— **Regular Article** —

Development of a SYBR Green Real-time Polymerase Chain Reaction Assay for Quantitative Detection of Human *N*-methyl-D-aspartate Receptors Subtype 1 Splice Variants

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(Received February 23, 2010; Accepted July 7, 2010; Published online July 23, 2010)

N-methyl-D-aspartate receptors (NMDAR) belong to the ionotropic glutamate receptor subclass and are widely distributed in the vertebrate brain. Molecular cloning has revealed the existence of seven NMDAR subunits: one NMDAR1 (NR1), four different NMDAR2 (NR2A-D), and two different NMDAR3 (NR3A,B). Alternative splicing of the single NR1 gene generates eight isoforms with distinct functional properties. So far, the transcripts of the NR1 splice variants have been discriminated by Northern blot, *in situ* hybridization, or competitive polymerase chain reaction (PCR) methods all of which have their intrinsic limitations. In this study, we have developed a method to quantify the mRNAs of the NR1 splice variants by real-time PCR with the double-stranded DNA-binding dye SYBR Green I. The implementation of this assay will allow a better understanding of the regulatory mechanisms of the NR1 splice variants, and hence, their role in neuronal disease pathogenesis.

Key words —— *N*-methyl-D-aspartate, splice variants, real-time polymerase chain reaction

INTRODUCTION

Glutamate receptors are the primary mediators of excitatory synaptic transmission in the mammalian brain.[1] The *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptor has been implicated in several critical central nervous system functions, including learning and memory.[2] NMDA receptor (NR) abnormalities may underlie a number of pathological conditions.[3] The molecular cloning studies showed that NR consists of three subunits named NR1,[4] NR2A-D,[5,6] and NR3A,B.[7–9] Furthermore, functional studies have revealed that NR1 subunits need to be coexpressed with NR2 subunits in order to produce cation transport activity.[10]

The human NR1 (hNR1) subunit mRNA is alternatively spliced at three exons to form eight splice variants.[11,12] Splicing of the *N*-terminal cassette (exon 4) results in NR1-a (absence) or NR1-b (presence) variants. In the *C*-terminal tail, individual splicing of the C1 (exon 20) or C2 (exon 21) cassette results in transcripts designated as NR1-2 or NR1-4, respectively. The presence or absence of both C1 and C2 cassettes results in NR1-1 and NR1-3 splice variants, respectively. Furthermore, the deletion of the C2 cassette alters the reading frame, generating an unrelated sequence of 22 amino acids designated as C2′.[13] These alternatively spliced regions of NR1 regulates the sensitivity to physiological pH,[14] and influences protein interactions[15] and intracellular trafficking.[16] Various studies have also shown that these splice variants vary in Alzheimer disease,[17,18] schizophrenia,[19] aging,[20] and exposure to various chemicals.[21–23] Hence, examination of the expression of the NR1 splice variants is crucial for determining their potential relevance in neurological diseases.

Evaluation of NR1 splice variants expression in these conditions presents critical restrictions and has been limited to research protocols partly due to analytical difficulties. Methods employed so far include Northern blotting,[21] *in situ* hybridization,[23]

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Table 1. Sequence of Oligonucleotides Used as Primers

mRNA target	Primer name	Sequence	Amplicon size
NR1-1	NR1-1/2F	5′-CTGGGATCTTCCTCATTTTCATC-3′	128 bp
	NR1-1R	5′-CAGTGGGATGGTACTGCTGC-3′	
NR1-2	NR1-1/2F	5′-CGGGATCTTCCTGATTTTCATC-3′	123 bp
	NR1-2R	5′-CCCCCGGTGCTCTGCA-3′	
NR1-3	NR1-3/4F	5′-GATAGAAAGAGTGGTAGAGCAGAGC-3′	122 bp
	NR1-3R	5′-ACCCCCGGTGCTCGTG-3′	
NR1-4	NR1-3/4F	5′-GATAGAAAGAGTGGTAGAGCAGAGC-3′	126 bp
	NR1-4R	5′-CAGTGGGATGGTACTGCGTG-3′	
NR1-a	NR1-aF	5′-GGAGCGTGAGTCCAAGGC-3′	117 bp
	NR1-aR	5′-GGCAGAAAGGATGATGACCC-3′	
NR1-b	NR1-bF	5′-AACTATGAAAACCTCGACCAACTG-3′	83 bp
	NR1-bR	5′-GGTCCCTGGGTCAAACTGC-3′	

and competitive PCR.[24] These methods provide only qualitative or semi-quantitative information, and a truly quantitative and reproducible evaluation of NR1 splice variants expression is still needed.

