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Research report

Cloning and characterization of the chick NMDA receptor subunit-1 gene

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Abstract

The *N*-methyl-D-aspartate family of glutamate receptors (NMDARs) are tetrameric cation channels including NR1, NR2, and possibly NR3 subunits. The physiological properties of the receptor are directly related to the subunit composition of the oligomer. Whereas NR1 is essential for the formation of functional channels, NR2 and NR3 play a modulatory role. This work reports, for the first time, the cloning of a non-mammalian NR1 gene, including the 5′-regulatory region. The chick gene spans 31 kb of genomic DNA sequence composed of 22 exons interrupted by 21 introns. The exon/intron organization and the deduced amino acid sequence up to the end of exon 19 showed 85% homology to mammalian NR1 cloned genes. Significant differences from mammals were found at the C-terminal region which in the chick gene, includes a novel exon (exon 20) previously identified at the mRNA level in the chick retina. The basal promoter activity was shown to reside within the proximal 377 bp of 5′-regulatory region. The transcriptional activity of the 5′-flanking region of the chick NR1 gene was shown to be higher in neuronally-differentiated PC12 cells and in chick retinal neurons, than in non-differentiated PC12 cells and Müller glia. Comparison of the 5′-flanking region of chick NR1 gene with mammalian NR1 genes suggests that, in spite of significant differences in the nucleotide sequence, they share common DNA binding sites such as RE1, SP1, AP2, CREB, NFκB, and MEF2; therefore, some of the molecular mechanisms involved in transcriptional regulation of NR1 gene expression could be conserved among species.

Theme: Excitable membranes and synaptic transmission,
Topic: Ligand-gated ion channels

Keywords: Avian; Gene regulation; Glutamate receptors.

1. Introduction

Glutamate is the main excitatory neurotransmitter in the CNS, acting through distinct ionotropic and metabotropic receptor populations. The family of *N*-methyl-D-aspartate ionotropic glutamate receptors (NMDARs) are calcium-permeable heteroligomeric ligand-gated cation channels which play an important role in synaptic transmission [12]. NMDARs have the unique property of functioning as coincidence detectors for correlated activity, which constitutes an important characteristic required for the refinement of synaptic connections during development, and for the activity-dependent potentiation or depression of synaptic inputs [30]. The participation of NMDARs in developmental and adult synaptic plasticity, as well as in the excitotoxicity which accompanies multiple pathological conditions, underlines the relevance of NMDAR-mediated glutamate transmission within the CNS [9]. Molecular cloning and functional expression studies have identified at least seven members of the NMDA receptor channel subunits, included in three families: NR1, NR2 (A–D), and NR3 (A and B) [9]. The functional diversity of NMDARs resides in the multiplicity of receptor subunits and their differential assembly into tetramers [24]. The NR1 subunit is essential for the formation of the channel, whereas different combinations of NR2 and NR3 subunits play a modulatory role conferring distinct properties to the receptor such as sensitivity to glutamate, peak open probability, and response kinetics [23].

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Further increase in NMDAR diversity derives from the post-transcriptional and post-translational processing of the subunits [12]. Seven possible NR1 isoforms have been cloned from mammalian c-DNA libraries [37]; however, the genomic sequence has only been determined for human (GenBank accession number NT_024000), rat (NW_047651), mouse (NT_039206), and zebra fish (AL831768). The rat NR1 gene includes 22 exons, with at least nine possible splice variant transcripts derived from the alternative splicing of pre-mRNA [12], one of which codes for a truncated protein with a stop codon on exon 3, unlikely to be functional. The remaining eight isoforms arise from the insertion or deletion of exons 5 and 21 and/or from the use of an alternative exon/intron acceptor site present within exon 22 [37].

Exon 5 in the rat NR1 gene contains 63 bp and codes for the N-terminal protein cassette N1, important in the regulation of channel properties such as the sensitivity to spermine, to pH, and to zinc [9]. The C-terminal region is shown to be essential for NMDAR membrane targeting, anchoring, and clustering. The 111-bp exon 21 encodes the alternatively spliced C-terminal protein cassette C1, involved in NR1 regulation by PKC as well as in the calmodulin-dependent inhibition of currents [10]. This region has also been shown to participate in the clustering of NMDARs and their interaction with neurofilaments [11], and to play an important role in the regulation of NMDAR translocation to the membrane, since it carries an ER retention signal [32].

