-Yotiao) was used at the following dilutions: Western blot (1/1000), immunoprecipitation (1/200) and immunostaining (1/1600). Rabbit anti-RII polyclonal antibodies were previously described [4]. For immunoprecipitation experiments, a mixture of anti-RIIα and anti-RIIβ polyclonal antibodies was used. Rat brain and synaptosomal membrane proteins from whole rat brain were prepared as described [14]. Protein kinase A assays were performed as described [6].

2.4. Northern gel analysis

Total RNA was extracted from rat tissues as previously described [7].

2.5. Immunofluorescence

Cerebellum sections from Sprague Dawley rats (4 weeks of age) and rat primary striatal neurons were double stained with anti-RIIβ or anti-Yotiao polyclonal antibodies and monoclonal antibodies against NR1 were carried out as described [6].

3. Results

A cDNA library derived from rat thyroid mRNAs was inserted into the yeast vector pGAD10 and screened by using as 'bait' a cDNA encoding a full-length RII in the yeast plasmid, pGBD10 (pGBD-RII) (Fig. 1A). The interaction screen yielded multiple isolates of different types of cDNAs. One clone was isolated and named RBP64. Coexpression of pGAD-RBP64 with pGBD-RII vectors in yeast strongly activated transcription of the His reporter gene (Fig. 1B). The open reading frame of RBP64 cDNA clone is 1182 bp in length and encodes a 394 amino acid polypeptide. Sequence and Southern blot analyses indicate that RBP64 cDNA is a portion of the rat homolog of human Yotiao cDNA, corresponding to the region 3904–5086 bp [14]. Yotiao belongs to a multiple spliced family of proteins coded by a single gene sequence and binds the C1-exon specific splice variant of the NR1 subunit of NMDA receptors [14,15]. RBP64 cDNA detected an 11 kb mRNA in brain and heart, which corresponds to the size of the Yotiao transcript [14]. Yotiao is also expressed in testes, thyroid and a thyroid-derived cell line (FRTL-5, 9) (Fig. 1C). Yotiao contains a putative RII binding site, common to all AKAPs. Amino acids with large aliphatic side chains that are essential for high affinity binding of RII appear at the same positions in Yotiao, AKAP75 and AKAP120 proteins [16,17]. Deletion mutagenesis of the clone RBP64 cDNA was employed to map the RII binding site. cDNA fragments including all or part of the putative RII binding site were subcloned in the pRSET-A plasmid and recombinant His-tagged fusion proteins were expressed in *E. coli*. RII overlay binding assays disclosed that residues 202–219 of the RBP64 protein (residues 1452–1469 of human Yotiao) mediate RII binding (Fig. 2B,C). Two recombinant fragments of RBP64 were tested in the ligand-blot analysis by probing denatured electroblots with [^{32}P]-RIIβ, in the presence or absence of unlabelled RIIβ. As shown in Fig. 2D, unlabelled RIIβ inhibited [^{32}P]-RII binding in a dose-dependent manner. The results demonstrated that recombinant fragment of rat Yotiao binds RII, with a K_d ranging between 50 and 90 nM for both recombinant fragments in vitro. To further analyze the affinity between RII and Yotiao, a synthetic

