ORIGINAL ARTICLE

Localization and Expression of TREK-1, TREK-2, and TRAAK Channels in Rat Medial Vestibular Nuclei

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Objective: The aim of this study was to examine the localization and expression of TREK-1, TREK-2 and TRAAK channels in rat medial vestibular nuclei by immunohistochemical staining, reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analysis.

Materials and Methods: Twenty eight male Sprague-Dawley rats were used in this study.

Antibodies for TREK-1, TREK-2 and TRAAK channels were used. Tissues containing medial vestibular nuclei were selectively for RT-PCR and Western blot analysis.

Results: TREK-1, TREK-2 and TRAAK channels immunopositive neurons were distributed throughout the medial vestibular nuclei. RT-PCR data showed TREKs/TRAAK mRNAs were expressed in the medial vestibular nuclei. Western blot results also showed TREKs/TRAAK protein expression in the medial vestibular nuclei.

Conclusion: This study may provide additional speculation into the role of K2p channels in the pathway between migraine and vestibular system.

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Introduction

TWIK-related K+ channel (TREK)-1, TREK-2 and TRAAK (TREKs/TRAAK) are members of the two-pore domain K^+ (K_{2p}) channel family. TREKs/TRAAK subunits as mechano-gated K_{2p} channels share the closest sequence identity, ranging from 63% to 78%. The genomic organizations of TREK-1 (1q41), TREK-2 (14q31) and TRAAK (11q13) are also very similar, suggested that they might have arisen by gene duplication from a common ancestor.[1] The mRNAs and proteins of these channels are widely detected in the peripheral and central nervous systems.[2-4] TREKs/TRAAK channels have a particularly abundant and overlapping expression in certain regions of the brain, with the highest levels found in the caudate nucleus, the putamen and the hippocampus.[1] Furthermore, mRNAs of these channels are expressed in the dorsal root ganglion (DRG) neurons and mRNA for TREK-1 has also been detected in the neurons in the trigeminal ganglion (TG), geniculate ganglion and petrosal ganglion.[5] Meanwhile, the presence of K_{2p} channel in migraine- or migrainous vertigo-related structures has received little attention, although it was recently reported that TRESK, a member of the K_{2p} subfamily, is specifically expressed in DRG, TG and parasympathetic neurons. Therefore, the TRESK channel has emerged as a novel and interesting component of migraine pathogenesis pathway and may lead to new approach to treat migraine headache.[6]

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A close relationship between migraine and the vestibular system has been increasingly acknowledged in the pathogenesis of both migraine and vertigo. Several studies conducted in the last decade have increased our understanding of the link between migraine and vertigo.[7-9] However, an exact pathophysiology of migraine or migrainous vertigo has not yet been determined.

We hypothesized that the presence of the TREKs/TRAAK channels in the rat medial vestibular nuclei, in concert with TRESK channels, functions in the pathogenetic mechanism of migraine or migrainous vertigo. The aim of this study was to investigate the localization and expression of TREKs/TRAAK channels in rat medial vestibular nuclei by immunohistochemical staining, reverse transcriptase-polymerase chain reaction (RT-PCR), and Western blot analysis.

Materials and methods

2.1. Animals

Twenty eight male Sprague-Dawley rats (50-100 g; Orient Bio Animal, Seongnam, South Korea) were used in this study. All experimental protocols complied with the Guidelines of the National Institute of Health and the Declaration of Helsinki and were approved by the Committee on the Use and Care of Animals at Gyeongsang National University.

2.2. Immunohistochemical procedures

The rats were deeply anesthetized with sodium pentobarbital (100 mg/kg, i.p.) and perfused transcardially with phosphate-buffered saline (PBS; 0.9% NaCl in 50 mM phosphate buffer, pH 7.3) followed by paraformaldehyde-lysine-periodate (PLP) fixative. The heads were removed, skinned, and then the brains were carefully removed and placed in 30% sucrose-PBS at 4 ºC for 48-72 h. Thirty five micrometer-thick sections were cut on a freezing-slide microtome, and sets of every sixth section were placed in PBS containing 30% sucrose and 30% ethylene glycol, and stored at –20 ºC prior to immunohistochemistry.

