Formation of Novel TRPC Channels by Complex Subunit Interactions in Embryonic Brain*§

Carsten Strübing‡§, Grigory Krapivinsky‡, Luba Krapivinsky, and David E. Clapham†

From the Howard Hughes Medical Institute, Department of Cardiovascular Research, Children's Hospital and Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115

Mammalian short TRP channels (TRPCs) are putative receptor- and store-operated cation channels that play a fundamental role in the regulation of cellular Ca\(^{2+}\) homeostasis. Assembly of the seven TRPC homologs (TRPC1–7) into homo- and heteromers can create a large variety of different channels. However, the compositions as well as the functional properties of native TRPC complexes are largely undefined. We performed a systematic biochemical study of TRPC interactions in mammalian brain and identified previously unrecognized channel heteromers composed of TRPC1, TRPC4, or TRPC5 and the diacylglycerol-activated TRPC3 or TRPC6 subunits. The novel TRPC heteromers were found exclusively in embryonic brain. In heterologous systems, we demonstrated that assembly of these novel heteromers required the combination of TRPC1 plus TRPC4 or TRPC5 subunits along with diacylglycerol-sensitive subunits in the channel complexes. Functional interaction of the TRPC subunits was verified using a dominant negative TRPC5 mutant (TRPC5DN). Co-expression of TRPC3DN suppressed currents through TRPC5- and TRPC4-containing complexes; TRPC3-associated currents were unaffected by TRPC5DN unless TRPC1 was also co-expressed. This complex assembly mechanism increases the diversity of TRPC channels in mammalian brain and may generate novel heteromers that have specific roles in the developing brain.

Cellular Ca\(^{2+}\) signaling is dependent on ubiquitously expressed receptor- and store-operated cation channels (ROCs1 and SOCs). These channels mediate Ca\(^{2+}\) influx in response to hormones and other stimuli that activate phospholipase C (PLC) isoenzymes (1–3). The widespread expression and functional diversity of ROCs and SOCs is reflected by the large variety of these channels with diverse biophysical properties and regulation mechanisms.

There is growing evidence that members of the TRPC cation channel family can form ROCs and SOCs. Belonging to the larger superfamily of mammalian TRP channels, seven TRPCs (TRPC1–7) have been identified (4, 5). Heterologous expression of the highly homologous TRPC3, 6, and 7 gave rise to diacylglycerol (DAG)-activated ROCs (6, 7). TRPC1, 4, and 5 constitute a second structural TRPC subfamily. Whereas TRPC4 and TRPC5 were activated by an unknown PLC-dependent mechanism (8, 9), TRPC1 was characterized as a SOC (10). Other studies indicated that TRPC3, TRPC4, and TRPC5 might also contribute to SOCs activated by depletion of intracellular Ca\(^{2+}\) stores (11–14).

Based on their structural similarity with voltage-dependent K\(^{+}\) channels, functional TRPC complexes are presumed to be tetramers. Many of the disparate results regarding TRPC function and regulation could be reconciled by assuming that TRPC subunits can assemble into heteromorphic channels with diverse properties. Montell and co-workers demonstrated that TRPC1 and TRPC3 formed complexes based on co-immunoprecipitation studies of tagged proteins (15). We reported that TRPC1 co-assembled with TRPC4 and TRPC5 in rat brain (16). The biophysical properties of TRPC1(+4) and TRPC1(+5) heteromers are surprisingly distinct from those of the channel homomers. Whereas TRPC4 and TRPC5 channels expressed alone were doubly rectifying, TRPC1(+4) and TRPC1(+5) heteromers outwardly rectified, similar to native G\(_{q}\)-coupled receptor-activated cation currents in hippocampus and frontal cortex (17–19). An elegant study confirmed the interaction of TRPC1 with TRPC4 and TRPC5 using intermolecular fluorescence resonance energy transfer (FRET) measurements in heterologous expression systems but found no interaction between members of the DAG-responsive TRPC subfamily (TRPC3 and TRPC6) and other TRPC subunits (20). A similar interaction pattern was recently reported for TRPC channel heteromers in rat brain synaptosomes (21).