This study aimed to develop a reliable and accurate real-time PCR method using SYBR Green I dye that allows cost effective measurement of NR1 splice variants expression levels.

MATERIAL AND METHODS

cDNA Synthesis —— The human hippocampus PolyA$^+$ RNA (500 ng) from Clontech (Mountain View, CA, U.S.A.) was reverse transcribed using SuperScript III Reverse transcriptase and random hexamers (Invitrogen, Carlsbad, CA, U.S.A.). In addition, total RNA (5 μg) from a normal hippocampus and Alzheimer disease hippocampus obtained from BioChain (Hayward, CA, U.S.A.) were reverse transcribed using High-Capacity cDNA Archive Kit (Applied BioSystems, Foster City, CA, U.S.A.).

Primer Design —— Primers were purchased from Invitrogen and were designed using the Primer Premier software (PREMIER Biosoft International, Palo Alto, CA, U.S.A.). Two forward primers designated hNR1-1/2F and hNR1-3/4F, and four reverse primers designated hNR1-1R (four bases annealing to the 3′ end of exon 19), hNR1-2R (five bases annealing to the 3′ end of exon 19), hNR1-3R, and hNR1-4R (four bases annealing to the 3′ end of exon 20), were designed to permit discrimination between the C-terminal splice variants. Two forward primers designated hNR1-aF and hNR1-bF, and two reverse primers designated hNR1-aR and hNR1-bR (two bases annealing to the 5′ end of exon 5) were designed to permit discrimination between the *N*-terminal splice variants. The real-time PCR primer sequences are shown in Table 1.

Plasmid Standard —— A 164-bp NR1-1 sequence (GenBank accession no. NM_000832.5, 2478–2658 bp); 230-bp NR1-2 sequence (GenBank accession no. NM_021569.2, 2478–2706 bp); 341-bp NR1-3 (GenBank accession no. NM_007327.2, 2478–2817 bp); 274-bp NR1-4 (GenBank accession no. U08106.1, 2478–2769 bp); 257-bp NR1-a (GenBank accession no. NW017008, 439–694 bp); and 320-bp NR1-b (GenBank accession no. NW017008, 439–757 bp) were amplified from the hippocampus cDNA by PCR. The PCR products were then separated on 1% agarose gel, and the bands were excised, purified using the MinElute Gel Extraction Kit (Qiagen, Valencia, CA, U.S.A.), and cloned in the pCR4-TOPO vector of TOPO TA Cloning Kit (Invitrogen) as per the standard protocols. Plasmid cDNA constructs were prepared with the QIAprep Spin Miniprep Kit (Qiagen) and their identity was verified by DNA sequencing on a 3700 sequencer (Applied BioSystems). Plasmid cDNA concentrations were measured by UVmini-1240 absorbance spectrophotometer (Shimadzu, Kyoto, Japan). Serial dilutions of the extract were prepared containing 10^7–10^3 copies of the plasmid.

Real-time Quantitative PCR Using SYBR Green I —— Real-time PCR was performed on a SDS7000 (Applied BioSystems). The dilution series of the standard plasmid DNA for each hNR1 splice variant and 100 ng cDNA (total RNA equivalent) of samples was amplified in a 50 μl reaction containing 1 × SYBR Green I Master Mix (Applied BioSystems) and 50 nM of each primer and nuclease-free water. We performed