After washing with distilled water, free-floating sections of rat brain tissue were incubated for 10 min with 0.9% hydrogen peroxide (H_2O_2) in PBS to remove endogenous peroxidase activity. After washing with distilled water followed by PBS, free-floating sections were treated for 1 h with blocking buffer consisting of PBS containing 2% bovine serum albumin (BSA) and were incubated for 48 h at room temperature in the presence of 1:500 dilution of rabbit TREK-1 antibody, rabbit TREK-2 antibody or TRAAK antibody (all from Alomone Labs, Jerusalem, Israel).

Following extensive washing with PBS, the sections were treated for 1 h with a 1:100 dilution of biotinylated goat anti-rabbit antibody (Vector Laboratories, Burlingame, CA, USA) in PBS containing 2% BSA. After three washes with PBS, sections were incubated in avidin-biotin-peroxidase reagent (Vectastain ABC Elite Kit; Vector Laboratories) for 1 h, washed repeatedly with PBS, and transferred to 50 mM Tris buffer (pH 7.2) for 10 min. The sections were then processed using immunoperoxidase procedures with 3.3'-diaminobenzidine tetrahydrochloride (DAB) as the chromogen (Vectastain ABC Elite Kit; Vector Laboratories) to visualize the cellular localizations of TREK-1, TREK-2 and TRAAK in the medial vestibular nuclei. The sections were rinsed with distilled water and mounted on gelatin/chrome alum-subbed slides. After air-drying overnight, the sections were dehydrated through a graded ethanol series, cleared with xylene, and coverslipped with DPX Mountant (Fluka, Milwaukee, WI, USA). The images obtained were adjusted for brightness and contrast, and cropped using Adobe Photoshop 7.0 (Adobe Systems, San Jose, CA, USA). Specificity for TREK-1, TREK-2 and TRAAK proteins was confirmed by omitting the primary antibody, and processed sections using immunoperoxidase procedure described above.

2.3. RT-PCR for TREK-1, TREK-2 and TRAAK mRNA expression

The rats were also sacrificed for RT-PCR by decapitation under deep anesthesia. Brains were rapidly isolated and mounted on a rat brain matrix in ice-cold PBS. Coronal sections were cut to isolate the brain stem at 2 mm and 4 mm rostrally from caudal margin of the 4th ventricle. The location of the medial vestibular nucleus was the most easily recognized and therefore was selected for RT-PCR. Tissue containing the medial vestibular nuclei was selectively isolated from brain stem slices using a tissue punch (1.8 mm internal diameter; Fine Science Tools, Foster City, CA, USA). The landmark used for the medial vestibular nuclei was the lateral border of the 4th ventricle and the brown-colored borderline between the prepositus hypoglossal nucleus and the dorsal paragigantocellular nucleus. One tissue sample (1.8 mm

in -diameter) of each medial vestibular nucleus was isolated in the caudo-rostral direction.[10] DRG and TG were also harvested for comparison with the medial vestibular nuclei. Total RNA was extracted from dissected tissues using QIAzol Lysis Reagent (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized from total RNA isolated from the DRG, TG and medial vestibular nuclei using oligo (dT) (DiaStar™ RT Kit; SolGent, Daejeon, South Korea) and was then used as a template for PCR amplification. Specific primers for TREK-1 and TRAAK (TREKs)/TRAAK channels were used in PCR reactions with *Taq* polymerase (G-Taq™; Cosmo Genetech, Seoul, South Korea). Table 1 lists the DNA sequences of the primers used to detect the expression of TREKs/TRAAK channels. PCR was conducted in a final reaction volume of 20 μl under the following PCR conditions: initial denaturation at 94°C for 5 min, followed by 32 cycles of 94°C for 30 sec, 55°C for 50 sec, and 72°C for 50 sec and a final extension step at 72°C for 10 min. The PCR products were directly sequenced with a PRISM® 3100-Avant genetic analyzer (Applied Biosystems, Foster City, CA, USA).

