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DOWN-REGULATION OF TRPM5s DURING THE DEVELOPMENT OF THE RAT NEOCORTEX AND HIPPOCAMPUS

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The transient receptor potential (TRP) superfamily of cation membrane channels includes a large number of recently identified protein molecules that share the sequence homology with the *Drosophila* protein named after a phototransduction mutant of this insect called trp. Believing that one or more TRP channels might be involved in the process of neural development, we examined the possibility by searching the full profile of TRPC1-7, TRPV1-6, TRPM1-8, and TRPA1 channels during the development of the neocortex and hippocampus of rats (from the embryonic state to adults). The RT-PCR experiment was performed with mRNA isolated from the above-mentioned cerebral structures. Developmental changes were identified in transcripts for the profiles of TRPC1-7, TRPV1-6, TRPM1-8, and TRPA1. Down-regulation of exclusively TRPM5, whose mRNA expression level gradually decreased in the course of pre- and post-natal development of both neocortex and hippocampus, was the most striking finding. Our results suggest that TRPM5s might have some important function in neural development. There is a possibility that TRPM5s are key components of the physiological function for sensing the environmental temperature in the developmental period of rats.

Keywords: transient receptor potential channels (TRPMs), TRPM5s, development, neocortex, hippocampus, transcripts, PCR.

INTRODUCTION

The transient receptor potential (TRP) superfamily of cation membrane channels includes a large number of identified protein molecules that share the sequence homology with the *Drosophila* protein named after a phototransduction mutant of the respective insect called *trp*. Mammalian TRP channel proteins form cation-permeable channels with six transmembrane domains and cytoplasmic N- and C-terminals. These channels may be further divided into subfamilies according to the sequence similarities, such as the four main subfamilies, namely TRPC (canonic), TRPV (vanilloid), TRPM (melastatin), and TRPA1 (ankyrin). The TRP channels generally mediate the transmembrane flux of cations according to electrochemical gradients of the latter, thereby raising intracellular Ca^{2+} and Na^{+} concentrations, respectively, and depolarizing the cell with polymodal activation properties [1-4].

It is widely accepted that TRP subunits are activated following phospholipase C activation and form cation-selective ion channels. By integrating multiple concomitant stimuli, as well as by coupling activity to downstream cellular signal amplification via calcium permeation and membrane depolarization, TRP channels are involved in diverse cellular functions, including receptor- and store-operated Ca^{2+} entry, Ca^{2+} transport, trace metal detection, and temperature and osmolarity sensations [5-8].

During neural development (which includes the processes of generating, shaping, and reshaping of the nervous system), a number of factors affect these events from the earliest stages of embryogenesis to the final stage of life. Believing that one or more TRP channel types might be involved in the process of neural development, we examined these possibilities by searching the full profile of the four main subfamilies of TRP channels during ontogenetic development of rats. The neocortex and hippocampus were chosen as the research objects because these

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structures are essential for encoding new memories and spatial learning, as well as for certain aspects of contextual learning. Some work in this direction was carried out by our research group earlier [9].

In this study, we have identified transcripts for the profiles of TRPC1-7, TRPV1-6, TRPM1-8, and TRPA1 channels in isolated preparations of both neocortex and hippocampus of the rat.

METHODS

Animals. Sprague-Dawley rats were supplied by the Laboratory Animal Center, Southern Medical University, China [Certificate No. SCXK (Yue) 2006-0015]. The experimental procedures were approved by the Experimental Animal Welfare and Ethics Committee at the Southern Medical University and were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of the People's Republic of China (Guidance Suggestions for the Care and Use of Laboratory Animals, 2006-09-30). All efforts were made to minimize animal suffering and discomfort and to reduce the number of animals used.

The day on which a vaginal plug was confirmed in pregnant females was defined as embryonic day 0 (E0), and the day of birth of pups was defined as postnatal day 0 (P0). The neocortex and hippocampus samples were taken from rats at postnatal days 1 to 90 (P1, P7, P14, P21, P45, and P90), and the embryonic brain was taken at embryonic day 18 (E18). Neonatal and adult rats were euthanized by cervical dislocation. Pregnant rats were anesthetized by i.p. administration of chloral hydrate (35 mg/100 g body mass), and the embryos were removed by cesarean section.