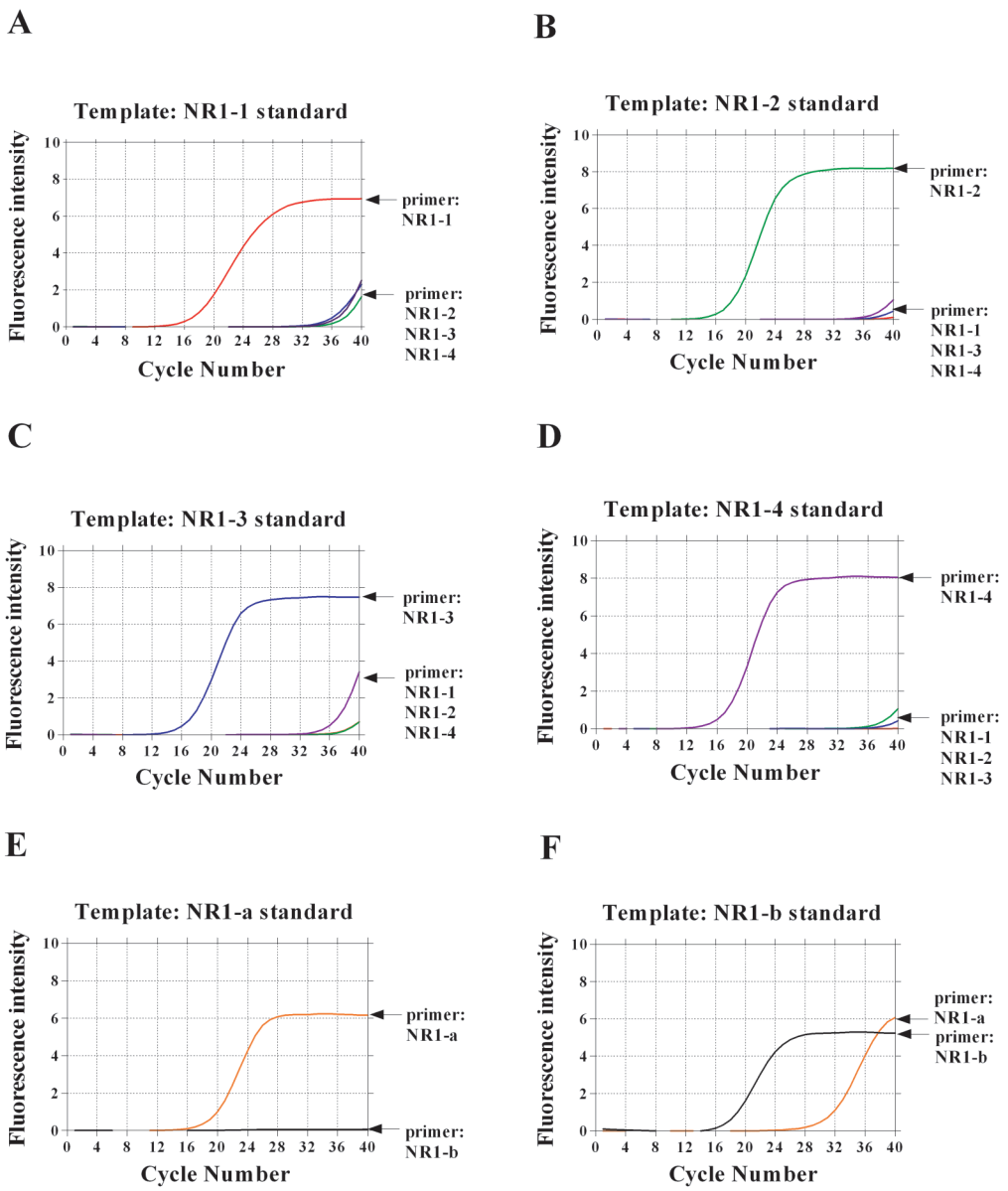

Fig. 1. Amplification Plots of SYBR Green I, Real-time PCR for Human NR1 Splice Variants

PCR was carried out using primers specific for NR1-1 (A), NR1-2 (B), NR1-3 (C), NR1-4 (D), NR1-a (E) and NR1-b (F) in the presence of each standard plasmids containing each splice variant specific cDNA sequence.