2.4. Western blot analysis

The medial vestibular nuclei were homogenized in PRO-PREP™ protein extraction solution (iNtRON Biotechnology, Seongnam, South Korea) containing 50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.5% NP-40, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM EGTA, 1 g/ml leupeptin, 1 g/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride and 10.5 g/ml aprotinin. Each mixture was incubated for 60 min on ice with intermittent vortexing. Extracts were clarified by centrifugation at 13,000 rpm (16,609 g) using a Micro 17TR apparatus (Hanil, Incheon, South Korea) for 20 min at 4°C. The resulting supernatant was separated by 10% SDS-polyacrylamide gel electrophoresis and the resolved proteins were transferred to a polyvinylidene fluoride (PVDF) membrane for 30 min using a semi-dry transfer (Bio-Rad, Hercules, CA, USA). Equal amounts (30 μg) of total protein were loaded. The membranes were blocked with 5% fat-free dry milk and then incubated with 1:500 dilution of anti-TREK-1, anti-TREK-2 or anti-TRAAK antibody (Alomone Labs), or with 1:10 000 dilution of anti-α-tubulin or anti-β-actin antibody. These were followed by incubation with a 1:10 000 dilution of horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Assay Designs, Ann Arbor, MI, USA). Immuno-positive bands were visualized by enhanced chemiluminescence (ECL Plus kit; ELPIS, Taejon, South Korea), following the manufacturer's instructions.

2.5. Culture of neurons in the medial vestibular nuclei and immunocytochemistry

The medial vestibular nuclei neurons isolated from the rats were rapidly washed in ice-cold (4°C) PBS and incubated for 15 min in a solution containing 0.0125% trypsin (Gibco, Grand Island, NY, USA) and 0.01% collagenase at 37°C. After digestion, the tissue was washed twice with PBS and triturated using a pipette tip. The isolated neurons were centrifuged at 1000 rpm (194 g) using a Labofuge 400 (Heraeus, Osterode, Germany) for 10 min. The pellet was resuspended in NeuroBasl medium (Invitrogen, Rockville, MD, USA) supplemented with B-27 (10 μL ml^{-1}, Invitrogen) and 5% fetal bovine serum (FBS, Invitrogen). The cells were plated on glass coverslips coated with poly L-lysine. The cells were cultured for 24 h at 37°C in a humidified incubator gassed with a 95% air/5% CO_2 mixture (v/v).

Table 1. Primer sequences used for RT-PCR.

Gene Name (Channel name)	Primer sequences (5'-3')	GenBank Accession numbers	Expected size (bp)
GAPDH	Forward : CTAAAGGGCATCCTGGGC		
	Reverse : TTACTCCTTGGAGGCCATG	NM_017008	201
KCNK2 (TREK-1)	Forward : GGAACTGACTCCATGTAGGA		
	Reverse : ACATTAGGAGAGGGGAAG	AF325671	434
KCNK10 (TREK-2)	Forward : CAGCCCAAGAGTGCCACTAA		
	Reverse : GGATCCCAAAGATGGCGTAT	NM_023096	493
KCNK4 (TRAAK)	Forward : CACCACTGTAGGCTTTGGCGATTATG		
	Reverse : ACTCTGCGTGTCTGAGGACTCGTCG	NM_053804	445

After an incubation to allow cell attachment, the cells were washed and used for immunocytochemistry.

The cultured medial vestibular nuclei neurons on round cover slips coated with poly-L-lysine were fixed with 4% paraformaldehyde in 0.1 M PBS for 30 min, washed and incubated in a blocking buffer containing 1% normal goat serum and 0.1% Triton X-100 for 2 h at room temperature under gentle rotation. The neurons were incubated with a 1:100 dilution of affinity-purified polyclonal antibodies for TREKs/TRAAK proteins and a 1:300 dilution of neuronal nuclear antigen (NeuN; BD Biosciences, San Diego, CA, USA) in PBS overnight at 4°C. After incubation, the neurons were washed with PBS three times and then incubated in the dark for 1.5 h with a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG for TREKs/TRAAK and cyanine 3 (Cy3)-conjugated anti-goat IgG for NeuN. Stained cells were wet-mounted on glass slides and observed using a confocal laser scanning microscope (Olympus, Tokyo, Japan).