Despite the progress in analyzing TRPC subunit interactions in heterologous expression systems, it is unclear whether the same principles apply to TRPC channel assembly in vivo. We used TRPC isoform-specific antibodies to identify heterotypic TRPC interactions in rat brain. Our findings demonstrate a developmentally regulated expression of TRPC subunits. In prenatal brain, various endogenous TRPC heteromers were detected, including complexes containing TRPC3 or TRPC6 and the DAG-insensitive TRPC1, TRPC4, or TRPC5. When expressed heterologously, the formation of these TRPC complexes was critically dependent on the presence of TRPC1 in conjunction with TRPC4 or TRPC5.
**EXPERIMENTAL PROCEDURES**

**Cell Culture, Cloning, and Transfection**—HM1 cells, a HEK 293 line stably expressing the muscarinic M1 receptor (22), and HEK293T cells were used in this study. Cells were grown at 37°C in Dulbecco’s modified Eagle’s medium/F12 (1:1) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin and a hypoxanthine and thymidine (HT) supplement (1:100) (Invitrogen) in 5% CO2, G418 (0.5 mg/ml) was added to the HM1 growth medium. The cDNAs for hTRPC1 (accession number U31110), hTRPC3 (NM 003305), hTRPC4 (AJ136296), hTRPC5 (AF029983), and hTRPC6 (AF060394), eGFP (pGreenLantern, Invitrogen), and DeRed2 (pDeRed2-N1, BD Clontech) were transfected using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s instructions. In biochemical co-expression experiments 5 μg of cDNA of each TRPC isoform was transfected per 10-cm plate of confluent cells. The total amount of transfected cDNA was adjusted to 15 μg by supplementing with empty vector.

For electrophysiological and imaging experiments, cells were transfected with the indicated amounts of cDNA in 35-mm dishes and plated onto coverslips 12–24 h after transfection. Cells were used 24–48 h after plating. If not indicated otherwise, 0.4 μg of eGFP was co-transfected as an expression marker for patch clamp experiments.

**Immunoprecipitation and Western Blot**—For immunoprecipitation experiments, combinations of TRPCs were expressed in HEK293T cells for 48 h. Rat brain microsome preparation, protein solubilization, immunoprecipitation, and Western blots were performed essentially as described (16). Brain microsomes were isolated from embryos (embryonic days 16–18) or 6 week-old rats. Antibodies—TRPC1 and TRPC5 antibodies were characterized previously (16). A rabbit polyclonal antibody was raised against rat TRPC4 C-terminal amino acids 956–977 (23) and affinity purified. TRPC3 and TRPC6 antibodies were purchased from Alomone Labs, Jerusalem, Israel. All antibodies used were tested by Western blot using TRPC1, 3, 4, 5, and 6 proteins expressed in HEK293T cells. All antibodies specifically recognized their antigenic proteins and did not cross-react with any of the tested TRPCs (data not shown). In experiments with brain microsomes, the specificity of the protein recognition on Western blot was confirmed by antibody pre-absorption with antigenic peptide. TRPC1, TRPC4, and TRPC5 antibodies were used for immunoprecipitation.

**Construction of TRPC5DN-eGFP**—The full-length mTRPC5 cDNA was cloned into pIREs-eGFP (Clontech). Replacement of the TRPC5 stop codon with a NotI site and deletion of the internal ribosome entry site (IRES) sequence yielded a TRPC5-eGFP fusion protein containing a 10-amino acid linker (AAAGDPPVAT). The nonfunctional, dominant negative TRPC5-eGFP (TRPC5DN-eGFP) was constructed by alanine replacement of a conserved LFW motif (amino acids 575–577) within the TRPC5 pore region. An insert containing the mutated residues was confirmed by two-step PCR. Mutated fragments were amplified with primers F1 (5′-CCAAAGCTGAACAGACGCGGCGGCCGACTGAAGGGTTTC-3′) and L2mut (5′-GGGAGGCCAAAGACAGACGCGGCGGCCGACTGAAGGGTTTC-3′) and F2mut (5′-CCAAAGCTGAACAGACGCGGCGGCCGACTGAAGGGTTTC-3′) and F2mut (5′-GGGAGGCCAAAGACAGACGCGGCGGCCGACTGAAGGGTTTC-3′). The fragments were then used as templates, and full-length inserts were made by PCR with primers F1/R2. The insert was cut with NheI/NotI and ligated to a corresponding fragment of TRPC5-eGFP. The identity of the mutations was confirmed by sequencing.

