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Research report

Behavioral effects of elevated expression of human equilibrative nucleoside transporter 1 in mice

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ABSTRACT

Adenosine concentrations are regulated by purinergic enzymes and nucleoside transporters. Transgenic mice with neuronal expression of human equilibrative nucleoside transporter 1 (hENT1) have been generated (Parkinson et al., 2009 [7]). The present study tested the hypothesis that mice homozygous and heterozygous for the transgene exhibit differences in hENT1 mRNA and protein expression, and in behavioral responses to caffeine and ethanol, two drugs with adenosine-dependent actions. Real time polymerase chain reaction (PCR) was used to identify mice heterozygous and homozygous for the transgene. Gene expression, determined by real time PCR of cDNA reverse transcribed from cerebral cortex RNA, was 3.8-fold greater in homozygous mice. Protein abundance, determined by radioligand binding assays using 0.14 nM [^{3}H]S-(4-nitrobenzyl)-6-thioinosine ([^{3}H]NBTI), was up to 84% greater in cortex synaptosome membranes from homozygous than from heterozygous mice. In western blots with an antibody specific for hENT1, a protein of approximately 40 kDa was strongly labelled in cortex samples from homozygous mice, weakly labelled in samples from heterozygous mice and absent from samples from wild type mice. In behavioral assays, transgenic mice showed a greater response to ethanol and a reduced response to caffeine than wild type littermates; however, no significant differences between heterozygous and homozygous mice were detected. These data indicate that the difference in ENT1 function between wild type and heterozygous mice was greater than that between heterozygous and homozygous mice. Therefore, either heterozygous or homozygous hENT1 transgenic mice can be used in studies of ENT1 regulation of adenosine levels and adenosine dependent behaviors.

1. Introduction

Adenosine is an important modulator for brain activity. It can function in the nervous system as a homeostatic modulator as well as a neuromodulator at the synaptic level [1]. It regulates brain activity through activation of G-protein coupled adenosine A_1, A_{2A}, A_{2B} and A_3 receptors [2]. These receptors are located throughout the brain and their activation has an important role in neuronal excitability, neuroinflammation, locomotor activity, and arousal [2].

Adenosine receptor activation is dependent upon local adenosine levels and adenosine formation. Adenosine can be formed intracellularly or extracellularly and is transported through cell membranes via nucleoside transporters [3,4]. Nucleoside transport in neurons and glia is predominantly mediated by equilibrative nucleoside transporters 1 and 2 (ENT1, ENT2) [5,6]. To examine the role of ENTs in regulating adenosine levels and adenosine receptor activity, transgenic mice that express human ENT1 (hENT1) under the control of a neuron specific enolase promoter have been developed [7].

Previously, we reported that heterozygous transgenic mice with neuronal expression of hENT1 were more sensitive to ethanol and less sensitive to caffeine than wild type mice, indicating that the

Abbreviations: PCR, polymerase chain reaction; hENT1, human equilibrative nucleoside transporter 1; mAK, mouse adenosine kinase; TAE, Tris acetate–EDTA; DMSO, dimethylsulfoxide; dNTPs, deoxynucleoside triphosphates; RT, reverse transcription; DTT, dithiothreitol; [^{3}H]NBTI, [^{3}H](S-(4-nitrobenzyl)-6-thioinosine; PMSF, phenylmethyl sulfonyl fluoride; ECL, enhanced chemiluminescence; LORR, loss of righting response; C_T, threshold cycle; GABA, γ-amino butyric acid; AMPA, α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; NMDA, *N*-methyl-D-aspartate; i.p., intraperitoneal.

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presence of hENT1 in neuronal cell membranes affects endogenous adenosine levels [7]. In the present study we used quantitative real time PCR to genotype homozygous transgenic, heterozygous transgenic and wild type littermates from three founder lines of hENT1 transgenic mice. We tested the hypothesis that homozygous mice have greater hENT1 RNA and protein abundance than heterozygous mice and show significantly different behavioral and pharmacological responses to heterozygous mice.

2. Methods

2.1. Transgenic mice

Transgenic mice with neuronal expression of hENT1 were generated on a CD1 background as described previously [7]. For the purpose of producing wild type, heterozygous and homozygous mice, heterozygous mating was performed. All procedures and data analyses were performed by an individual who was blinded to the genotype of the mice. All procedures with animals were in accordance with guidelines set by the Canadian Council on Animal Care and approved by the University of Manitoba Animal Protocol Management and Review Committee.

