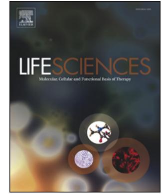
Life Sciences 153 (2016) 50–54

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie



Subacute activation of mGlu4 receptors causes the feedback inhibition of its gene expression in rat brain

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ARTICLE INFO

Article history:
Received 17 August 2015
Received in revised form 2 March 2016
Accepted 17 March 2016
Available online 4 April 2016

Keywords:
mGlu4
GRM4
α-Subunit of the $GABA_A$
GABRA1
Gene expression
TCN 238
Hippocampus

ABSTRACT

Aim: The present study aimed to understand the relationship between pharmacological activation of mGlu4 receptors and regulation of its gene in the hippocampus.
Main methods: The expression level of the *GRM4* gene, encoding mGluR4 receptors, was studied in the hippocampus and frontal cortex of rats after pharmacological activation of the receptor with positive allosteric modulator (E)-4-(2-Phenylethenyl)-2-pyrimidinamine (TCN 238). The drug was injected subcutaneously four times at a dose of 2 mg/kg. The animals were previously trained with hippocampal-dependent task and after the treatment were tested for memory retrieval. The expression level of *GRM4* was determined by qRT-PCR in control and experimental groups of animals one and five days post-treatment.
Key findings: We found that TCN 238 did not affect the performance of the learned task. However, the expression level of *GRM4* in the hippocampus was reliable down-regulated five days after treatment with TCN 238. In addition, we showed that the expression level of *GABRA1*, encoding $GABA_A$ α-subunit was downregulated five days after the treatment in the frontal cortex.
Significance: Subacute pharmacological intervention in mGluR activity by the selective positive modulator TCN 238 has led to adaptive rearrangements of transcription processes in the hippocampus. Moreover, this regulation affected GABA system, confirming importance of the brain excitation-inhibition balance. Since the pharmacological influence on mGluR activity can be regarded as a promising tool aimed to correct brain dysfunction, the properties of mGluR modulators should be studied in more detail, including the level of gene transcription.
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1. Introduction

Glutamate is the main excitatory neurotransmitter in the brain which realizes its action through ionotropic and metabotropic receptors. The metabotropic receptors (mGluRs) associated with G-proteins are expressed by virtually all neurons and glial cells [27], indicating the universal role of glutamate in the regulation of cellular functions. Based on the similarity of the nucleotide sequences of genes, eight subtypes of mGluRs are divided into three groups. Receptors of all groups contain seven hydrophobic segments of the transmembrane domain, which penetrate cytoplasmatic membrane, while their C-termini, are located in the intracellular space and are responsible for the individual properties of the receptor. mGluRs are regarded as important and promising targets for new pharmacological agents aimed at a treatment of various neurological diseases [9,10,20,22]. Over the past few decades a large number of specific ligands to these receptors were synthesized. Some of these ligands showed neuroprotective properties by preventing neuronal death on *in vivo* and *in vitro* models. For example, VU0155041, a positive allosteric modulator of mGluR4, significantly reduced neuronal loss, decreased neuroinflammation, and improved motor functions in rats on 6-hydroxydopamine model of Parkinson disease [7]. Likewise, (R, S)–PPG, the agonist of group III mGluRs, reduced excitotoxic brain damage caused by the infusion of NMDA into the striatum [8]. Moreover, neuronal loss was also reduced in wild-type mice, but this effect was not shown in the knockout mice without mGluR4. The authors demonstrated that NMDA infusion into the striatum causes an increase in extracellular glutamate to a higher level in wild-type mice than in mice with *GRM4* knockout [8], suggesting an important role of mGluR4 in maintaining a homeostatic level of extracellular glutamate.