Total RNA Isolation. To assess relative levels of TRP channel transcripts during the development of rats, we used a modified semi-quantitative noncompetitive method for the polymerase chain reaction (PCR) [10-12]. This allowed us to compare the amount of a target sequence against the amount of the constitutively expressed reference sequence. The subsaturating level of cDNA template that is needed to produce a dose-dependent amount of the reaction product was defined empirically in initial experiments by testing ranges of the template concentrations. The relative intensity of the target sequence product visualized with ethidium bromide can then be interpreted as reflecting the relative abundance of the target mRNA in the original total RNA pool. In our investigation, the housekeeping β-actin gene served as an internal standard control alongside the experimental samples for the relative quantitative comparisons.

Tissues of the neocortex or hippocampus from two to ten rats were combined to generate each total RNA sample. Neocortex samples were obtained from four P1 rats and also from two P7, P14, P21, P45, and P90 animals each. Hippocampus samples were obtained from 10 P1 rats, four P7 animals, and two P14, P21, P45, and P90 rats each. Embryonic brains (E18) were obtained from four animals.

Three sets of total RNA samples were prepared at each developmental time point. Total RNA was extracted from neocortical or hippocampal tissues using Trizol reagent (Invitrogen, Life Technologies, USA) according to the manufacturer's protocol. The purity and concentration of the extracted RNA were determined by absorption measurement at 260 and 280 nm using a PharmaSpec UV-1700 spectrophotometer (Shimadzu, Japan). Two milligrams of each sample were used for reverse transcription-PCR (RT-PCR).

PCR Primers. Aliquots of the reverse-transcribed cDNA preparations were used for PCR amplification with the synthesized oligodeoxyribonucleotide primers. PCR primers were specifically designed based on the published sequences of TRPC1 (NM_053558), TRPC2 (NM_022638), TRPC3 (NM_021771), TRPC4 (AF288407), TRPC5 (NM_080898), TRPC6 (NM_053559), TRPC7 (XM_001067646), TRPV1 (NM_031982), TRPV2 (NM_017207), TRPV3 (AY325813), TRPV4 (NM_023970), TRPV5 (NM_053787), TRPV6 (NM_053686), TRPM1 (NM_001037734), TRPM2 (NM_001011559), TRPM3 (XM_001079904), TRPM4 (XM_001080721), TRPM5 (XM_001065110), TRPM6 (XM_001078158), TRPM7 (XM_001078325), TRPM8 (NM_134371), TRPA1 (NM_207608), and β-actin (NM_031144) obtained from the GenBank Sequence Data Library (http://www.ncbi.nlm.nih.gov/). The optimal primers were selected utilizing Oligo version 6.0 Software package (National Biosciences, USA) except for TRPC4 primers that were described previously [13] (Table 1). The fidelity and specificity of all designed primers were screened using the Basic Local Alignment Search Tool program (http://blast.ncbi.nlm.nih.gov/). All primers were synthesized by either Takara Biotechnology (Japan) or Invitrogen (Life Technologies, USA).

Products of RT-PCR. Reverse transcription and subsequent amplification by PCR were performed utilizing a commercially available RNA PCR Kit Ver.

Oligonucleotide Primer Sequences for Reverse Transcription-Polymerase Chain Reaction

Послідовності олігонуклеотидних праймерів для зворотної транскрипції – полімеразної ланцюгової реакції