2.2. Genomic DNA extraction

Genomic DNA was extracted from tail snips using the Wizard® Genomic DNA Purification Kit (Promega Corporation), following the manufacturer's protocol. The DNA concentration of each sample was measured using a GeneQuant Pro spectrophotometer (Biochrom, Ltd.) and diluted to working concentrations of 6.25 ng/μL and 3.125 ng/μL. All dilutions were stored at −20 °C until use.

2.3. Standard curve preparation

PCR was performed using DNA extracted from transgenic mice, or cDNA reverse transcribed from RNA isolated from transgenic mice. For the mouse adenosine kinase (mAK) gene, the forward and reverse primers were 5′-CACTTGCAGAGGTGGCAGTAG-3′ and 5′-GTGTCACCACCATGGTCAATC-3′. For the mouse β-actin gene, the forward and reverse primers were 5′-GTGACGTTGACATCCGTAAA-3′ and 5′-CTCAGGAGGAGCAATGATCT-3′. For the hENT1 gene, the forward and reverse primers were 5′-TGTCCCAGAATGTGTCCTTG-3′ and 5′-GATGCAGGAAGGAGTTGAGG-3′. These primer pairs produced products of 179, 148 and 177 base pairs, respectively. Ready To Go™ PCR beads (GE Healthcare) and 500 nM of primer mix (forward and reverse) were used to amplify 1 μL of DNA (or cDNA in the case of the mouse β-actin gene). A PTC-100™ Programmable Thermal Controller (MJ Research, Inc.) was used for the amplification. The 'slow-down PCR' program [8] was used because it had been found previously to reduce primer–dimers and give cleaner results.

A 1% agarose gel, containing ethidium bromide for visualization, was run in Tris acetate–EDTA (TAE) buffer (40 mM Tris acetate and 1 mM EDTA) for 30–90 min at 105 V and the PCR products were cut from the gel while being illuminated by a FBTI-88 Transilluminator (Fisher Scientific). The DNA was extracted from the gel using a QIAquick Gel Extraction Kit (Qiagen Inc.), following the manufacturer's protocol.

The concentration of the DNA produced was measured by running a 1% agarose gel in TAE buffer. Two microliters of DNA mixed with 0.5 μL of loading buffer was run alongside a Low DNA Mass Ladder (Invitrogen) and the gel was imaged using a Gel Logic 100 Imaging System (Kodak). The DNA samples were diluted to 10^{10} copies and ten-fold serial dilutions were performed; copy numbers between 10^7 and 10^1 were used to construct the standard curves.

2.4. Real time PCR of genomic DNA

The gene of interest in the real-time PCR reaction was the gene encoding hENT1. The mAK gene was used as an internal control since it is expressed in the genome at a constant level, irrespective of transgene status. Both of these reactions were run independently on the same plate. Real time PCR reactions were conducted in a final volume of 50 μL, which consisted of 1 μL of DNA template, 1× PCR buffer (Invitrogen), 0.5 μM primers, 2.5 mM $MgCl_2$, 1 μL dimethylsulfoxide (DMSO), 0.2 mM deoxynucleoside triphosphates (dNTPs) (*i.e.*, dATP, dCTP, dTTP, and dGTP) (Invitrogen), 0.1X SYBR Green I (Sigma–Aldrich), 0.25 U Platinum *Taq* polymerase (Invitrogen), and 10 nM fluorescein standard calibration dye (Bio-Rad Laboratories). The real time PCR reaction was performed in triplicate using a Bio-Rad iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories), which consisted of an initial denaturation at 95 °C for 4 min, then 50 cycles of denaturation at 95 °C for 15 s, annealing at 64 °C for 15 s, and elongation at 72 °C for 30 s. A melt curve analysis was then performed, by raising the temperature from 64 °C to 95 °C at a rate of 0.5 °C/10 sec. In all cases, melt curve analysis indicated a single PCR product in each reaction.

2.5. Real time PCR of cDNA

RNA was isolated from cortex samples using the TRIzol™ method (Invitrogen). The concentration of RNA was measured using a GeneQuant Pro spectrophotometer (Biochrom, Ltd.) and diluted to 1.5 μg/μL using ddH_2O. The diluted RNA was stored at −80 °C until use.

Reverse transcription (RT) was performed by first treating 1.5 μg RNA with 1 U DNase (Invitrogen) in 20 mM Tris–HCl (pH 8.4), 2 mM $MgCl_2$ and 50 mM KCl at room temperature for 15 min. Then, 500 ng olig$(dT)_{12–18}$ primer and was added and heated to 65 °C for 5 min. The final step consisted of the addition of Moloney-murine leukemia virus reverse transcriptase (200 U), dNTPs (0.5 mM), dithiothreitol (DTT; 10 mM), Tris–HCl (50 mM; pH 8.3), KCl (75 mM) and $MgCl_2$ (3 mM) in a final volume of 60 μL, then incubated at 37 °C for 1 h followed by 90 °C for 5 min. All cDNA samples were stored at −20 °C until use.