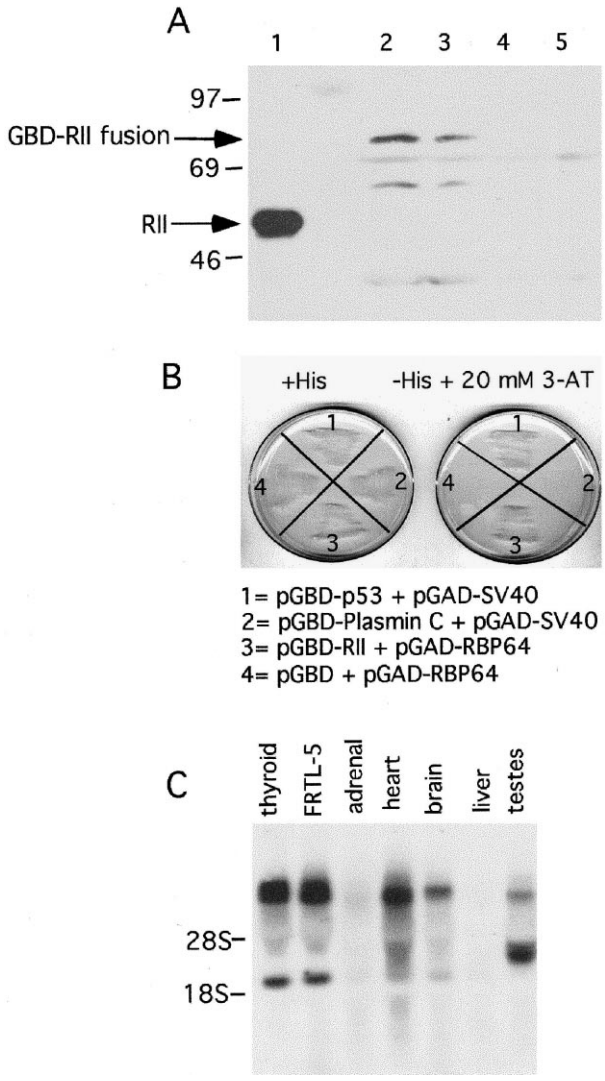

Fig. 1. Identification of a cDNA clone (RBP64) that interacts with the PKA regulatory subunit, RII, in a yeast two-hybrid system. A: 200 ng of His-tagged RII purified protein (lane 1) or total protein extracts (100 μg) from control YRG-2 yeast strain (lanes 4, 5) or from YRG-2 cells expressing recombinant pGBD-RII fusion protein (lanes 2, 3) were loaded on a 10% SDS-PAGE gel and immunoblotted with anti-RIIα polyclonal antibodies. B: YRG-2 cells were co-transformed with the indicated yeast expression vectors and grown for 2 days on selective plates. A histidine analog 3-aminotriazole (3-AT) was added to His-plates. C: Total RNA (20 μg) from several rat tissues was size-fractionated on a 0.9% agarose gel, transferred to an N-Hybond filter and hybridized with ^{32}P-labelled RBP64 cDNA probe.

peptide spanning the RII binding site of human Yotiao was used in a competition curve with [^{32}P]-RIIβ and RBP64-2F recombinant fragment. Fig. 2D shows that the peptide competed the binding of [^{32}P]-RIIβ to RBP64-2F with an apparent IC_{50} between 70 and 150 nM. A synthetic peptide mutated in Ala^{1457} > Pro and Val^{1459} > Pro, that disrupt the amphipathic helix, did not compete the binding of [^{32}P]-RIIβ to RBP64-2F recombinant polypeptide [3].

Immunoprecipitation of Triton X-100 solubilized membrane proteins from whole rat brain with anti-RII antibody resulted in the co-isolation of Yotiao (Fig. 3A). Anti-RII or anti-Yotiao antibody also efficiently co-precipitated NR1 subunit (Fig. 3B). The results suggest that Yotiao, NR1, and RII