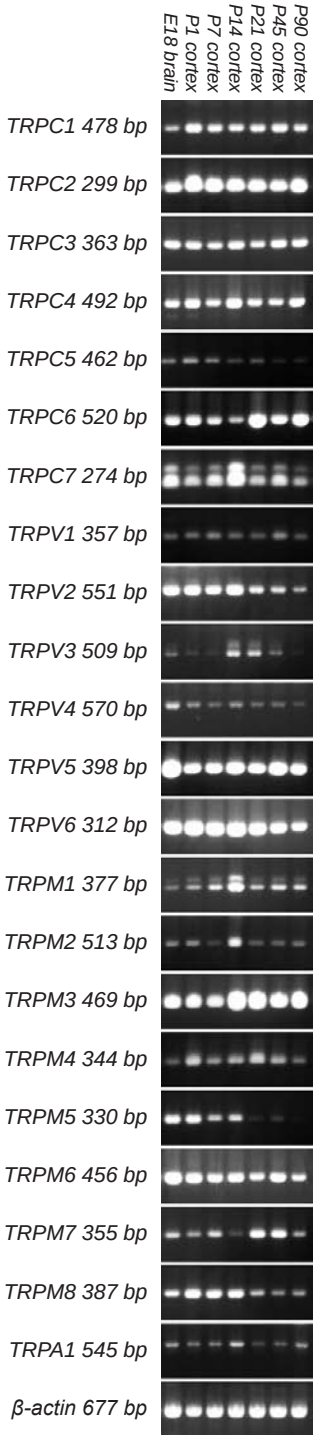
Target gene	Direction	Primer sequence	Predicted length of the product
TRPC1	Forward	5’AAAGTGGTGGCTCACAACAAGT3’	478 bp
	Reverse	5’CGACATTGTAAGTTCCGACA3’	
TRPC2	Forward	5’CCAGCGGTAGTGCGTCGTCT3’	299 bp
	Reverse	5’CGGTAGGTGTTGATTCGGGATA3’	
TRPC3	Forward	5’TCTCAATCAGCCAACACGAT3’	363 bp
	Reverse	5’CGCATGGTGAAGGTATTAACAC3’	
TRPC4 [13]	Forward	5’GCCTACACCTTTCAATGTCATCCC3’	492 bp
	Reverse	5’CTTAGGTTATGTCTCTCGGAGGC3’	
TRPC5	Forward	5’GGGACTATGCCACCGTGAAGC3’	462 bp
	Reverse	5’GTCGGGACCTTGAATGACGTA3’	
TRPC6	Forward	5’ACACTGGGGGACAACGTCAAATACTAC3’	520 bp
	Reverse	5’ACTTCCACTCCACATCCGCATCA3’	
TRPC7	Forward	5’CTCAATTTCAACTGCGTGGACT3’	274 bp
	Reverse	5’GCGTGCCATCCTCGTCGTAG3’	
TRPV1	Forward	5’GCCGTCATCCCAGGACTCCGTC3’	357 bp
	Reverse	5’ATGTTCCGCCGTTCAATG3’	
TRPV2	Forward	5’TCGCCTGCTACTTGGTCTACAT3’	551 bp
	Reverse	5’CGTGGGCTGTTCCGTCACT3’	
TRPV3	Forward	5’TTTGTTAAGCGCATGTATGACA3’	509 bp
	Reverse	5’AGGAGCTGAAGCCAACTCATCTTATGT3’	
TRPV4	Forward	5’GCCCATCCTCTTTGACATCGT3’	570 bp
	Reverse	5’AACTTGGTGTTCTCTCGGGTGT3’	
TRPV5	Forward	5’CGCCAGCAAGGACGTTGTATT3’	398 bp
	Reverse	5’GCGCGTGTGTGGGATCTATG3’	
TRPV6	Forward	5’ACCTGATGCAGAAGCGGAAACA3’	312 bp
	Reverse	5’GGTTGGTGCGGTTAGTGATCCT3’	
TRPM1	Forward	5’CCCTACTGGATGATCTACGG3’	377 bp
	Reverse	5’CTGAGACGCATAACGATGATG3’	
TRPM2	Forward	5’TCTGCCTGTTTGCCTACGTGCTCA3’	513 bp
	Reverse	5’GCCCGAAGATGGTAAGGTAAG3’	
TRPM3	Forward	5’ACAAAGATGACATGCCCTATATGA3’	469 bp
	Reverse	5’GCGATGAGGTCCGTGACGTT3’	
TRPM4	Forward	5’CGGGATCGAATTAGACGTTACTTC3’	344 bp
	Reverse	5’GAGATGAGCAAGCGCACAAA3’	
TRPM5	Forward	5’GTGTTCACACTTCGGCTCAT3’	330 bp
	Reverse	5’CCAGTTGGCATAGAGATTAGGG3’	
TRPM6	Forward	5’GCAATGGCTTGGGATAGAAT3’	456 bp
	Reverse	5’CAGTGTGCTTTCCGAAGACTC3’	
TRPM7	Forward	5’GGACGGCTGAATATGAG3’	355 bp
	Reverse	5’GAAATCCTAAGTATGCCAATG3’	
TRPM8	Forward	5’AATGAGTATGAGACCCGAGCAG3’	387 bp
	Reverse	5’GGCGATGTAGAAGACCACGTTC3’	
TRPA1	Forward	5’TACGCCTCTCCATTATGCTTG3’	545 bp
	Reverse	5’CCACTTCCTTGCGCTTATTGT3’	
β-actin	Forward	5’ATTGAACACGGCATTGTCA3’	677 bp
	Reverse	5’TTGGCATAGAGGTCTTTACGGA3’	