The density of mGluR4 is high in the cerebral cortex, in the basal ganglia, in the thalamic nuclei, in the hippocampus, and they are localized mainly on the presynaptic membranes [12]. These receptors are potential targets of drugs to interfere with the development of excitotoxicity and seizure activity in temporal lobe epilepsy [28]. Experiments with PHCCC (positive allosteric modulation of mGluR4) confirmed this supposition because this ligand enhanced spontaneous and evoked absence seizures in rats [26].

The expression level of many receptor genes, including *GRM4*, depends on functional state of the brain or external environmental

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http://dx.doi.org/10.1016/j.lfs.2016.03.033
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influences [16,17]. Variability in the expression of *GRM4* supports optimal synaptic activity of glutamate. Bidirectional shifts in the expression level of *GRM4*, for instance, have been shown in the hippocampus and frontal cortex of rats after kainate treatment [5,6]. An involvement of mGluR2/3 and mGluR4 has been detected in response on intrahippocampal microinjection of kainate, which upregulated *GRM2* and *GRM3* in the hippocampus one week after the microinjection. Four weeks after the kainate treatment the expression levels of these genes drop down to the control values, but *GRM4* was upregulated at this time point [5]. Complicated individual dynamics of transcription in the brain must therefore be taken into account because the treatment supposed to be used for therapeutic purposes.

Obviously, of particular significance are those genes, whose expression level is the most labile in pathological state, or in response to an adverse effect on the brain, such as ischemia and hypoxia, mechanical traumas, neurotoxins, and neurotoxicants. The effects of drug treatment also should not be ignored, as both acute and chronic treatments can affect the transcription processes in yet unpredictable manners. Since mGluRs ligands may be used for therapeutic purposes, we explored the expression level of *GRM4* in rat brain after pharmacological activation of mGluR4. For this, we used the selective positive allosteric modulator of mGluR4, TCN 238. *In vivo* pharmacokinetic profiling of TCN 238 revealed that the modulator is good penetrating through the blood-brain barrier; *in vitro* assay revealed TCN 238 EC_{50} is 1 μM in the rat [14,15]. Additionally, TCN 238 was run in a receptor screening panel of 68 targets and no activity was observed at ≥50% at 10 μM for any of the receptors. The receptor panel included adenosine A_{2A}, $5HT_{1A}$ and adrenergic α2A receptors. Furthermore, examination of TCN 238 in the haloperidol-induced catalepsy model revealed a dose dependent effect with ED_{50} ~1 mg/kg [14].

2. Materials and methods

Male Wistar rats (n = 22), weighting 200–220 g were used for experiments, that were carried out in accordance with the guidelines of the Institutional Animal Care and Use Commission of ITEB RAS and the Council of the European Community (EC Directive of 1986). Rats were kept on a 12/12 h light/dark schedule with free access to water, and on a feeding schedule, according to which, they were fed during training and immediately after the experiment once a day.

2.1. Pharmacological treatment

Positive allosteric modulator (E)-4-(2-Phenylethenyl)-2-pyrimidinamine (TCN 238, Tocris) was dissolved in isotonic NaCl solution supplemented with DMSO (30%), as recommended by manufacturers of the drug. TCN 238 was administered subcutaneously at a dose of 2 mg/kg (volume of 0.5 ml) four times in two days (morning and evening). Control animals were treated with solvent in the same time.

2.2. Behavioral test

The animals were trained with a spatial appetitive-motivated task in an experimental chamber (60 × 80 × 60 cm) containing a start box and four shelves placed at different heights and ladders (Fig. 1). To reach the goal shelf with moistened ball of bread, animals had to climb the ladder. Before the beginning of the training, all animals were habituated including handling and ascending the ladders for 6 days. A daily session of the training consisted of 10 trials. The learning was estimated by the measure of trial time (latency), i.e. the time that took the animals to run the distance from the start box to the goal shelf (**G**) displayed at a height of 25 cm over the floor. During the training period the latency decreased and on the fifth day became stabilized to 5 ± 4 s. The details of the learning procedure and properties of used task were described previously [2,4]. After the training all rats were divided into two groups: control (solvent-treated, n = 10) and experimental (TCN 238-treated, n = 12). Retrieval of the task was tested 30 min after the first and third injections of TCN 238, and 5 days after the last injection of the substance. During the retrieval test the animals were placed to the start box, the door was opened, and the latent period of response was registered.