Real time PCR of the cDNA was performed to quantify expression of the genes encoding hENT1 (the gene of interest) and mouse β-actin, the internal control. The procedure followed was as described above for real time PCR of genomic DNA but with the following differences: a final volume of 25 μL, 1 μL of cDNA template or water, and the use of a Realplex^2 Mastercycler (Eppendorf). The thermocycler program used was the same as that for the genomic DNA.

2.6. [^{3}H] S-(4-nitrobenzyl)-6-thioinosine (NBTI) binding assays

For radioligand binding assays, cortex samples were collected from mice after the end of behavioral experiments. Synaptosomes were prepared from cerebral cortex samples using the method published previously [7], with slight variation. Cortices were homogenized in approximately 10 volumes of ice-cold 0.32 M sucrose and then centrifuged at 1000 × *g* for 10 min at 4 °C. The pellets were washed twice and pooled supernatants were then centrifuged at 20,000 *g* for 1 h at 4 °C to obtain pellets. The pellets were resuspended in HEPES buffer composed of 110 mM NaCl, 25 mM glucose, 68.3 mM sucrose, 5.3 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, and 20 mM HEPES, pH 7.4. Protein concentrations were determined with a dye binding assay (Bio-Rad Laboratories).

[^{3}H]NBTI binding assays were performed in an assay volume of 0.9 mL with 30 μg/assay synaptosomes, 0.14 nM [^{3}H]NBTI, and 0.5 U adenosine deaminase (Sigma–Aldrich). Vacuum filtrations were performed through GF/B filters after 1-h incubations at 22 °C. The filters were placed in scintillation vials with 5 mL of scintillation cocktail and radioactivity was counted after at least 12 h.

2.7. Western blot

Cerebral cortex samples were dissected and stored at −80 °C until use. Membrane proteins were extracted as described previously [9]. Briefly, partially thawed tissue was lysed in buffer containing 100 mM Tris–HCl (pH 7.2), 300 mM sucrose, 1 mM EDTA, 0.02% NaN_3, 0.3 mM phenylmethyl sulfonyl fluoride (PMSF; Sigma–Aldrich), 0.5 mM DTT and protease inhibitors (Roche). After homogenization with a Dounce glass grinder, the tissue was centrifuged at 1000 × *g* for 10 min at 4 °C. Supernatants were centrifuged at 150,000 × *g* for 45 min at 4 °C. The resulting pellets were resuspended in lysis buffer and homogenized again, then centrifuged at 150,000 × *g* for 45 min at 4 °C. The resulting pellets were resuspended in buffer containing 20 mM Tris–Citrate (pH 7.5), 1 mM EDTA, 0.02% NaN_3, 0.3 mM PMSF, 0.5 mM DTT and protease inhibitors, then frozen in liquid nitrogen and stored at −80 °C until use. Protein content was determined with a protein assay kit (Bio-Rad Laboratories) and 50–100 μg of proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and transferred onto PVDF membranes. The electroblotted membranes were incubated with 1:1000 dilutions of supernatant from hybridoma 10D7G2, which produces monoclonal antibodies against hENT1 amino acid residues 254–271 [9], or mouse monoclonal anti-Na^+/K^+ATPase antibody (Sigma–Aldrich). Following incubations with horseradish peroxidase conjugated goat anti-mouse IgG (Sigma–Aldrich) at 1:10,000 dilution, immunoreactive bands were visualized on X-ray film by an enhanced chemiluminescence (ECL) detection kit (Roche). Human red blood cells were used as a positive control for detection of hENT1.

2.8. Wheel running

Mice, 8–10 weeks of age, were housed individually in fresh plastic cages equipped with food and water and provided with voluntary access to wireless running wheels (diameter: 15.5 cm; Med Associates Inc.). Cages were placed in an environmentally controlled room with 12/12 h light–dark cycle in which lights were on between 6:00 a.m. and 6:00 p.m. and off between 6:00 p.m. and 6:00 a.m. In the experiment, mice were placed in the cages at 10:00 a.m. and the counts of wheel rotations were collected for 46 h by the SOF-860 Wheel Manager system.

2.9. Ethanol sensitivity

Using the method described previously [7], mice, 8–10 weeks of age, were injected with ethanol (3.6 g/kg; 20%, v/v in saline). Each mouse was gently placed on its back when the locomotion ceased. The time duration of the loss of righting