Footnotes. TRP) Transient receptor potential. Primers described in the literature [13] are indicated. All other primers were newly designed.

3.0 (Takara Biotechnology, Japan) in accordance with the manufacturer's instructions. Polymerase chain reactions for each individual target TRP channel gene and internal standard β-actin gene were performed in the same reaction tube. Thermal cycling was performed in an Eppendorf Mastercycler Epradient (Eppendorf, Germany) using the following amplification profile: an initial denaturation at 94°C for 120 sec, 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 60 sec; the final elongation step at 72°C lasted 10 min. Amplification products were then subjected to 1.5% (w/v) agarose gel electrophoresis and hybridization with 0.5 μg/ml ethidium bromide, visualized under ultraviolet transillumination in a MultiImage Light Cabinet (Alpha Innotech Corp., USA) and photographed using digital imaging FluorChem software. The size of each cDNA product was estimated by comparison with a DNA size marker (DNA Marker DL2000, Takara Biotechnology, Japan). The PCR product of each amplified gene was sequenced by Invitrogen Biotechnology (Life Technologies, USA).

Semi-quantification. For semi-quantification, RT-PCR products of the target TRP genes were grouped and analyzed simultaneously. Regions were sampled by Gel-Pro Analyzer Version 3.1 (Media Cybernetics, USA) from an imaged gel to evaluate relative levels of target gene expression. The value of the band of the internal standard β-actin in each lane was used as the baseline gene expression of the sample, and the relative value of the band in the same lane was calculated for each target TRP gene amplified in that reaction. Results were obtained from three sets of total RNA preparations based on three independent PCRs carried out for each pair of both individual target TRP primers and β-actin primers. For quantitative comparison, the ratios were expressed as arbitrary units. The performed negative control reactions did not contain any cDNA.

Statistical Analyses. Numerical values were expressed as means ± s.e.m. Differences among the studied groups were statistically evaluated by one-way analysis of variance (ANOVA) followed by the *post-hoc* least significant difference test using SPSS 10.0 (SPSS, USA), and differences were considered significant with $P < 0.05$.

