NeuroToxicology 39 (2013) 138–145

Contents lists available at ScienceDirect

NeuroToxicology

Methamphetamine alters reference gene expression in nigra and striatum of adult rat brain

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ARTICLE INFO

Article history:
Received 11 June 2013
Accepted 22 August 2013
Available online 14 September 2013

Keywords:
Methamphetamine
Housekeeping genes
Nigra
Real-time PCR
Reference genes
Striatum

ABSTRACT

The nigrostriatal dopaminergic system is a major lesion target for methamphetamine (MA), one of the most addictive and neurotoxic drugs of abuse. High doses of MA alter the expression of a large number of genes. Reference genes (RGs) are considered relatively stable and are often used as standards for quantitative real-time PCR (qRT-PCR) reactions. The purpose of this study was to determine whether MA altered the expression of RGs and to identify the appropriate RGs for gene expression studies in animals receiving MA. Adult male Sprague-Dawley rats were treated with high doses of MA or saline. Striatum and substantia nigra were harvested at 2 h or 24 h after MA administration. The expression and stability of 10 commonly used RGs were examined using qRT-PCR and then evaluated by geNorm and Normfinder. We found that MA altered the expression of selected RGs. These candidate RGs presented differential stability in the striatum and in substantia nigra at both 2 h and 24 h after MA injection. Selection of an unstable RG as a standard altered the significance of tyrosine hydroxylase (TH) mRNA expression after MA administration. In conclusion, our data show that MA site- and time-dependently altered the expression of RGs in nigrostriatal dopaminergic system. These temporal and spatial factors should be considered when selecting appropriate RGs for interpreting the expression of target genes in animals receiving MA.

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1. Introduction

Quantitative real-time PCR (qRT-PCR) is one of the most commonly used techniques to examine gene expression in biomedical research. The relative quantification method is often used to analyze qRT-PCR data, whereby relative gene expression is obtained by normalization with one or several reference genes (RGs) (Bustin et al., 2009) or previously known as housekeeping genes (HKGs). Increasing evidence, however, suggests that the expression of RGs can be altered after injury, such as oxidative stress, inflammation, apoptosis, and proliferation. It is likely that the outcome of target gene expression may differ depending on the choice of RGs (Ferguson et al., 2010; Ullmannova and Haskovec, 2003). Selection of the stable RGs is, thus, critical for accurately measuring gene expression after injury.

Methamphetamine (MA) is a dopaminergic neurotoxin. High doses of MA cause neurodegeneration through suppression of neurotrophic factors, like BMP7 (Shen et al., 2008) and GDNF (Wang et al., 2001), and upregulation of apoptotic genes (Bachmann et al., 2009; Shen et al., 2008; Wang et al., 2001). Many reference genes (RGs), i.e. 18s rRNA (Krasnova et al., 2011), Hmbs (Luo et al., 2010), Hprt (Luo et al., 2010), Oza1 (Martin et al., 2012), have been used to examine the expression of these target genes after MA treatment. However, limited studies have been conducted to examine the changes of these RGs in the nigrostriatal pathway of adult rats receiving MA. It is thus possible that the expression of target genes may have been mis-interpreted after MA injury if stability of RGs is not validated.

The purpose of this study was to investigate the effect of MA on RGs in rat striatum and nigra and to identify the stability of RGs for qRT-PCR quantification. We examined the expression of 10 frequently used RGs, according to the literature for candidate internal references, at two time points after MA exposure. We found that RGs' expression was altered by MA in a time-dependent and tissue-specific manner. Our data identified relatively stable RGs that can be potentially useful as the standards for target gene expression in striatum and substantia nigra after MA injury.

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0161-813X/$ – see front matter. Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.neuro.2013.08.009

2. Materials and methods

2.1. Candidate reference gene selection

A total of 10 candidate RGs were chosen for this study based on published reports involving MA actions in the nigrostriatal dopaminergic pathway (Table 1).

2.2. Experimental groups and sampling

Adult male Sprague-Dawley rats (n = 32, Charles River Laboratories Inc., Wilmington, MA), weighting 200–225 g, were used in this study. Animals were housed individually in cages with free access to food and water. Animals were acclimated for 1 week in our temperature-controlled animal facility prior to drug treatment. All the animal procedures were done according to guidelines of National Institute of Health. Animals were treated with subcutaneous injections of 0.9% saline (1 mL/kg, every 2 h, 4× times, n = 16) or MA (5 mg/kg every 2 h, 4× times, n = 16). Animals were sacrificed by decapitation at 2 h and 24 h after the last dose of MA or saline. Brain tissues were collected and frozen at −80 °C until use.