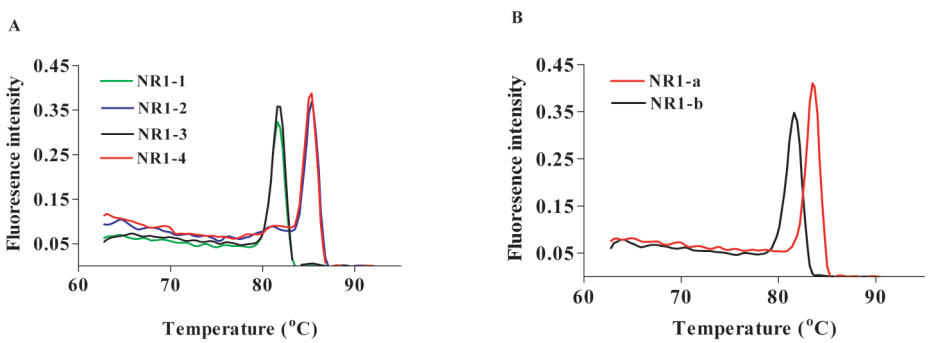

Fig. 2. Dissociation Curve Analysis of hNR1 Splice Variant Amplicons

A C-terminal splice variant amplicon (A) and *N*-terminal splice variant (B) is subjected to melting curve analysis and a plot of fluorescence versus temperature is indicated. The presence of a single peak is consistent with the formation of a single amplicon. It also indicates the lack of primer-dimer formation.

each amplification three times. The thermal profile consisted of one cycle at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and at 62°C for 1 min. Melting curves were generated after amplification. Real-time PCR efficiencies for each reaction were calculated using the formula: efficiency (E) = $[10^{(1/\text{slope})}] - 1$, from the slope values given in the SDS7000.

Quantification and Data Analysis —— For each run, data acquisition and analysis was done by the SDS7000 System software. The relative mRNA level of each hNR1 splice variants was determined by interpolating the threshold cycle (Ct) values of the unknown samples to each standard curve and the obtained values were normalized to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) mRNA levels in same samples as determined by a TaqMan human GAPDH Control Reagent kit (Applied BioSystems).

RESULTS AND DISCUSSION

Specific detection of different hNR1 splice variants was achieved with the following primer pairs: NR1-1, primer pair NR1-1/2F-NR1-1R; NR1-2, primer pair NR1-1/2F-NR1-2R; NR1-3, primer pair NR1-3/4F-NR1-3R; NR1-4, primer pair NR1-3/4F-NR1-4R; NR1-a, primer pair NR1-aF-NR1-aR; and NR1-b, primer pair NR1-bF-NR1-bR. For every splice variant, 10^7 copies of standard plasmid cDNA were detectable with the appropriate primer sets with a mean Ct of 16. Real-time reverse transcription (RT)-PCR with "nonspecific" primer set for NR1-1, NR1-2, NR1-3, NR1-4, and NR1-a, and 10^7 copies of standard plasmid cDNA did not generate any reporter fluorescence signal even after 35 PCR cycles. On the other hand, real-time RT-PCR for NR1-b standard plasmid cDNA generated a reporter fluorescence signal in 28 cycles, and achieved a 8000-fold specificity with standard plasmid cDNA (Fig. 1). Specificity was also confirmed in a melting curve analysis performed on the SDS7000 (Fig. 2). Dissociation curves showed a single peak corresponding to a melting temperature of 81.6°C for NR1-1 and NR1-3, and corresponding to 85.3°C for NR1-2 and NR1-4 splice variants (Fig. 2A). Similarly, dissociation curves showed a single peak corresponding to melting temperatures of 83.5 and 81.6°C for NR1-a and NRI-b, respectively (Fig. 2B). These results demonstrate specific amplification and the absence of primer dimers.

To quantify the number of molecules of each hNR1 splice form, we constructed six different standard curves (Fig. 3). A linear relationship was observed between the initial copy numbers and each 10-fold dilution from 10^7–10^3 copies per reaction mixture, and the Ct values for each standard curve. A regression analysis of the Ct values generated by the log10 dilution series resulting in a correlation coefficient (r^2) value > 0.99 was performed for each standard curve. The efficiency of the PCR reaction was typically > 87%.