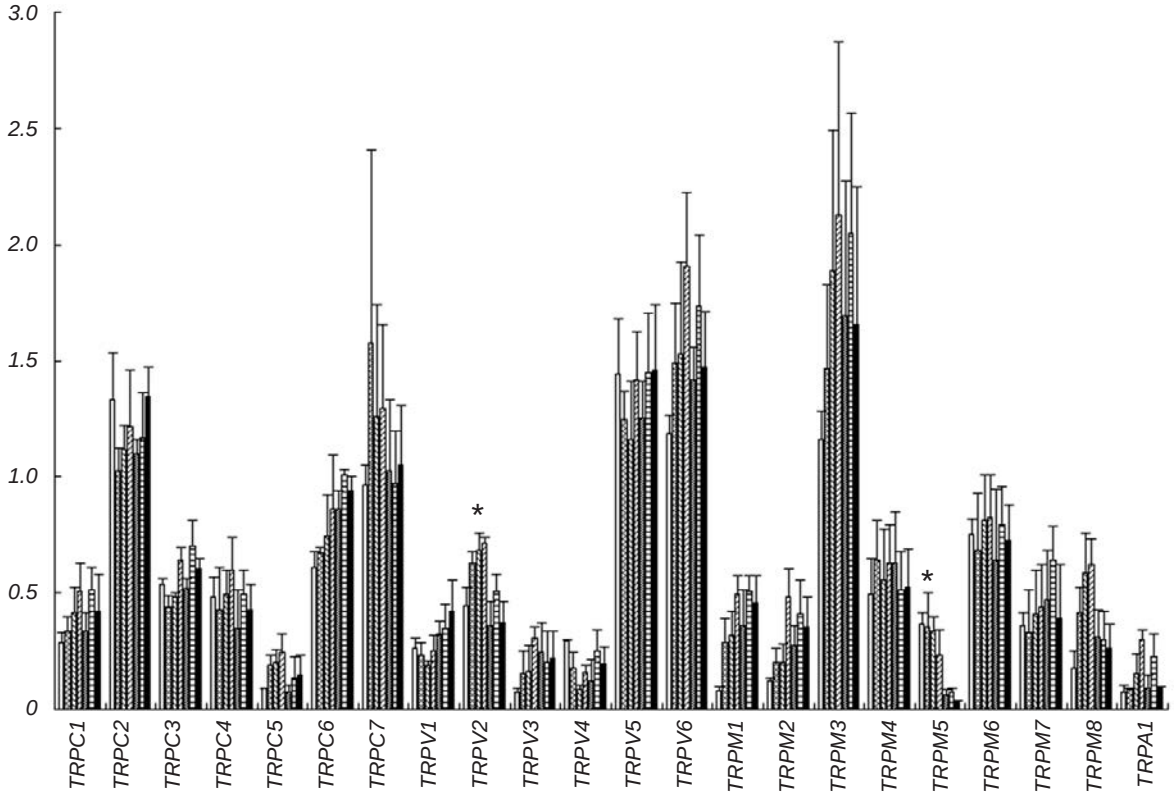


F i g. 1. mRNA expression of TRPC1-7, TRPV1-6, TRPM1-8, and TRPA1 channels during the development of the rat neocortex. This figure shows the developmental profiles of RT-PCR products of TRPC1-7, TRPV1-6, TRPM1-8, and TRPA1 channel subunits and RT-PCR product of β-actin (internal RT-PCR control) with mRNA isolated from the rat embryonic brain (embryonic day E18) and neocortex (postnatal days P1, P7, P14, P21, P45, and P90). It can be seen that mRNA of all TRPC1-7, TRPV1-6, TRPM1-8, and TRPA1 channel subunits is expressed from E18 to P90.

Рис. 1. Експресія мРНК каналів TRPC1-7, TRPV1-6, TRPM1-8 та TRPA1 у перебігу розвитку неокортексу щура.

RESULTS AND DISCUSSION

We investigated the developmental expression of TRPC1-7, TRPV1-6, TRPM1-8, and TRPA1 channels that are members of four subfamilies of TRP channels. To determine whether the mRNAs encoding these channel proteins are present, an RT-PCR experiment was performed with mRNA isolated from the neocortex or hippocampus of rats ranging from newborns to adults