**Electrophysiology, Ca2+ Imaging, and Confocal Microscopy**—Whole cell currents were recorded from eGFP- or eGFP/DeRed2-positive cells at room temperature with an Axopatch 200A patch clamp amplifier and pClamp 8 software (Axon Instruments, Union City, CA). Patch pipettes with resistances of 2–5 MΩ in standard extracellular buffer were pulled from borosilicate glass. The holding potential was set to −70 mV, and currents were continuously sampled at 220 Hz. Currents during 160-ms voltage ramps from −100 to +60 mV were sampled with 6.6 kHz. All recordings were filtered at 1 kHz.

Standard external buffer contained 140 mM NaCl, 5.4 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM glucose, and 10 mM HEPES, and the pH was adjusted to 7.4 with NaOH. The standard pipette buffer contained 120 mM CsOH, 120 mM gluconic acid, 2 mM MgCl2, 3 mM CaCl2, 5 mM CsCl, 5 mM Cs2-BAPTA, and 10 mM HEPES, and the pH was adjusted to 7.3 with gluconic acid. Free [Ca2+]i was calculated to be ~200 nM using the CaBuF program (G. Droogmans, Katholieke Universiteit, Leuven, Belgium). Receptor-activated currents were elicited in HM1 cells by the application of 10 μM carbachol. The phosphatidylcholine-specific PLC from Clostridium perfringens used to raise intracellular DAG levels was from Fluka (Buchs, Switzerland).

All statistical data is expressed as means ± S.E. Statistical analysis was performed using SigmaPlot (SPSS, Chicago, IL). Data groups contained only results from matched experimental series that were measured on the same day. Results were pooled and analyzed using the Mann-Whitney rank sum test. The significance level was set to p < 0.05.

**RESULTS**

**Differential Expression of TRPC Subunits in Embryonic and Adult Brains**—Subunit interactions require spatial and temporal overlap of the expression of the potential interaction partners. Although mRNA for all TRPC isoforms can be found in brain (4), the temporal expression pattern of TRPC proteins has only begun to be examined. Rat brain TRPC3 protein was expressed only within a narrow temporal window between embryonic day 18 and postnatal day 20 (24), suggesting that TRPC channel subunit composition might also be developmentally regulated. Here we compare the expression of TRPC proteins in the brain of embryonic (embryonic day 18) and adult (postnatal days 40–45) rats. All proteins tested (TRPC1, 3–6) were recognized by Western blot in embryonic brain preparations. In adult brain TRPC1, TRPC4, and TRPC5, proteins were detected only at significantly lower levels than in embryonic brain tissue (Fig. 1). We were not able to detect TRPC3 and TRPC6 in adult brain. Hence, membranes from embryonic tissue were used for further experiments.

**Identification of TRPC Heteromers in Embryonic Brain**—To investigate possible interactions of TRPC isoforms between the TRPC1, 4, and 5 and the TRPC3, 6, and 7 subfamilies, we immunoprecipitated proteins from solubilized rat brain membranes with anti-TRPC1, anti-TRPC4, or anti-TRPC5 antibodies and probed the immunoprecipitates with anti-TRPC1 as well.
as anti-TRPC3 and anti-TRPC6 antibodies (Fig. 2A). In these experiments, TRPC1, TRPC3, and TRPC6 were consistently detected in TRPC4 and TRPC5 immunoprecipitates from embryonic brain. Immunoprecipitates from adult brain did not contain detectable TRPC3 or TRPC6 (Fig. 2A). Notably, this lack of TRPC3 and TRPC6 was not due to low expression levels of the immunoprecipitated subunits, because comparable amounts of TRPC1, 4, and 5 were precipitated from embryonic and adult brain (Fig. 2B). Co-immunoprecipitation of the TRPC subunits was not the result of nonspecific binding, because TRPC3 and TRPC6 were not detected in anti-TRPC1, anti-TRPC4, or anti-TRPC5 precipitants from cells expressing TRPC3 or TRPC6 (Fig. 3). These interactions suggest that TRPC3 and TRPC6 form molecular complexes with TRPC1, TRPC4, and TRPC5 in embryonic brain.

