



Cloning and sequencing of c-DNA encoding N-methyl-D-aspartate receptor subunit-1 in the hypothalamus of male sheep

Ana Zuley Ruiz¹

¹ Departamento de Ciências Biomédicas, Cátedra de Fisiología Animal, Facultad de Ciencias Veterinarias, Universidad Central de Venezuela, Maracay, estado Aragua, Venezuela.

ABSTRACT - The objective of the present study was to report the molecular cloning and determination of the sequences N-methyl-D-aspartate subunit-1 receptor (NMDAR-1) in the hypothalamus of sheep. The re-amplified DNA template for NMDA -1 from the hypothalamus of male sheep was cloned using pBluescript-Sk-plasmids (pBSK, 2958 bp). Purified plasmids containing the NMDA receptor c-DNA were sequenced using the dye-terminator chemistry at the University of Nebraska-Lincoln, Nebraska, USA. Results were entered into the National Center for Biotechnology Web site as accession number AY434689. The sequence of *Ovis aries* (sheep) NMDAR-1 mRNA from hypothalamus of male sheep has 94-97% homology with *Homo sapiens*, 94-100% homology with *Sus scrofa*, and 88-90% homology with *Rattus norvegicus* NMDAR-1 mRNA sequence, and 94-97% homology with *Homo sapiens* glutamate receptor and transcript variant NR1-2 and NR1-1 mRNA sequence. These results show high evolutionary conservation of NMDA receptor subunit-1 across species.

Key Words: cloning, NMDA receptor, *Ovis aries*, ram, sequencing

Introduction

The excitatory amino acids (EAA), aspartate and glutamate, are considered the major endogenous neuroactive substances involved in excitatory neurotransmission in the brain. Electrophysiological and radioligand studies have shown that the stimulatory effects of EAA are used in a variety of different postsynaptic receptor subtypes. The two main classes of glutamate receptors are ionotropic and metabotropic. Receptors in the ionotropic glutamate receptor classification are composed of: 1) N-methyl-D-aspartic acid (NMDA) receptors; 2) kainic acid (KA) receptors; 3) 2-amino-3-hydroxy-5-methyl-4-isoxazol propionic acid (AMPA) receptors; and 4) amino-4-phosphobutyric acid (L-AP₄) receptors. Molecular biological techniques have revealed that the NMDA-receptor-channel-complex comprises two subunits. The NMDA receptor is formed by the subunit NMDAR-1 in combination with NMDAR-2A through NMDAR-2D. Conventional NMDA receptors are composed of obligate NMDAR-1 subunits and of subunits NMDAR-2A to NMDAR-2D (Monaghan et al., 1989; Monyer et al., 1992; Matsuda et al., 2003; Furukawa et al., 2005).

The NMDA receptor is the major EAA receptor that mediates many neuronal functions in glutamate neurotransmission including synaptic plasticity, habituation and neurodegeneration in the central nervous system (Riedel et al., 2003; Schmidt et al., 2008; Hardingham and Bading, 2010; Hsieh et al., 2012; Kalev-Zylinska et al., 2013). These EAA play an important role in regulation of luteinizing hormone releasing-hormone (LHRH) secretion from the hypothalamus and subsequent release of luteinizing hormone (LH) from the anterior pituitary in many species (Brann, 1995; Maffucci et al., 2008). One approach to understanding the complex properties of the NMDA receptor involves the cloning and characterization of the genes encoding this receptor (Durand et al., 1992). The NMDA receptor has been extensively characterized electrophysiologically in the *Xenopus oocyte* system. The objective of the present study was to report the molecular cloning and determination of the sequence NMDA subunit-1 receptor in the hypothalamus of sheep using purified plasmids.

Material and Methods

The University of Nebraska-Lincoln-USA Institutional Animal Care and Use Committee approved all the experimental and surgical procedures. After sodium pentobarbital-induced euthanasia (Sleepaway, Fort Dodge Labs, Inc, Fort Dodge, IA, USA), the hypothalamic from four (4) peripuberal spring-born crossbred blackface intact

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Corresponding author: ruizz11@yahoo.com

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rams (6-7 months of age) were removed. The hypothalamus was placed in cryo-vials and frozen in liquid nitrogen within 5 min and stored at -70°C .