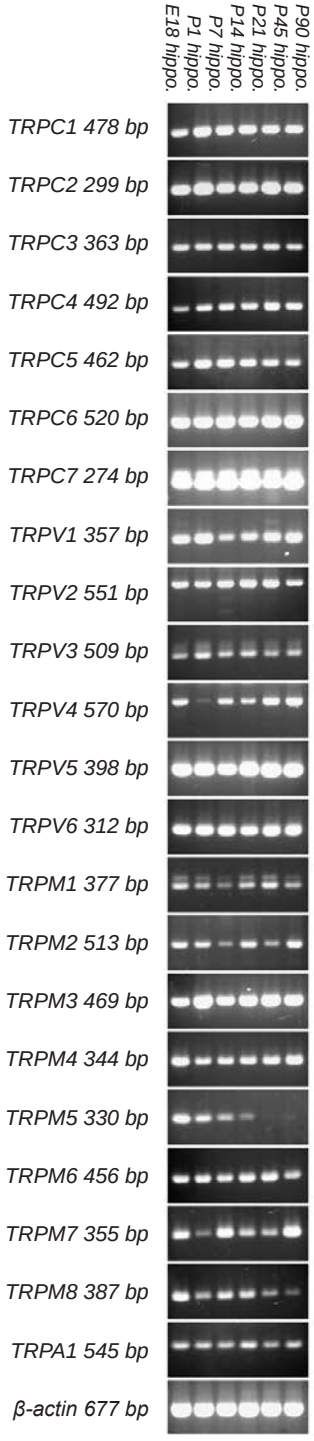


F i g. 2. Down-regulation of TRPM5 during the development of the rat neocortex. Vertical scale) mRNA expression (arbitrary units). E18) Embryonic day 18; P1, P7, P14, P21, and P90) postnatal days 1, 7, 14, 21, and 90. There is only a gradual decrease for TRPM5 expression (from E18 till adulthood). Data are means ± s.e.m. from three independent experiments (*$P < 0.05$, as determined with ANOVA).

Р и с. 2. Негативна регуляція наявності TRPM5 у перебігу розвитку неокортексу щура.

(postnatal days P1, P7, P14, P21, P45, and P90), as well as from the embryonic brains (embryonic day E18). Using the respective primers, we detected constitutive levels of TRPC1-7, TRPV1-6, TRPM1-8, and TRPA1 channel subunit mRNAs and β-actin mRNA in the tissues; β-actin was used as an internal control [14, 15]. The optimal protocol, i.e., amplification of all genes simultaneously for 35 cycles, generated very consistent results in repeated assays. The results showed that mRNAs of all TRPC1-7, TRPV1-6, TRPM1-8, and TRPA1 channel subunits were expressed, and β-actin mRNA served as the positive control. The amplified cDNA sizes corresponded to the expected size. The RT-PCR products of TRPC1-7, TRPV1-6, TRPM1-8, and TRPA1 channel subunit genes and also that of β-actin were of the same size, regardless of whether the performance was in coamplification or in a single-gene reaction. No specific product was obtained from controls that contained no RT enzyme.

The identity of each RT-PCR product was confirmed by direct nucleotide sequencing commercially. The obtained sequences of TRPC1-6, TRPV1-6, TRPM1-2, TRPM8, TRPA1, and β-actin were found to match the well-known rat cDNA sequences of TRPC1 (NM_053558), TRPC2 (NM_022638), TRPC3 (NM_021771), TRPC4 (AF288407), TRPC5 (NM_080898), TRPC6 (NM_053559), TRPV1 (NM_031982), TRPV2 (NM_017207), TRPV3 (AY325813), TRPV4 (NM_023970), TRPV5 (NM_053787), TRPV6 (NM_053686), TRPM1 (NM_001037734), TRPM2 (NM_001011559), TRPM8 (NM_134371), TRPA1 (NM_207608), and β-actin (NM_031144). The obtained sequences of TRPM3-5 and TRPM7 were found to match the known predicted rat cDNA sequences of TRPM3 (XM_001079904), TRPM4 (XM_001080721), TRPM5 (XM_001065110), and TRPM7 (XM_001078325). The obtained sequences of TRPC7 and TRPM6 were found to display a high level of homology with the known predicted rat cDNA sequences of TRPC7 (XM_001067646) and TRPM6 (XM_001078158). Furthermore, as the new partial encoding region of mRNA of the rat cDNA sequences



F i g. 3. mRNA expression of TRPC1-7, TRPV1-6, TRPM1-8, and TRPA1 channels during the development of the rat hippocampus. This figure shows the developmental profiles of RT-PCR products of TRPC1-7, TRPV1-6, TRPM1-8, and TRPA1 channel subunits and β-actin (internal RT-PCR control) with mRNA isolated from the rat hippocampus (postnatal days P1, P7, P14, P21, P45, and P90). It can be seen that mRNA of all TRPC1-7, TRPV1-6, TRPM1-8, and TRPA1 channel subunits is expressed from P1 to P90.