**Reconstitution of Inter-subfamily Subunit Interactions in Vitro**—This finding was somewhat surprising, because previous work in heterologous systems has demonstrated that only TRPC subunits from the same subfamily (i.e. TRPC1, 4, and 5 or TRPC3, 6, and 7) form molecular complexes with each other (20). We, therefore, tested the interaction of recombinant TRPC isoforms expressed in HEK293T cells and found that, in agreement with this previous work, TRPC3 did not co-immunoprecipitate with TRPC4 or TRPC5 (Fig. 3A). TRPC6 interacted very weakly with TRPC4 and not at all with TRPC5 (Fig. 3B).

We hypothesized that an additional protein(s) missing in the heterologous system linked TRPC4 and TRPC5 with TRPC3 and TRPC6 subunits in neurons. A candidate for this function was TRPC1. We have shown earlier that TRPC1 co-assembled with TRPC4 and TRPC5 in rat brain as well as in expression systems (16). TRPC1 had also been reported to bind to TRPC3 subunits in vitro (15, 25). Although the latter interaction was not confirmed by other studies (20, 21), we decided to test whether TRPC1 was able to tether DAG-sensitive and -insensitive subunits in heteromeric complexes. When TRPC1 was co-expressed in addition to combinations of TRPC4 or 5 and TRPC3 or 6, we observed the formation of mixed heteromers containing DAG-sensitive and -insensitive subunits (Fig. 3). The weak interaction of TRPC6 and TRPC4 was significantly enhanced by TRPC1 co-expression (Fig. 3). Therefore, TRPC1 could reconstitute the subunit interactions found in rat brain in heterologous cells.

In light of the contradictory reports on the ability of TRPC1 to bind DAG-sensitive TRPCs, we next examined the hypothesis that TRPC1 acts as a universal mediator of TRPC subunit interactions. Therefore, TRPC1 was transfected with either TRPC3 or TRPC6 in HEK293T cells, and the formation of
heteromeric complexes was tested by co-immunoprecipitation. TRPC3 or TRPC6 were not detected in TRPC1 immunoprecipitates (Fig. 4), making it unlikely that TRPC1 represents a simple linker with binding affinity to TRPC4 and 5 as well as to TRPC3 and 6 isoforms.

Functional Assessment of TRPC Interactions—Physical interactions between TRPC subunits can result in the formation of channel pores with novel properties. To test this possibility, we studied whole cell currents in HM1 cells co-transfected with different combinations of TRPC subunits. Channels of the TRPC1, 4, and 5 and also the TRPC3, 6, and 7 subfamily respond to activation of PLCβ (6–9). In cells co-expressing TRPC1, 3, and 5, stimulation of PLCβ by the muscarinic agonist carbachol elicited outwardly rectifying currents that were almost identical to TRPC(1+5) (Fig. 5A). However, current rectification in triple (TRPC1, 3, and 5) transfected cells was less pronounced than in TRPC(1+5), allowing significantly larger currents in the inward direction (Fig. 5B). To test whether the TRPC3 subunit could confer DAG sensitivity to channel heteromers in TRPC1-, 3-, and 5-transfected cells, we raised the intracellular DAG concentration by application of bacterial phosphatidylinositol-specific PLC (10 units/ml) (26). This procedure robustly activated TRPC3 and TRPC6 homomeric channels in HM1 cells.2 Unlike the effect of carbachol, stimulation of triple transfected cells with phosphatidylinositol-PLC did not produce outwardly rectifying currents but rather activated small currents only. Mean maximum currents amounted to 664 ± 85 pA for TRPC1, TRPC3, and TRPC5 only. Mean maximum currents amounted to 664 ± 85 pA for TRPC1, TRPC3, and TRPC5 and 562 ± 86 pA for TRPC1- and TRPC5-transfected cells. The gray areas correspond to the variation (S.E.) of the normalized currents.