Exon 22 in the rat NR1 gene encodes the C-terminal protein cassette C2. The use of an alternative acceptor site in this exon results in the deletion of the first 356 bp which gives rise to the protein cassette C2′, composed of 22 amino acids. The presence of a PDZ binding domain in the C2′ cassette facilitates receptor surface expression of the homomeric and heteromeric NMDAR channels [25] which include NR1 splice variants lacking C1 and containing C2′ [34]. The C-terminal region however, is not conserved among species, which suggests that the regulation of surface expression and synaptic location of NMDARs must involve different mechanisms, additional domains, or possibly inter-subunit interactions which await clarification [8,18].

The understanding of the complex mechanisms which direct the tissue- and region-specific expression as well as the exchange of NMDAR subunits in the CNS during development and its modulation by epigenetic factors requires the analysis of the promoter region of the NMDAR subunits. Among NMDAR subunits, the promoter region of the rat NR1 gene is the most extensively studied. It has been shown to contain regulatory elements such as RE1 (repressor element-1), SP1, MEF2C (Myocyte-specific Enhancer-binding Factor-2C), MAZ (Myc-associated zinc finger protein), and NFκB (Neuronal κB-Binding Factor) [3–5,15,20,26]. Although the extent to which regulatory sites are conserved among species might ease the identification of mechanisms underlying the transcriptional control of NMDARs, the NR1 promoter region from non-mammalian receptors has not been characterized.

NMDARs in the chick retina have been shown to differ biochemically and pharmacologically from those described in the rat brain [7,21,28]. This divergence could derive, at least in part, from the expression in this tissue of distinct NR1 splice variants which could in turn, modify the activity of the heteromer. The expression in the mature retina of exon-5-containing NR1 isoforms, contrary to findings in the brain [13], as well as the developmental expression of a novel C-terminal isoform containing a novel splice cassette supports this assumption [18]. In addition to this evidence, C-terminal variants containing C1 and C2 cassettes have been identified at the mRNA and protein level in the chick retina, which suggest that NMDAR membrane targeting mechanisms differ in mammalian and avian species.

The aim of the present study is the elucidation of the structure of an avian NR1 gene for the first time, and to compare its exon/intron organization and 5′-regulatory sequences with its mammalian counterparts. Our results demonstrate that the structure of the chick NR1 gene shares most of the exon/intron organization with mammalian NR1 genes, except for the last three exons at the C-terminal region (exons 20–22). We also confirmed that the novel amino acid sequence expressed in a new NR1 variant in the chick retina is encoded by exon 20 in the NR1 chick gene. The 5′-flanking region of the chick NR1 gene was cloned, partially characterized, and promoter constructs were shown to carry transcriptional activity in neuronally-differentiated PC12 cells and in chick retina neurons and Müller glia.

2. Materials and methods

2.1. Materials

All enzymes for molecular biology procedures were purchased from New England Biolab (Beverly MA, USA) and Invitrogen (Rockville, MD, USA). [γ-^{32}P]ATP and [α-^{32}P]dCTP were obtained from Dupont, NEN (Boston, MA, USA). All DNA oligonucleotides were synthesized in the Molecular Biology Unit from the Instituto de Fisiología Celular, UNAM, México. Fetal bovine serum and horse serum were purchased from Invitrogen (Rockville, MD, USA). Plasmids pGL3-basic, pGL3-promoter, pRL-CMV, dual luciferase assay kit and passive lysis buffer were purchased from Promega (Madison, WI, USA). GeneScreen-plus nylon membranes and nitrocellulose membranes were purchased from NEN Life Science products. Nerve growth factor (NGF 2.5S) was a gift from Alomone Lab. (Israel). All other chemicals were from Sigma (St. Louis, MO, USA).

2.2. Genomic library screening

A chick genomic library from Clontech in the vector EMBL3 SP6/T7 with a titer of 2×10^7 pfu/μl, carrying