Рис. 3. Експресія мРНК каналів TRPC1-7, TRPV1-6, TRPM1-8 та TRPA1 у перебігу розвитку гіпокампа щура.

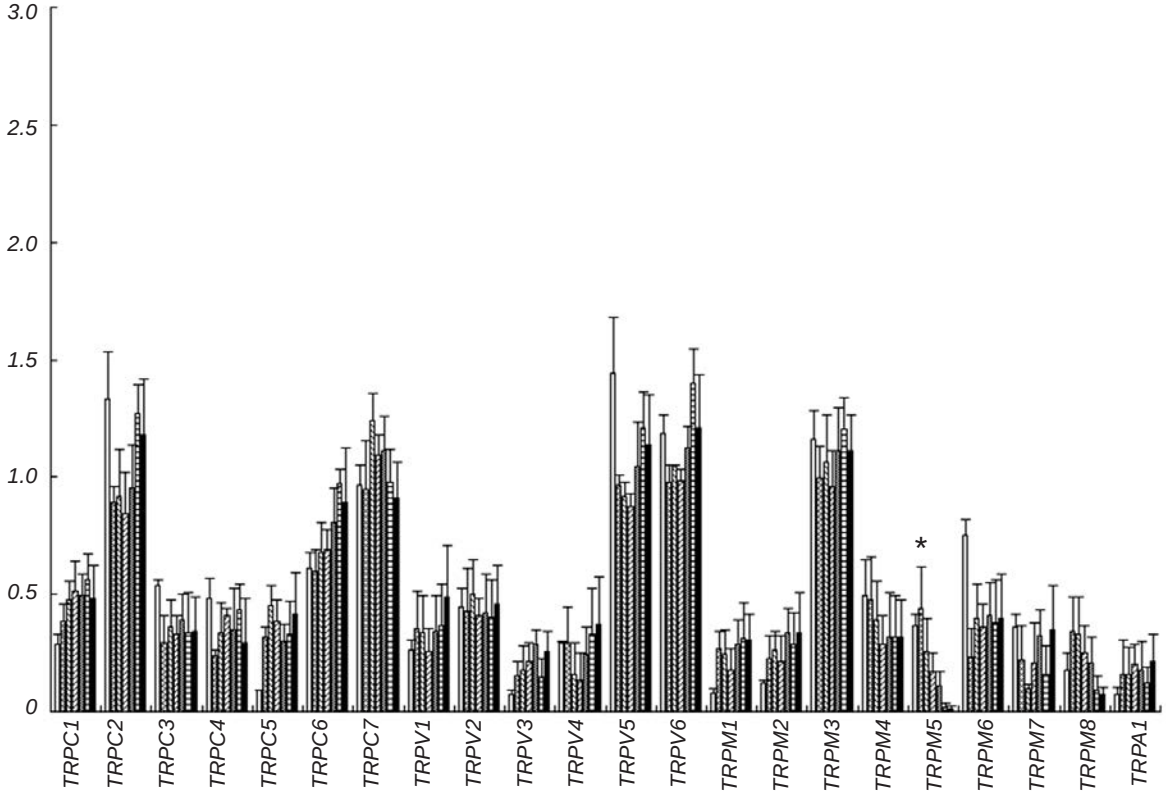
of TRPC7 and TRPM3-7 had been originally submitted to the GenBank, the recorded database accession numbers are TRPC7 (EF673687), TRPM3 (EF673689), TRPM4 (EF673691), TRPM5 (EF673692), TRPM6 (EF673693), and TRPM7 (EF673694).

Developmental changes were identified in transcripts for the profiles of TRPC1-7, TRPV1-6, TRPM1-8, and TRPA1 during maturation of the rat neocortex and hippocampus (from E18 till adulthood). During this period, the most striking pattern for both studied cerebral structures was found only for down-regulation of TRPM5, whose mRNA expression level decreased gradually within this period (Figs. 1-4).