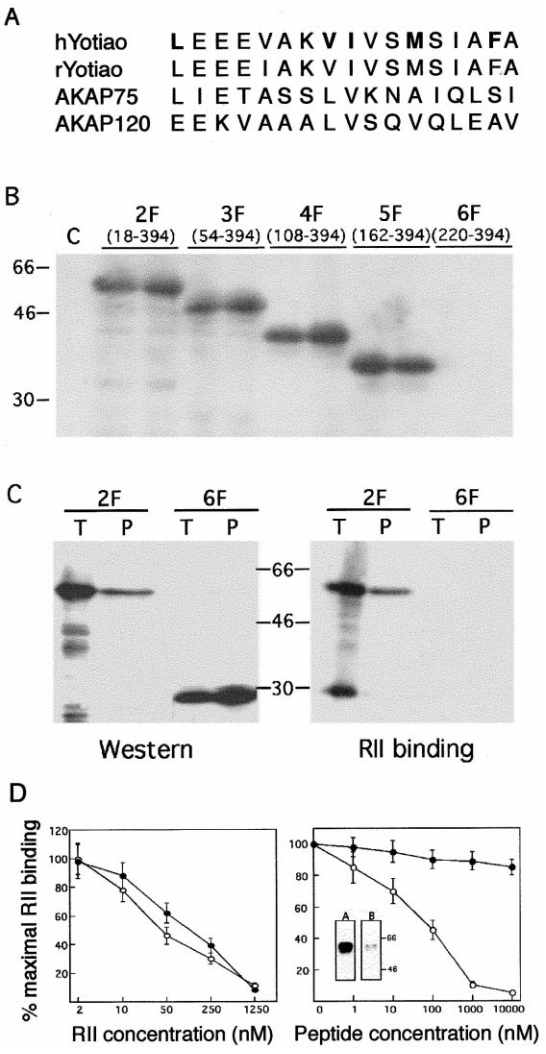

Fig. 2. Characterization of the RII binding site of Yotiao. A: Amino acid sequence alignment of the RII binding regions of two AKAPs with the putative RII binding sites of human Yotiao (hYotiao, residues 1452–1469) and that derived from the rat homolog (rYotiao, residues 202–219 of the clone RBP64). In bold are the conserved hydrophobic residues essential for RII binding activity. B: His-tagged recombinant proteins of the rat clone RBP64 were size-fractionated by SDS-PAGE, transferred to Immobilon P and blotted with full-length ^{32}P-labelled RIIβ. C: Total protein (T) or 100 ng of affinity purified RBP64 fragments (P) from *E. coli* expressing the RBP64-2F or RBP64-6F deletion mutants, were immunoblotted with anti-Xpress (Invitrogen) polyclonal antibodies (left panel) or ligand-blotted with ^{32}P-labelled RIIβ (right panel). D: Left panel: Electroblots containing 100 ng of either RBP64-2F (open circles) or RBP64-4F (closed circles) were probed with ^{32}P-labelled RIIβ in presence or absence of increasing concentrations of unlabelled RIIβ protein. Right panel: The RII binding of RBP64-2F fragment was analyzed using ^{32}P-labelled RIIβ preincubated with increasing concentrations of synthetic peptide spanning the α-helical RII binding site of the human Yotiao (open circles) or with a mutant peptide (close circles). Inset: Immunoblots containing 100 ng of recombinant RBP64-2F peptide were probed with [^{32}P]-RIIβ in the absence (A) or presence (B) of 5 μM of Yotiao peptide (residues 1450–1472).

interact in a stable manner in vivo. Immunoprecipitation of either Yotiao or NR1 from whole rat brain resulted in a 7.3 ± 1.2- and 6.9 ± 1.9-fold enrichment of cAMP-dependent protein kinase activity in the immune complexes over the control serum. Kinase A activity was fully inhibited by a specific PKA inhibitor peptide (PKI), demonstrating that NR1/Yotiao interact in vivo with the kinase. We estimate that PKA activity immunoprecipitated from whole rat brain with NR1 or Yotiao antibodies represents about 1.2 ± 0.3% of the total PKAII immunoprecipitated with anti-RII antibodies (Fig. 3C). PKA activity recovered in the immunoprecipitates of NR1 or Yotiao of synaptosomal membranes is enriched 3–4-fold compared to that of whole rat brain (Fig. 3D). These

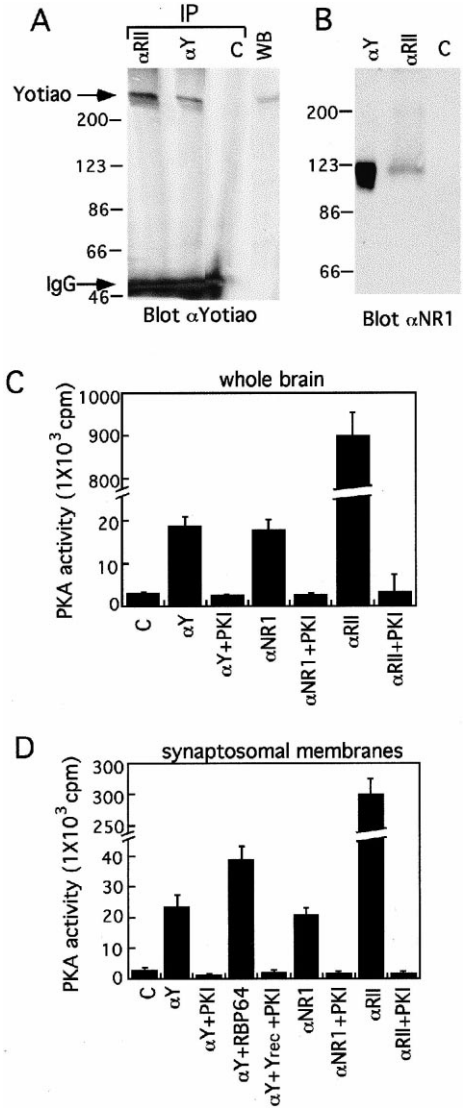

Fig. 3. RII and Yotiao coimmunoprecipitate with NR1. A: Total proteins (6 mg) extracted from whole rat brain were immunoprecipitated with anti-RII (α and β) or anti-Yotiao antibody. Immunoprecipitates (IP) and 400 μg of proteins from whole rat brain (WB) were immunoblotted with anti-Yotiao antibody. B: Detection of NR1 in the immunoprecipitates of Yotiao or RII immunoblotted with affinity purified anti-NR1 monoclonal antibodies. C, D: PKA activity of immunoprecipitates from rat brain. Total proteins (2 mg) were solubilized with Triton X-100 from either whole rat brain or synaptosomal membranes and subjected to immunoprecipitation either with anti-Yotiao (αY), anti-NR1 (αNR1), anti-RII (αRII) or pre-immune sera (C). PKA activity on immunoprecipitates was assayed in absence or presence of a PKA specific inhibitor (PKI). Where indicated, 500 ng of recombinant Yotiao polypeptide (Yrec) were added to brain extracts before immunoprecipitation. The data represent a mean of four independent experiments ± S.E.M.