Extraction of total RNA was performed on the hypothalamus. The hypothalamic tissue was placed in Trizol LS reagent (0.75 mL of Trizol per each 100 mg of tissue, GIBCO BRL, Life Technologies, Gaithersburg, Maryland) and total RNA was extracted according to the recommendations of the manufacturer. Prior to conversion to c-DNA, RNA samples were treated with Dnase-I (GIBCO BRL, Life Technologies, Gaithersburg, Maryland, USA) in presence of ribonuclease inhibitor (40 U/ μL) to eliminate any potential DNA contamination by standard procedures. Glyceraldehyde-3-phosphate dehydrogenase (G_3PDH) was used as a positive control to determine the integrity of c-DNA from the RNA sample. The set of primers used was forward primer 5'ACCACAGTCCATGCCATCAC and reverse primer 5'TCCACCACCCTGTTGCTGTA. Thermal cycling conditions were denaturing at 94°C for 2 min, followed by 29 cycles starting with denaturing at 94°C for 1 min and 30 s, annealing at 58°C for 3 min, extension at 72°C for 4 min, with a final extension period of 72°C for 10 min. The PCR reaction products were electrophoresed on a 1% agarose gel at 100 volts for 30 min with ethidium bromide and visualized with UV light (Hitachi Genetic Systems, Alameda, CA, USA) to verify an expected G_3PDH product of 496 base pairs.

Reverse transcription of RNA was conducted to obtain c-DNA for the NMDA receptor. First-strand cDNA synthesis was set up in a 20 μL reaction using 0.2 μg RNA. This mixture was incubated for 10 min at 25°C , 60 min at 42°C , followed by 15 min at 70°C and kept at 4°C in a thermal cycler (MJ Research, ICN, Waltham, MA, USA) with a heated lid. All c-DNA samples were stored at -20°C .

The re-amplification reaction had a total reaction volume of 35 μL that consisted of 3 μL of c-DNA (150 ng/mL), 5 U of *Taq* polymerase (Promega, Madison, WI, USA), 1.5 mM MgCl_2 , 200 μM dNTP, 1X MgCl_2 -free supplied buffer and 200 nM of NMDAR-1 primer mix (forward primer 5'AACGACCACTTCACTCCCAC3', and reverse primer 5'GCCATTGTAGATGCCCACTT 3') of the coding sequence of NMDA receptor from *Homo sapiens*. Thermal cycling conditions for NMDA receptor were denaturing at 95°C for 3 min, followed by 34 cycles starting with denaturing at 95°C for 30 s, annealing at 58°C for 1 min, extension period at 72°C for 1.5 min, and with a final extension condition for PCR products of 72°C for 10 min. The re-amplified DNA template was cloned using pBluescript-Sk plasmids (pBSK, 2958 bp, Stratagene, La

Olla, CA, USA). A polishing reaction was performed in the re-amplified DNA template for NMDAR-1 to create blunt ends on PCR products using T4 DNA polymerase (7.7 U/ μL , Fisher Scientific, Fair Lawn, NJ, USA). T4 DNA ligase treatment (5 U/ μL , MBI Fermentas, Enzymatic™, Hanover, MD, USA) was applied to 4 different polished inserts: vector ratios (30:1, 10:1, 0:1, and no insert-no enzyme control) in which a 1:1 ratio contained 6.8 ng of polished insert with 25 ng/ μL of pBluescript plasmid. The four mixtures were incubated overnight at 15°C and then incubated at 65°C for 15 min. A 100 μL aliquot of *E. coli* cells (DH5 ∞ Strain, RbC1 cells, GIBCO, Grand Island, NY, USA) was mixed with 10 μL of each plasmid DNA ratio (blunt-end ligation reaction) and 400 μL SOB (0.5% yeast extract, 2.0% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl_2 , and 10 mM MgSO_4) media, and incubated with shaking at 225 rpm and 37°C for one hour. Then, 200 μL of each preparation was placed on LB+ampicilin agar plates spread with 60 μL (20 mg/mL) X-Gal and 10 μL (100 mM) IPTG to perform blue/white screening. Subsequently, all plates were incubated overnight at 37°C . Each single bacterial colony was inoculated with 2 mL of sterile LB+ampiciline media and grown overnight at 37°C . Two milliliters of overnight culture were used to purify plasmid DNA (Wizard® Plus SV Minipreps DNA purification System, Promega, Madison, WI, USA). Double enzymatic digestion of the purified plasmids was performed using *Bam*HI (10 U/ μL , Promega, Madison, WI, USA) and *Hind*III (10 U/ μL , MBI Enzymatic Fermenta, Hanover, MD, USA) at 37°C for 1 hour. Incubation reaction products were subjected to electrophoresis to determine the presence and size of the PCR products. Purified plasmids containing the NMDA receptor c-DNA were sequenced (Becker/Coulter CEQ2000XL8 capillary DNA sequencer) using the F3 (5' CAATTAACCCTCACTAAAGG) and F7 (5' TAATACGACTCACTATAGGG) primers and dye-terminator chemistry at the University of Nebraska DNA Core Facility (Lincoln, NE, USA). The results were entered into the web site of the National Center for Biotechnology (<http://www.ncbi.nlm.nih.gov/>), using the advanced blast function for sequence identification.