TRP channels are widely distributed in a variety of mammalian organisms, tissues, and cell types. These channels sense local changes in the parameters of various stimuli (from light, olfaction, temperature, pH, and osmolarity to mechanical, chemical, and metabolic stress). The properties and activation mechanisms of TRP channels have been extensively studied in heterologous expression systems. In contrast, relatively little information is available on their role in native cells or for certain native tissues [16, 17].

The TRPM5 type was identified during functional analysis of the chromosomal region (11p15.5), which is associated with loss of heterozygosity in a variety of childhood and adult tumors [18]. Recent reports suggested that TRPM5 is a component of the channel that mediates taste transduction in vertebrates. TRPM5s are highly expressed in taste buds of the tongue, where they play a key role in the perception of sweet, umami, and bitter tastes [19, 20]. In addition TRPM5s are highly temperature-sensitive and heat-activated channels. Inward TRPM5-related currents increase steeply at temperatures between 15 and 35°C; an increase in temperature between 15 and 35°C markedly enhances the gustatory nerve response to sweet compounds in wild-type but not in TRPM5-knockout mice [21].

The main finding of our study is that only the mRNA expression level of TRPM5 decreases gradually during the development of both neocortex and hippocampus of the rat. The mRNAs of all TRPC1-7, TRPV1-6, TRPM1-8, and TRPA1 channel subunits were expressed in isolated neocortical or hippocampal preparations. Developmental changes in transcripts for the profile of TRPC1-7, TRPV1-6, TRPM1-8, and



F i g. 4. Down-regulation of TRPM5 during the development of the rat hippocampus. There is only a gradual decrease for TRPM5 expression (from E18 till adulthood). Data are means ± s.e.m. from three independent experiments (**P* < 0.05, as determined with ANOVA). Designations are the same as in Fig. 2.

Р и с. 4. Негативна регуляція наявності TRPM5 у перебігу розвитку гіпокампа щура.

TRPA1 channels were identified. The observation that TRPM5 is continually down-regulated during the development of the rat neocortex and hippocampus suggests that TRPM5 might have some important function in neural development. There is a possibility that TRPM5s are a key component of the physiological function for sensing the environmental temperature in the developmental period of the rat.

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НЕГАТИВНА РЕГУЛЯЦІЯ НАЯВНОСТІ КАНАЛІВ TRPM5 У ПЕРЕБІГУ РОЗВИТКУ НЕОКОРТЕКСУ ТА ГІПОКАМПА У ЩУРІВ

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Р е з ю м е

Суперсімейство мембранних катіонних каналів транзієнтного рецепторного потенціалу (TRP) включає в себе велику кількість нещодавно ідентифікованих канальних протеїнів, у котрих первинні послідовності є гомологічними такій у протеїну мутанта дрозофіли, отриманого в результаті фототрансдукції та названого trp. Вважаючи, що один або більше типів TRP-каналів можуть бути задіяними в процес розвитку нервової системи, ми намагалися дослідити повні

часові профілі представленості каналів TRPC1-7, TRPV1-6, TRPM1-8 та TRPA1 у перебігу розвитку неокортексу та гіпокампа у щурів (від ембріонального стану до дорослого віку). Зворотна рескриптаза – полімеразні ланцюгові реакції (ЗР-ПЛР) були проведені зі зразками мРНК, виділеними зі згаданих структур. Пов'язані з розвитком зміни були ідентифіковані в транскриптах для профілів TRPC1-7, TRPV1-6, TRPM1-8 та TRPA1. Найбільш вражаючим випадком була негативна регуляція (down-regulation) тільки кількості TRPM5; рівень експресії їх мРНК поступово зменшувався в перебігу пре- та постнатального розвитку як неокортексу, так і гіпокампа. Наші результати дають досить переконливі підстави вважати, що TRPM5 можуть виконувати якусь важливу функцію в онтогенетичного розвитку нервової системи. Розглядається можливість того, що TRPM5 є ключовими компонентами в системі сприйняття температури середовища в початковий період онтогенезу щурів.

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