2.3. qRT-PCR analysis

Expression of *GRM4* (mGluR4) was evaluated in 24 h (n = 4) and in five days (n = 8) after the last injection of TCN 238. The expression level of *GABRA1* (α-subunit of $GABA_A$) was estimated in five days after the last administration of TCN 238. Animals of the control group (n = 10) were injected with isotonic NaCl solution supplemented with DMSO (solvent of the drug). To assess the expression level of the target genes, the frontal cortex and the hippocampus were extracted from the brain and placed in a denaturing buffer (4 M guanidine thiocyanate, 25 mM $Na_3O_7C_6H_5$, 0.5% *N*-laurylsarcosine sodium salt, 0.1 M 2-mercaptoethanol) for homogenization. Total RNA was isolated from homogenates using a single-step method of extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture [11]. To remove residual DNA, the samples were treated with DNase I (New England Biolabs) according to the manufacturer's instructions, and the quality of the RNA preparations was verified by electrophoresis in 3% agarose gel. The concentration of purified RNA was measured using the spectrophotometer ND-1000 (NanoDrop Technologies Inc., USA).

GenBank database was used to select the gene-specific primers. The primer for reverse transcription of *GRM4*-mRNA (mGlu4) was: 5′-TGAG AAGTTGACGTTCCTGA-3′, and the counter primer added for the PCR was: 5′-AAGGTGCAGTTCGTGATTG-3′ (the product length was 156 bp). The primer pair used to assess the expression level of *GABRA1* (α1-subunit of the $GABA_A$ receptor) was, respectively: 5′-TACAGCAGTGTGCCATCCTC-3′ and 5′-CCGTGTCAGACCACGATATG-3′, giving a product length of 232 bp. The gene of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a reference sample. Content of *GAPDH*–mRNA in the brain tissues was evaluated with primers 5′-TTGAGGGTGCAGCGAACTT-3′ and 5′-ACAGCAACAGGGTG GTGGAC-5′, which gave a 252 bp-long amplicons.

Reverse transcription was performed with 20 U of M-MuLV reverse transcriptase according to the standard protocol developed by the manufacturer of the enzyme (Fermentas, Lithuania). The reaction was carried out in 25 μl with 2.5 μg of purified RNA. Two and four microliters of this reaction mixture were further used for PCR performed in 20 μl with 4 μl of premix qPCRmix-HS SYBR (Evrogen). This ready for use premix contained Taq DNA polymerase, all substrates and SYBR Green I for detection. Quantitative real time PCR (qRT-PCR) was carried out in the detection thermocycler "DT-322" (DNA Technology, Russia). The PCR program included 10-min of initial denaturation at 95 °C, followed by 33–35 cycles: 95 °C for 30 s, 55 °C for 30 s, and synthesis at 72 °C for 30 s. The fluorescence of SYBR Green I was measured for 15 s at the end of each cycle. Samples with RNA subjected to reverse transcription without reverse transcriptase were used as negative controls. The quality and the size of PCR products were verified by gel-electrophoresis. The amount of mRNA in the hippocampus and the frontal cortex was estimated by the threshold cycle, registered in qRT-PCR, with subsequent calculation by the $2^{-\Delta\Delta Ct}$ method [31].

2.4. Statistic analysis

Data are presented as means ± SD of the mean. Mann–Whitney *U* test was used for comparison of repeated measurements ($P < 0.05$ was considered to be statistically significantly).

3. Results

It is well known, that mGluR4 receptors localize mainly in the presynaptic membranes, i.e. in the active zone of synapses, and their activation reduces glutamate release into the synaptic cleft [12,27]. Thus, the

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