Results

3.1. Immunoreactivity of TREK-1, TREK-2 and TRAAK channels in the medial vestibular nuclei

Immunoreactivity of TREK-1, TREK-2 and TRAAK channels was demonstrated by the presence of 3.3′-diaminobenzidine tetrahydrochloride immunopositive products in medial vestibular nuclei sections. The nomenclature and boundaries defined in the rat brain atlas of Paxinos and Watson[11] were utilized throughout the present study. TREK-1, TREK-2 and TRAAK channels immunopositive neurons were distributed throughout the medial vestibular nuclei. Immunoreactivity of TREKs/TRAAK (Fig. 1) was primarily detected in

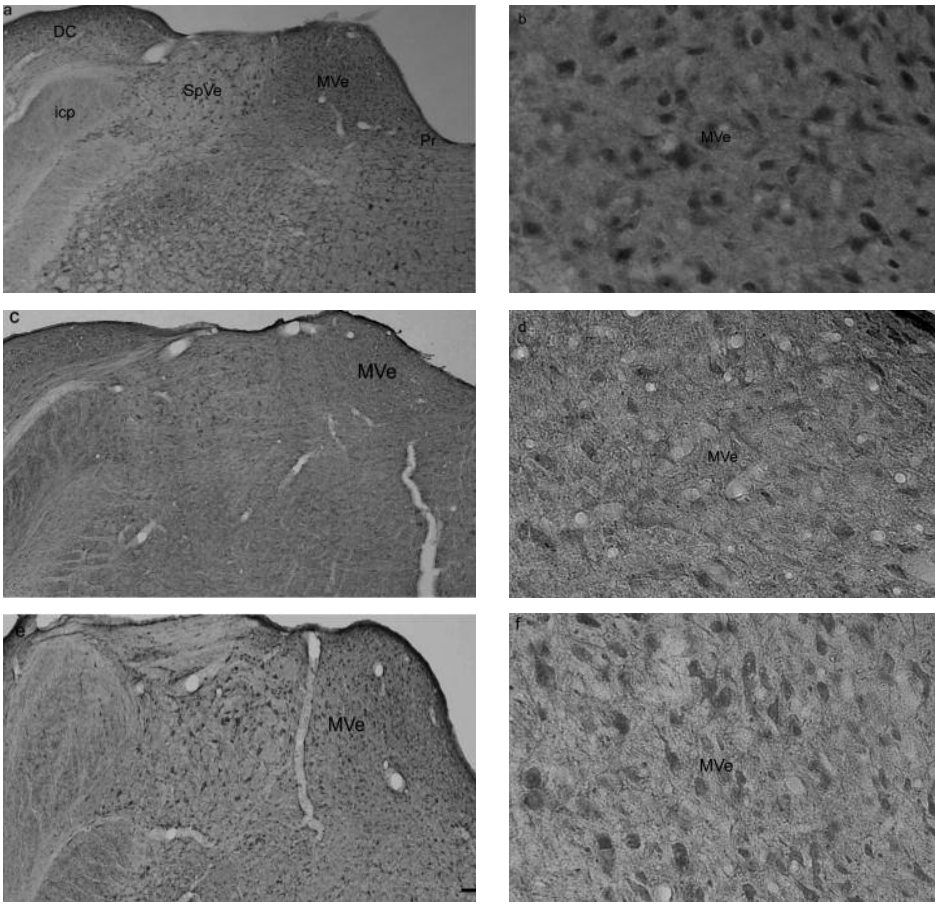

Figure 1. Photomicrographs of TREK-1, TREK-2 and TRAAK channels in the medial vestibular nuclei of rats. The photomicrographs in the right panel show high magnification views of those in the left panel (a+b, c+d, e+f). TREK-1, TREK-2 and TRAAK channel immunopositive neurons are observed throughout the medial vestibular nuclei (MVe). Abbreviations: icp, inferior cerebellar peduncle; Pr, prepositus nuclei; DC, dorsal cochlear nucleus; SpVe, spinal vestibular nucleus. Scale bars: 200 μm (in e for a and c); 25 μm (in f for b and d).

neuronal somata and their proximal dendrites. TREK-1 and TRAAK channels were moderately expressed in the medial vestibular nucleus (Figs. 1a and 1b, and 1e and 1f, respectively). On the other hand, TREK-2 channels were lightly expressed in the medial vestibular nuclei (Figs. 1c and 1d). In control sections not treated with primary antibody, no neuronal staining was observed (data not shown).