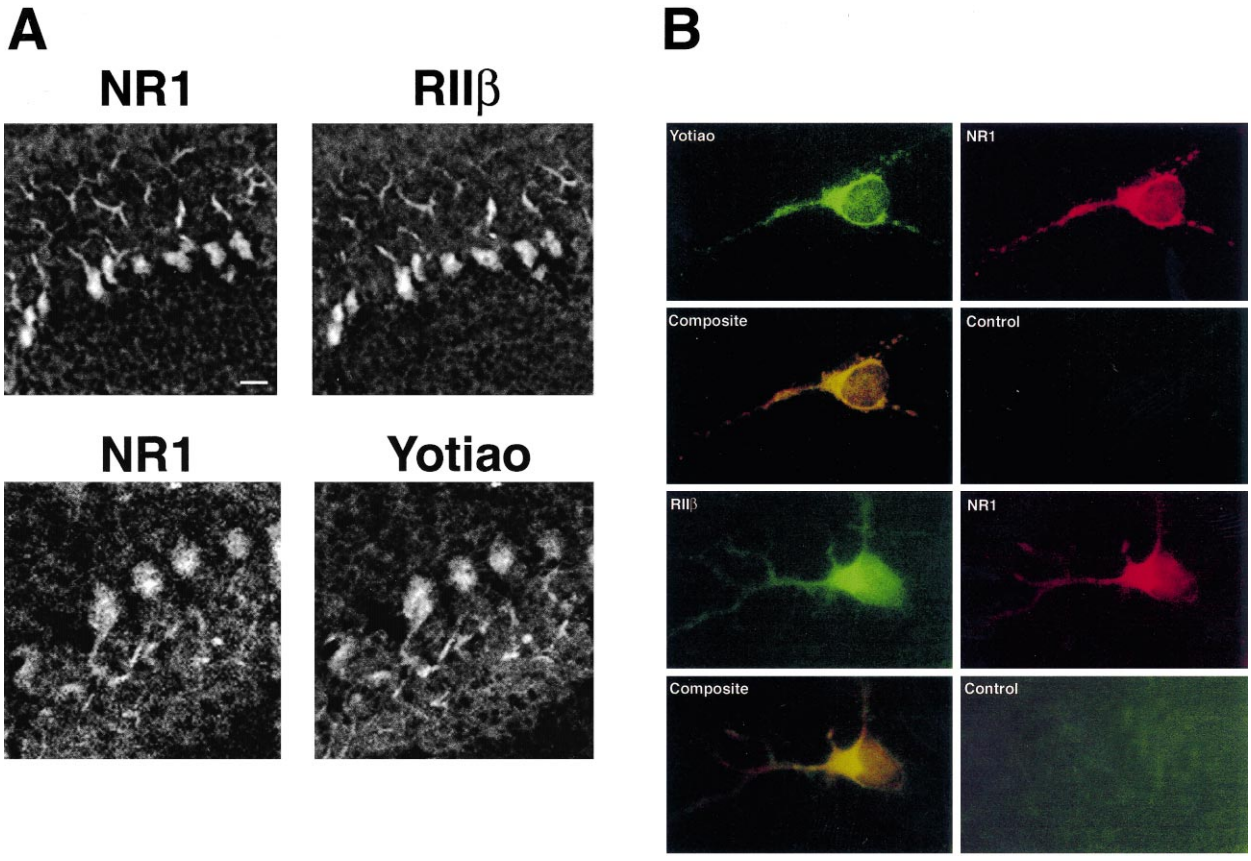

Fig. 4. Immunofluorescence analyses of Yotiao, RIIβ and NR1. A: Cerebellum sections were probed with either anti-Yotiao or anti-RIIβ polyclonal antibodies and monoclonal antibodies against NR1. Confocal microscopy was used to collect fluorescence signals after application of secondary antibodies tagged with fluorescein isothiocyanate or rhodamine. Size bar: upper panels, 45 μm; lower panels, 22 μm. B: Rat primary striatal neurons were probed with anti-Yotiao polyclonal antibodies or anti-RIIβ and monoclonal antibodies against NR1. Fluorescein isothiocyanate (Yotiao and RIIβ) or rhodamine (NR1) fluorescent signals were collected: a composite of two images (Yotiao/NR1 and RIIβ/NR1) is also shown. In control panels, the first antibodies (anti-Yotiao or anti-RIIβ) were preincubated with excess of Yotiao or RIIβ recombinant proteins, respectively.