We also examined the relative expression (%) of hNR1 splice variants, *i.e.*, the ratio of a specific variant to the sum of all hNR1 splice variant mRNAs in hippocampus from normal and Alzheimer disease patients. In normal hippocampus, the relative expression of C-terminal splice variants was 47, 6, 30, and 17% for NR1-1, NR1-2, NR1-3, and NR1-4, respectively; in hippocampus of Alzheimer patients, the relative expression was 32, 9, 37, and 21% for NR1-1, NR1-2, NR1-3, and NR1-4, respectively (Table 2), suggesting similar expression patterns between the two groups. This result was in accordance with those of previous studies that used a different technology namely competitive PCR.[24] On the other hand, we found different expression patterns of the N-terminal splice variants between the hippocampus from normal and Alzheimer patients (Table 3). We observed that, in normal patients the percentage of NR1-a and NR1-b, as part of total N-terminal splice variants, was 84 and 16%, while in the Alzheimer patient the percentage was 39 and 61%, respectively. However, it must be noted that these mRNA samples were from single donors and hence the issue of interindividual variation has not been addressed in this study.

As NR involvement has been implicated in a number of pathologic conditions and as these conditions may be reflected in altered patterns of NR1 mRNA expression, quantification of mRNA expression is essential for the assessment of NR1-mediated mechanisms. A number of different methods are used for the quantification of mRNA, such as competitive RT-PCR, Northern blotting, and *in situ* hybridization. Of all these methods, competitive RT-PCR has proven to be the most accurate and sensitive method to study NR1 splice variant mRNA expression in the brains of Alzheimer patients.[24] However, this method has some drawbacks such as post-PCR manipulations, impaired quantification due to either a lack of sensitivity in gel quantification, variations in PCR efficiencies between in-