3.2. Expression of TREK-1, TREK-2 and TRAAK channels in the medial vestibular nuclei

Dorsal root ganglion and trigeminal ganglion neurons expressed TREKs/TRAAK channels, in agreement with earlier studies.[12,13] RT-PCR, Western blot analysis and immunostaining were performed to identify whether the expression of these channels in the medial vestibular nucleus neurons. RT-PCR data showed TREKs/TRAAK mRNAs were expressed in DRG, TG and medial vestibular nuclei isolated from 3-week-old rats (Fig. 2a). Western blot results also showed TREKs/TRAAK protein expression in medial vestibular nuclei (Fig. 2b). The immunocytochemistry results demonstrated that TREKs/TRAAK channels were expressed in the cell bodies of the medial vestibular nuclei neurons together with NeuN, a neuronal nucleus marker (Fig. 2c). TREK-1 and TRAAK were highly expressed, while TREK-2 was faintly expressed in the medial vestibular nucleus neurons.

Discussion

The discovery of the K_{2P} channel provided a molecular basis for characterizing functional properties of leak K^+-channel subunits and localizing native sites of expression.[14] K_{2P} channels produce background K^+ currents that regulate cell excitability and. These channels are distributed widely in the central nervous system and periphery, with differential, but often overlapping, expression patterns.