To further test the hypothesis that TRPC3 subunits form heteromeric pores with TRPC1 and 5, we studied the effect of a dominant negative TRPC5 subunit on channel function. In contrast, co-expression of TRPC5DN-eGFP with TRPC3 had no significant effect on TRPC3 currents (Fig. 6). Although TRPC5DN-eGFP alone did not reduce TRPC3 currents, concomitant overexpression of TRPC1 and TRPC5DN-eGFP led to reduced TRPC3 currents (Fig. 1B). TRPC1 had no inhibitory effect per se, as TRPC3 current densities were not different in cells co-transfected with either TRPC1 or eGFP (Fig. 7B). TRPC1-mediated suppression by TRPC5DN was not the result of decreased expression or mislocalization of TRPC3 protein. When a TRPC3-yellow fluorescent protein (YFP) fusion protein was co-transfected with non-fluorescent TRPC5DN and TRPC1 or an equal amount of LacZ, cDNA membrane localization (determined as ratio of intracellular versus peripheral mean pixel intensity values) as well as total mean fluorescence intensities were unchanged (p > 0.05; n > 50 for each group; Supplemental Fig. 1, which is available in the on-line version of this article). To further control for nonspecific

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2 C. Strubing, unpublished data.

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Fig. 4. TRPC1 does not interact directly with TRPC3 and TRPC6. TRPC1 was co-expressed with TRPC3 or TRPC6 in HEK293T cells, and cell lysates were precipitated with TRPC1 antibody. The presence of TRPC3, TRPC6, and TRPC1 in the immunoprecipitates (IP) was assessed with appropriate antibodies on Western blots (WB). Protein expression is labeled as “….” below the respective column. The experiment was performed in triplicate and showed no co-immunoprecipitation of TRPC3 or TRPC6 with TRPC1.

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Fig. 5. Carbachol induced whole cell currents in HM1 cells co-expressing TRPC1, TRPC3, and TRPC5. A, representative whole cell recordings from cells, each transfected with 1.5 µg of TRPC1, TRPC3, and TRPC5 before (con) and after the application of 10 µM carbachol (carb). B, comparison of the current to voltage (I–V) relations (means of n = 10 cells each, normalized to the maximum current) of carbachol-induced currents in TRPC1-, TRPC3-, and TRPC5-expressing cells and cells transfected with 1.5 µg of TRPC1 plus 1.5 µg of TRPC5 only. Mean maximum currents amounted to 664 ± 85 pA for TRPC1, TRPC3, and TRPC5 and 562 ± 86 pA for TRPC1- and TRPC5-transfected cells. The gray areas correspond to the variation (S.E.) of the normalized currents.
effects of differential TRPC subunit expression on PLC-mediated signaling, we measured carbachol-induced Ca\textsuperscript{2+} release in Fura-2/AM-loaded HM1 cells. Changes in fluorescence ratios (\(R/R_0\)) were not significantly different between cells co-transfected with TRPC3, TRPC5 DN-eGFP plus LacZ (\(R/R_0 = 158\% \pm 12\%; n = 31\)), and cells transfected with TRPC1 instead of LacZ (\(R/R_0 = 173\% \pm 10\%; n = 28\)). Taken together, these results strongly support the hypothesis that TRPC1 linked TRPC5 DN-eGFP and TRPC3 in a molecular complex.

**DISCUSSION**

In this study we identified novel heterotypic TRPC channel oligomers in rat embryonic brain and delineated a new principle of TRPC channel assembly in vivo. Evidence suggests that all TRPCs can form functional homomeric channels. These channels, however, may represent only a minority of endogenous TRPC complexes, considering the number of channels that can arise from the combination of different TRPC subunits. Fluorescence resonance energy transfer measurements with fluorescently labeled TRPC subunits overexpressed in heterologous cells showed that TRPCs assemble only within their structural subfamilies, i.e. TRPC1, 4, and 5 versus TRPC3, 6, and 7. Mouse TRPC2 was unable to form complexes with any of the other TRPCs (20). Although there is little data on co-expression of native TRPC proteins in single cells, partially overlapping expression of TRPC subunits was detected in various brain areas; e.g. high levels of TRPC1, 3, and 5 mRNA were found in cerebellum (28). Moreover, cultured hippocampal neurons express TRPC1 (16), TRPC5 (29), and TRPC6 \(^3\) proteins. Thus, selective co-assembly of TRPC subunits may occur in discrete brain regions.