Results and Discussion

The mRNA of NMDA receptor was sequenced using the template DNA obtained by PCR-based amplification. The sheep NMDAR-1 subunit sequence was submitted to the National Center for Biotechnology as accession number AY434689 (Figure 1). The sequence homology analysis

revealed 94-100% homology with *Sus scrofa* and 88-90% homology with *Rattus norvegicus* NMDAR-1 mRNA sequence and 94-97% homology with *Homo sapiens* glutamate receptor ionotropic (GRIN1), transcript variant NR1-2 and NR1-1, mRNA sequence. Foldes et al. (1993) used several c-DNA clones encoding human NMDA receptor (hNR1) subunit polypeptides isolated from a human hippocampus library. The hNR1 c-DNAs demonstrated 84% to 90.8% nucleotide identity (nt) with the corresponding rodent c-DNAs. Additionally, the clone hNR1-4 isolated from a human hippocampus c-DNA library demonstrated 85.7% nucleotide (nt) identity to the corresponding portion of rat NR1 (rNR1) c-DNA. The nt sequence of hNR1-4 would encode a protein that has a 99.8% identity with the corresponding rNR1 subunit (Foldes et al., 1994). Likewise, Karp et al. (1993) reported 99% amino acid homology between human NMDAR-1 with the rat counterpart. Hieber and Goldman (1995) cloned the NMDA receptor in the degeneration of the goldfish optic nerve. The NMDA receptor has been



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1 acgaccactt cactcccacc cccgtctect acacagccgg cttctaccgc atccccgtcc
61 tggggctgac cacgcgatg tccatctact cagacaagag catccacctg agctctctgc
121 gcaccgtgcc gcctactcc caccagtcca gcgtctggtt cgagatgatg cgcgtctaca
241 gcctggagac gctgctggag gacgcgcgagt ccaagagtaa aaaaaggaac tatgacagcc
301 tcgaccaact gtctctatgac cacaagcgcg gccaccaaggc ggagraggtg ctgcgattcg
361 acccggggac caagaacgtg acggccctcg tgatggaggc gcgggagctg gaggccgggg
421 tcatcatctc ctcccagcag gaggacgacg ctgccaccgt gtaccgcgca gccgcatgac
481 tgaacatgac gggtcggggg tacgtgtggc tgggggggga gcgcgagatc tccgggaacg
541 ccctgcgcta cgccccggac ggcatcatcg ggctgcagct catcaacggc aagaacagct
601 cggcgccatc cagcgacgcc gtggcgctgg tggcccagcc cgtgcacgag ctgctcgaga
661 aagaagaacat caccgacccg ccgcccgggct gcgtgggcaa caccaacatc tgaagaccg
721 ggccgctctt caagaggggt ctgatgtctt ccaagtacgc agacgggggt accggccgcg
781 tggagttcaa tgaggacggg gaccggaagt ttgccaacta cagcatcatg aaactcgaga
841 accgcaagct gg