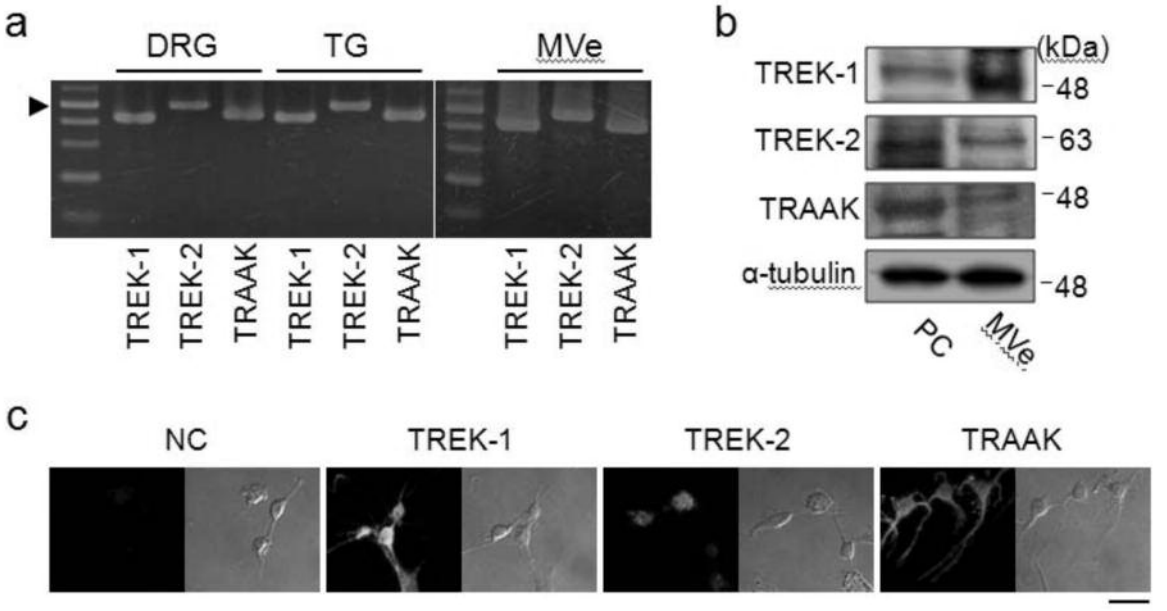

Figure 2. Expression of TREKs/TRAAK channel in the medial vestibular nuclei. (a) Expression of TREK-1, TREK-2, and TRAAK mRNAs in rat medial vestibular nuclei. TREKs/TRAAK specific primers were used to generate 434-bp (TREK-1), 493-bp (TREK-2), and 445-bp (TRAAK) PCR products, which correspond to the expected lengths. DRG and TG were used as positive controls for TREKs/TRAAK mRNA expression. The first lane shows a 1-kb DNA ladder, and arrow head indicates 500 bp. (b) Western blot analysis of TREKs/TRAAK proteins in the medial vestibular nuclei. Whole-cell lysate was used as total protein. The TREKs/TRAAK expression in rat medial vestibular nuclei was compared with that in PC12 cells transfected with TREK-1, TREK-2, or TRAAK. Equal amounts (30 μg) of total protein were loaded in each lane. Molecular weight is indicated on the left side of the blot. (c) Immunostaining for TREKs/TRAAK in the medical vestibular nuclei. Green and red fluorescence show TREKs/TRAAK proteins and NeuN, respectively. Negative controls (NC) were checked by omitting the primary antibody. No staining of green or red fluorescence was observed on the any of NC. Abbreviations: DRG, dorsal root ganglion; TG, trigeminal ganglion; PC, positive controls; NC, negative controls; MVe, medial vestibular nucleus. Scale bar; 20 μm.

A widespread distribution of K_{2p} channels in the central nervous system has been documented in two representative studies.[2,4] However, TREKs/TRAAK expression patterns in vestibular nuclei were different in the two studies. In one study, *in situ* hybridization did not reveal localization and expression of TREKs/TRAAK channels in the vestibular nuclei.[4] On the other hand, TREK-1 expression was observed in the rat vestibular areas in the other study.[2] These different detection methods (*in situ* hybridization and immunohistochemistry) may result in different TREKs/TRAAK expression levels. Gene and protein expression levels do not always correlate. A negative correlation between gene and protein expression levels could be resulted from a number of complex steps between transcription and translation. In the present study, TREKs/TRAAK channels were also moderately or lightly immunostained in the medial vestibular nucleus in agreement with the previous study.[2]

It is being increasingly recognized that migraine and balance disorders are interrelated.[15-17] Recent studies have shown that dizziness occurs in 28-30% and motion sickness in approximately 50% of migraine patients. Furthermore, migrainous vertigo has been reported in 9% of migraine sufferers. However, the pathophysiology of migraine-associated vestibular disorders is not well understood. The reciprocal connections[18] between the vestibular nuclei and the trigeminal nucleus caudalis implies that trigeminal and vestibular signals are likely to be simultaneously activated during the development of migraine or migrainous vertigo. Recent clinical studies are also consistent with the concept that, as in the case of trigeminal pathways in migraine, both peripheral and central vestibular mechanisms may contribute to co-morbid migraine and balance disorders. Drummond[19] reported that trigeminal pain, pain sensitivity in the fingers and photophobia are augmented in migraineurs by simultaneous optokinetic stimulation motion. Furthermore, motion sickness is augmented by simultaneous painful trigeminal stimulation in migraineurs.[20] Marano[21] reported that painful trigeminal stimulation has significant effects on nystagmus in migraine patients.

The prior studies have indicated the principle involvement of TREK-2 and TRESK channels as the major background K^+ channels in DRG neurons.[12,22,23] In addition, TREK-1, TREK-2 and TRAAK channels colocalize with thermosensitive transient receptor potential (TRP) channels in rat TG neurons.[5,13] The expression of TREKs/TRAAK in DRG and TG gives rise to an investigation of their expression in the medial vestibular nuclei. The present study demonstrates localization and expression of TREKs/TRAAK channels in rat medial vestibular nuclei. Given that TREK-1, TREK-2 and TRAAK channels are localized and expressed in rat medial vestibular nuclei, presumably these K_{2p} channels operate in parallel to the trigeminal pathways involved in migraine.

Conclusion

The present study demonstrates, for the first time, that TREK-1, TREK-2, and TRAAK channels are localized and expressed in rat medial vestibular nuclei. This study results could strengthen the speculated functional link of K_{2p} channels in the pathway between migraine and the vestibular system.

Conflict of interest: The authors declare that we have no vested interest that could be construed to have inappropriately influenced this study.

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