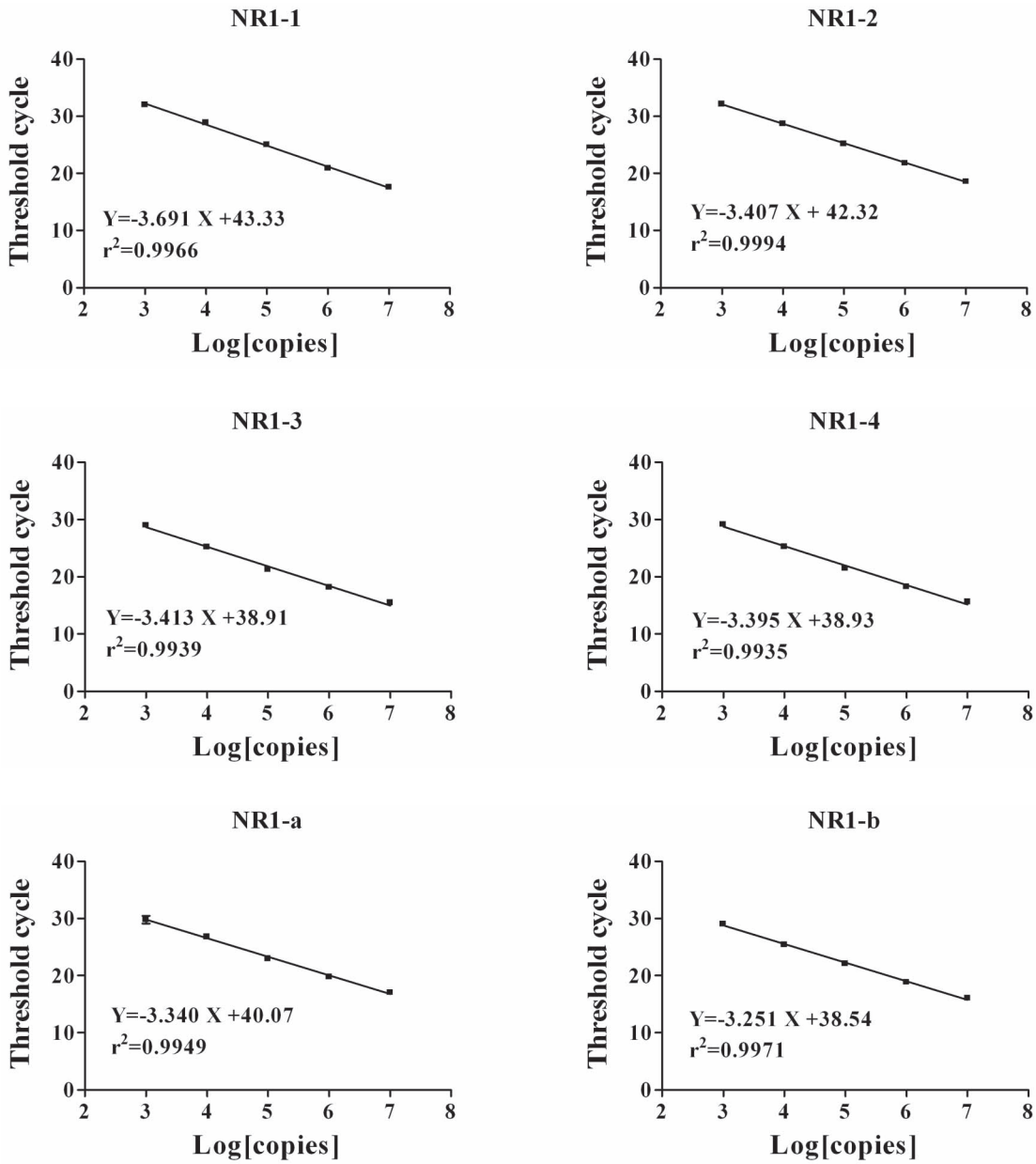

Fig. 3. Standard Curve for the Real-time PCR Amplification of Human NR1 Splice Variants

A plot of *Ct* values versus the logarithm of 10^3, 10^4, 10^5, 10^6, and 10^7 copies of standard plasmids containing the specific cDNA sequence for each splice variant is indicated.

Table 2. Expression of *C*-terminal NR1 Splice Variants mRNA in Normal and Alzheimer Patients Hippocampus

	Normal	Alzheimer
NR1-1	0.47 (47%)	0.32 (32%)
NR1-2	0.07 (6%)	0.09 (9%)
NR1-3	0.30 (30%)	0.37 (37%)
NR1-4	0.17 (17%)	0.22 (22%)

All data presented as the *C*-terminal NR1 splice variant: GAPDH mRNA ratio. The relative amounts of the individual splice variants related to the total sum are given in parentheses as percentages.

Table 3. Expression of *N*-terminal NR1 Splice Variants mRNA in Normal and Alzheimer Patients Hippocampus

	Normal	Alzheimer
NR1-a	0.63 (84%)	0.31 (39%)
NR1-b	0.12 (16%)	0.49 (61%)

All data presented as the *N*-terminal NR1 splice variant: GAPDH mRNA ratio. The relative amounts of the individual splice variants related to the total sum are given in parentheses as percentages.

ternal control and target cDNA, or the extended time requirement.[25] However, these disadvantages can be overlooked as real-time PCR allows for increased sensitivity and low variability.[26] In this study, we utilized a SYBR Green I dye, a nonspecific DNA binding dye, to quantitatively assess the levels of gene expression for NR1 splice variant transcripts in human tissue samples. We demonstrated that fluorescent detection with SYBR Green I dye in real-time RT-PCR applications can be used to quantify PCR products of NR1 splice variant with high sensitivity over a wide linear range. This procedure can be easily used with a high-throughput format, such as a 96-well microtiter plate, allowing several hundred reactions to be processed on a daily basis with the nonspecific nature of the dye being the only drawback, which precludes the use of multiplex reactions. Thus, the use of SYBR Green I dye for detection offers a method for routine quantification of human. NR1 splice variant.

Acknowledgements This work was supported in part by the Ministry of Education, Culture, Sports, Science, and Technology (MEXT), HAITEKU (2004–2008).

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