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Translation="DHFTPTPVSYTAGFYRIPVLGLTTRMSIYSDKSIHLSFLRTVPP
YSHQSSVWFEMMRVYSWNHVILLVSDDEHGAAQKRLETLEERESKSK
KRNYDSLQLSYDHLKRGPKAEXVLQFDPGKNTVALLMEARELEARVILS
ASEDDAATVYRAAAMLNMTGSGYVWLVGEREISGNALRYAPDGIHGLQLI
NGKNESAHISDAVGVAQAVHELLENITDPPRGCVGNTNIWKTGPLFK
RVLMSKYADGVTGRVEFNEDGDRKFANYSIM
NLQNRKL"

The 1-19 nucleotides correspond to the sequence of the NMDAR-1 forward primer used to amplify the template DNA for the NMDAR-1. Sequence submitted to the website of the National Center for Biotechnology (<http://www.ncbi.nlm.nih.gov/>) as accession number AY434689.

Figure 1 - Sequence of *Ovis aries* NMDAR-1 mRNA and its corresponding amino acid sequence isolated from the hypothalamus of male sheep.

extensively characterized electrophysiologically in *Xenopus oocyte* (Moriyoshi et al., 1991; Nakanishi, 1992; Sugihara et al., 1992; Ishii et al., 1993; Karp et al., 1993; Planells-Cases et al., 1993). Ishii et al. (1993) cloned and characterized the key subunit of the NMDA receptor (NMDAR-1) using a *Xenopus oocyte* expression system combined with electrophysiology. Gill et al. (2010) used the PCR2.1-TOPO TA-cloning vector with quick plasmid mini-preparation to produce a sea lion heart NMDAR-1 clone and found 94% homology with the dog (*Canis lupus familiaris lupus*) and only 88% with the rat (*Rattus norvegicus*), respectively. The ovine-cloned NMDAR-1 expressed in plasmids begins at 1388 nucleotide of the human NMDAR-1 sequence. These results showed extremely high evolutionary conservation of NMDAR-1 across species. This may be due to the crucial role of the NMDA receptor in both signal transmission at neuronal synapses and regulation of reproductive function. Exogenous activation of the NMDA receptors results in the release of luteinizing hormone releasing-hormone (LHRH) from the hypothalamus and subsequent release of LH from the anterior pituitary in rodents (Urbanski and Ojeda, 1987; Carbone et al., 1992), primates (Gay and Plant, 1987) and sheep (Bucholtz et al., 1996). Interestingly, the ability of NMDAR to stimulate LHRH release is significantly compromised in castrated animals. Ruiz and Kittok (2006) reported that castration reduced ($P < 0.05$) the expression of hypothalamic DNA for NMDAR compared with that in intact males. Dominguez (2009) found that the levels of glutamate increased in the medial preoptic area (MPOA) during mating, particularly with ejaculation. The predicted polypeptide from our research consists of 283 amino acid residues (Figure 1). Moriyoshi et al. (1991) reported the rat NMDAR1 polypeptide as a 938 amino acid protein with four putative trans-membrane segments followed by a large extra-cellular domain but using a *Xenopus oocyte* system. Additionally, Foldes et al. (1993) suggested that the nt sequences of hNR1-1, hNR1-2 and hNR1-3 would encode 885-, 901-, and 938-aa proteins, respectively, which had 99.1-99.8% identity with the corresponding rodent NR1 subunits. However, Hieber and Goldman (1995) reported the goldfish NMDA receptor as a 221 amino acid polypeptide. Thus, the amplitude of the ovine NMDAR-1 differs from rodent and fish NMDA receptors, which could suggest the existence of additional NMDA receptor subunits. Hedegaard et al. (2012) found that the binding sites and conformational changes leading to channel gating, in response to agonist binding, are highly conserved between human and rat NMDA receptors. GluN1 showed high sequence identity between rat and human NMDA receptors, with only seven non-identical amino acids out of 938.

Conclusions

The sequence of *Ovis aries* NMDA mRNA receptor subunit-1 determined in the present study using purified plasmids has 94-97% homology with *Homo sapiens*, 94-100% homology with *Sus scrofa* and 88-90% homology with *Rattus norvegicus* NMDAR-1 mRNA sequence, and 94-97% homology with *Homo sapiens* glutamate receptor, ionotropic (GRIN1), transcript variant NR1-2 and NR1-1 mRNA sequence, which could represent high evolutionary conservation of NMDAR-1 across species.

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