Using microsomes from whole embryonic brain, we showed that complexes containing one of the DAG-responsive TRPCs

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\(^3\) G. Krapivinsky, unpublished data.
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(Trpc3 or 6) along with trpc1, 4, or 5 exist in mammalian brain. The formation of mixed heteromers is likely to require additional cofactors, because co-immunoprecipitation studies with various combinations of pairwise co-expressed TRPC isoforms confirmed that only intra-subfamily interactions occur under these conditions. Our experiments revealed that combining three different subunits, including trpc1, trpc4, or trpc5 and trpc3 or trpc6 led to the co-assembly of DAGE-sensitive and -insensitive TRPCs. Thus, we were able to reconstitute subunit interactions that were also detected in brain. Although Hofmann et al. (20) did not study TRPC complexes in brain, they showed that trpc6 is excluded from trpc1(+4) assemblies based on co-immunoprecipitation experiments with tagged trpc1a (a trpc1 splice variant), trpc4, and trpc6. We expressed unspliced trpc1 and used antibodies against native TRPC proteins for in vitro co-immunoprecipitations. Hence, different detection sensitivities or variant-dependent protein interactions may account for the conflicting experimental observations.

Functional co-expression of trpc1, trpc3, and trpc5 in HM1 cells gave rise to M₄-receptor-activated currents that were similar but not identical to currents through heteromeric trpc1(+5) channels. Most importantly currents in triple-transfected cells were significantly less outwardly rectifying than trpc1(+5) currents. The unique current-voltage relation of the mixed TRPC heteromers will specifically modulate the activation threshold and, thus, the firing pattern of neurons expressing these channels. The trpc1(+5) current-voltage relation exhibits a negative slope at hyperpolarized potentials. Depolarization over this range leads to further increases in inward (depolarizing) current, similar to the behavior of the N-methyl-d-aspartate (NMDA) receptor channel. The trpc1(+5) channel will be excitatory preferentially at depolarized potentials and may play a role as a coincidence detector, preferentially enhancing depolarizing inputs. The trpc1(+5)5 channel, on the other hand, provides inward current even at very hyperpolarized potentials.

We hypothesize that mixed channel heteromers containing all three TRPC subunits assembled in HM1 cells. This assumption is supported by the finding that suppression of TRPC3 currents by trpc3DN requires co-expression of TRPC1. Moreover, the presence of TRPC1 as well as TRPC4 or TRPC5 was necessary for the association with TRPC3 or TRPC6. Because neither TRPC1 nor TRPC4 or 5 alone associated with TRPC3 or TRPC6, we propose two potential models for heteromer formation. First, the trpc1 + trpc4/trpc1 + trpc5 interaction allosterically modifies trpc1(+4)/trpc1(+5) heteromers to produce a binding site for trpc3 or trpc6. Alternatively, the formation of trpc1(+4/5/3/6) heteromers requires simultaneous binding of a DAGE-sensitive subunit to TRPC1 and TRPC4 or 5. Based on the assumption that TRPC are tetramers, sequential or concurrent interaction with a fourth subunit will be required to form a functional channel complex. Elucidation of the exact subunit stoichiometry of the mixed TRPC heteromers will involve further biochemical analysis of native channel complexes.

The finding that synaptosomes from adult rat brain did not contain mixed TRPC heteromers (21) raises further interesting questions, namely whether various sets of TRPCs exist in different cellular compartments and how the channel complexes are sorted and localized. At present there is little information about the subcellular distribution of TRPC channels in neurons. The only subunit examined in this regard is TRPC1 that was detected in cell bodies as well as processes of primary hippocampal neurons (16). It is an exciting possibility that particular TRPC1-containing heteromers may localize to synapses whereas others do not. Preliminary experiments revealed TRPC6 immunoreactivity in the cell bodies and processes of embryonic hippocampal neurons, further supporting the notion that TRPCs subunits are not restricted to synapses in immature neurons.

The developmental control of TRP channel assembly and localization is only beginning to be examined. Although their expression levels are generally lower in adult versus embryonic brain, TRPC proteins can still be detected in mature neuronal tissue and might be enriched at synapses (21). Mixed TRPC heteromers, however, are not present or expressed only at undetectable levels in adult brain. This developmental regulation may reflect specialized functions of these TRP channel subtypes, e.g., in neuronal growth, pathfinding, and differentiation. Our findings provide new details on the assembly and composition of native TRPC complexes. Such information is essential for the reconstitution of these channels in expression systems and for a better understanding of TRPC function and regulation in vivo.

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