2.3. RNA isolation

RNA isolation was performed in an AirClean 600 PCR Workstation (AirClean® Systems, Raleigh, NC) to protect the samples from potential contamination. All work surfaces, pipettes and gloves were kept free of RNase by spraying Ambion® RNaseZap® (Life Technologies, Carlsbad, CA) just before each experiment. 1 mL TRIzol® Reagents (Life Technologies, Carlsbad, CA) was added to each tissue sample in a 2 mL polypropylene microvial (Biospec Product, Bartlesville, OK) containing 0.5 g of 0.9–2.0 mm stainless steel beads (Next Advance, Averill Park, NY). Brain tissue was homogenized by vigorous shaking with a Mini-BeadBeater-16 (Biospec Product, Bartlesville, OK) for 2 × 30 s. Total RNA was isolated according to the manufacturer's instruction (TRIzol® Reagent). Briefly, 0.2 mL chloroform was used for separating the organic and aqueous phases by centrifugation at 12,000 × g for 15 min at 4 °C. RNA in the aqueous phase was then precipitated using 0.5 mL isopropanol at 12,000 × g for 10 min at 4 °C, washed with 1 mL of 75% ethanol, air-dried and redissolved in 30 μL DEPC-treated water (Ambion, Austin, TX). The quantity and purity of isolated RNA were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). RNA samples were stored at −80 °C until use.

2.4. Reverse transcription and qRT-PCR

All first-strand cDNA samples were synthesized from 2.5 μg total RNA per 20 μL reaction using SuperScript® VILO™ cDNA Synthesis Kit (Life Technologies, Carlsbad, CA), then diluted 1:10 with nuclease-free water. Pre-designed TaqMan® probes and primer sets (Life Technologies, Carlsbad, CA, see Table 1) were used to span exons to amplify target cDNA without amplifying genomic DNA. A total volume of 10 μL reaction mixture consisting of 5 μL TaqMan® Fast Advanced Master Mix (Life Technologies, Carlsbad, CA), 0.5 μL of a TaqMan® probe and primer set and 4.5 μL diluted cDNA was loaded to each single well of a 96-well plate. qRT-PCR was run on an Applied Biosystems 7500 Fast Real-Time PCR System under the thermal cycling conditions recommended in the manual for TaqMan® Fast Advanced Master Mix: 50 °C for 2 min, followed by 95 °C for 20 s, 40 cycles of denaturation at 95 °C for 3 s and annealing and extension at 60 °C for 30 s. Standard curve for amplification efficiency of PCR reaction was generalized from three replicates per each dilution. Eight biological replicates were used for each treatment group and two technical replicates for each biological replicate. No-reverse-transcription controls (no RT controls) and no template controls (NTCs) were used respectively to rule out genomic DNA contamination and reagent contamination.

2.5. Evaluation of the stability of the candidate reference genes

Two freely accessible Excel-based tools, geNorm v3.5 (http://medgen.ugent.be/~jvdesomp/genorm/) (Vandesompele et al., 2002) and Normfinder v0.953 (http://www.mdl.dk/publications-normfinder.htm) (Andersen et al., 2004) were used to evaluate the expression stability of the 10 candidate reference genes. Briefly, Ct values from qRT-PCR runs were transformed to linear scale expression quantities using comparative Ct method as described in the manuals. Gene expression stability or "M value" was calculated for each gene by geNorm. The RG with the highest M value was stepwise eliminated until only two RGs remained (Vandesompele et al., 2002). Genes with the highest M values represent the least stable genes, while genes with the lowest M values have the most stable expression. geNorm did not take the experimental conditions into account because this measure relies on the principle that the expression ratio of two ideal internal control genes is identical in all samples (Vandesompele et al., 2002). A pairwise variation value (V value) of each candidate genes was ranked according to stability of intragroup and intergroup variations [15]. The V value represents the levels of variation in average RG stability and was used to determine of the optimal number of RGs. The threshold of $Vn/n+1$ value was 0.15. An additional RG for endogenous controls was not required if $Vn/n+1 < 0.15$. The top-ranked candidate RGs were those with smallest V value.

Normfinder analysis, which takes information of groupings and samples into consideration, was used for assessing the stability of candidate RGs. A stability value of each gene was calculated using intra-group and inter-group variations (Andersen et al., 2004). The

Table 1
The panel of candidate reference genes.

Gene symbol	Gene bank ID#	Gene name	Reference	Assay ID#/part number
GAPDH	NM_017008.3	Glyceraldehyde 3-phosphate dehydrogenase	Pendyala et al. (2012)	4352338E
Act	NM_031144.2	Actin, beta	Nakahara et al. (2003) and Putzke et al. (2007)	Rn00667869_m1
18S	NR_046237.1	18S ribosomal RNA	Krasnova et al. (2011), Pendyala et al. (2012) and Wang et al. (2001)	4333760F
Tbp	NM_001004198.1	TATA box binding protein	Pendyala et al. (2012)	Rn01455646_m1
Hmbs	NM_013168.2	Hydroxymethylbilane synthase	Luo et al. (2010)	Rn00565886_m1
Hprt1	NM_012583.2	Hypoxanthine phosphoribosyltransferase 1	Luo et al. (2010)	Rn01527840_m1
Ubc	NM_017314.1	Ubiquitin C		Rn01499642_m1
B2m	NM_012512.2	Beta-2 microglobulin	Shilling et al. (2006)	Rn00560865_m1
Rps6	NM_017160.1	Ribosomal protein S6	Shilling et al. (2006)	Rn00820815_g1
Oaz1	NM_139081.1	Ornithine decarboxylase antizyme 1	Martin et al. (2012)	Rn01408148_g1