data suggest that the complex PKAII·Yotiao·NR1 accumulates specifically in synaptosomes. The addition of recombinant fragment of rat Yotiao to synaptosomal membrane extracts increased 12.5 ± 1.8-fold the cAMP-dependent kinase activity in the anti-Yotiao immunoprecipitates over the control serum. These findings indicate that Yotiao is limiting in vivo.

RIIβ and PKAIIβ are concentrated in the soma and dendrites of many types of neurons in olfactory bulb, basal ganglia, striatum, cerebral cortex, and other forebrain regions [18]. Yotiao is especially abundant in the soma and the dendritic arborizations of Purkinje cells, but the protein is also evident in neurons in most regions of the mammalian brain. The localization of Yotiao partly overlaps with that of NR1 [14]. To determine the distribution of Yotiao, RIIβ and NR1 in situ, sections of 4 weeks old rat cerebella were analyzed by immunofluorescence staining. Sections were probed with both monoclonal anti-NR1 and polyclonal anti-Yotiao antibodies or with monoclonal anti-NR1 and polyclonal anti-RIIβ antibodies (Fig. 4A). The fluorescence signals indicate that NR1 colocalizes with Yotiao in cell bodies and dendritic arborizations of Purkinje cells. RIIβ is likewise concentrated in cell bodies and dendrites and the distributions of RIIβ and NR1 partially overlap. The intracellular distributions of Yotiao and RIIβ are also similar. Furthermore, double immunofluorescence analysis revealed that Yotiao and NR1 immunoreactivity had overlapping staining patterns in the soma and dendrites of striatal neurons (Fig. 4B). RIIβ signal on the other hand overlaps only partially since it is present in other localizations inside the cell (Fig. 4).

4. Discussion

We have shown that Yotiao binds RII with high affinity and interacts in vivo with PKAII. Thus, Yotiao may serve as a scaffolding protein, anchoring PKA holoenzyme close to the NMDA receptor. The ability to sequester PKAII and co-localization with RII and NMDA receptors in neurons in situ suggest that Yotiao mediates incorporation of the anchored kinase into multi-component signaling modules. Our data complement a recent report showing that PKA and a phosphatase are present in the same complex in vitro with the NMR1 subunit, but the trimeric complex in vivo and the affinity of Yotiao and PKA have not been determined [19].

NMDA receptors are multimeric proteins that directly couple with calmodulin, calmodulin-activated protein kinase II, SynGAP and other cytoskeleton-associated adapter proteins that assemble and organize clusters of receptors. The nature and stoichiometry of proteins associated with NMDA receptors may markedly modulate receptor functions [20,21]. Ion

currents elicited by NMDA agonists are potentiated by cAMP analogs and forskolin [10,22]. Moreover, PKA efficiently phosphorylates Ser^{897} in NR1 in vitro and in vivo [13]. However, PKA also promotes NR1 phosphorylation at several sites (including Ser^{899}, Ser^{890}) by activating DARPP-32, an inhibitor of protein phosphatase 1 [23]. In addition, protein kinase C-mediated phosphorylation of Ser^{889} and Ser^{890} in NR1 regulates the clustering of NMDA receptors [13,24] and inhibits binding of calmodulin (a channel inhibitor) to the C-terminus of NR1 [21]. Thus, the precise role of PKA-catalyzed phosphorylation in the regulation of NMDA receptor functions remains to be elucidated.

We propose that the complex formed in the membrane containing PKAII, functions as a 'transduceosome' by physically transmitting the signals from cAMP/PKA to the NMDA receptor via Yotiao. Yotiao-mediated association of PKAII with post-synaptic NMDA receptors could have a variety of fundamentally important consequences. Activation of anchored PKAII might (a) directly alter NMDA channel activity in the context of a multi-protein effector complex; (b) modulate the clustering of receptors; (c) promote or inhibit recruitment of other proteins that interact with and regulate the receptor/channel; or (d) promote phosphorylation of co-localized AMPA receptors, which are an established target for anchored PKA.

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