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Molecular and functional characterization of *Xenopus laevis* N-methyl-D-aspartate receptors

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ABSTRACT

N-methyl-D-aspartate (NMDA) receptors of many different vertebrates have been characterized in the past. However, little information is available about amphibian NMDA receptors. Here, we investigated the South African clawed frog *Xenopus laevis* NR1 subunit at the molecular and functional level. In this subunit, which is obligatory for functional NMDA receptor complexes, we found three exons, the N1, C1, and C3 cassettes, being alternatively spliced. Combinations of these cassettes generated six different splice variants, which were functionally characterized in oocytes. The *Xenopus* NR1 isoforms generally showed the same functional properties as their mammalian homologs when coexpressed with rat NR2B. In coexpression with *Xenopus* NR2B, however, some properties changed significantly. This included a Zn^{2+}-mediated potentiation of current amplitudes for some subunit combinations which lasted for several minutes. This mechanism presents a novel form of *Xenopus* NMDA receptor modulation, possibly mediating a form of short-term potentiation in the *Xenopus* central nervous system.

Introduction

Ionotropic glutamate receptors of the N-methyl-D-aspartate (NMDA) subfamily are essential for synaptic plasticity in the brain of all vertebrates and, thus, play a key role for higher brain functions like learning and memory (Thompson, 2000; Dingledine et al., 1999; Collingridge and Lester, 1989). These NMDA receptors are functional exclusively as heteromeric receptor complexes (Schmidt and Hollmann, 2008) which assemble from NR1 subunits in combination with NR2 and/or NR3 subunits (Paoletti and Neyton, 2007).

While in vertebrates four separate genes exist that encode NR2 subunits (NR2A through NR2D) and two different genes code for NR3 subunits (NR3A and NR3B), a single gene encodes the NR1 subunit (Hollmann, 1999). However, the NR1 protein shows considerable molecular diversity, which is based on alternative splicing of the subunit-encoding mRNA. Thus, eight functional NR1 splice variants can be generated in mammalian vertebrates (Dingledine et al., 1999; Hollmann et al., 1993), and at least two severely truncated, non-ion channel-forming isoforms of unknown function have been predicted (Campusano et al., 2005; Sugihara et al., 1992). The eight functional NR1 splice variants differ in the absence or presence of three alternatively spliced exons one of which, the so-called N1 cassette, is located in the extracellular N-terminal domain of the NR1 subunit while the other alternatively spliced exons, the so-called C1 and C2 cassettes, are part of the intracellularly located C-terminus (Dingledine et al., 1999; Hollmann et al., 1993). However, experiments in lower vertebrates like fish (Tzeng et al., 2007; Cox et al., 2005; Dunn et al., 1999; Bottai et al., 1998) and birds (Zarain-Herzberg et al., 2005; Kurosawa et al., 1994) revealed that the mammalian splice pattern of the NR1 mRNA as described above is not conserved throughout all classes of vertebrates. The NR1 subunits of fish and birds showed alternatively spliced N1 and C1 cassettes just as observed in mammals; however, the avian C2 cassette has an amino acid sequence unrelated to mammalian sequences (Zarain-Herzberg et al., 2005; Kurosawa et al., 1994), while fish lacked a C2 cassette-homologous sequence altogether (Tzeng et al., 2007; Cox et al., 2005; Dunn et al., 1999; Bottai et al., 1998). Furthermore, in birds and fish additional splice cassettes have been identified. These splice cassettes are inserted directly after the so-called C0 cassette, which is the exon coding for the C-terminal part of the receptor's third transmembrane domain and which is present in every functional NR1 splice variant characterized so far. Thus, the insertion of a so-called C3 cassette in avian NR1 leads to a splice variant with a truncated intracellular C-terminus (Zarain-Herzberg et al., 2005), while in fish the C-terminus of NR1 can be elongated by alternative splicing of two short cassettes referred to as C1′ and C1″ (Tzeng et al., 2007; Bottai et al., 1998).

Although NMDA receptors, and especially the NR1 subunits of mammals, birds, and fish, have been well-described in the literature, there is little known about these receptors in amphibians. This is surprising as the South African clawed frog *Xenopus laevis* commonly serves as a model organism in developmental studies, including the investigation of the development of the central nervous system of vertebrates (Eagleson, 1996). In this process NMDA receptors play an important role in activity-dependent developmental brain plasticity (Contestabile, 2000). However, it is unknown if the molecular and

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functional characteristics of amphibian and mammalian NMDA receptors are identical and, therefore, if conclusions drawn from studies on *X. laevis* are also valid for mammals. We therefore set out to investigate NMDA receptors from *X. laevis* with an initial focus on the *X. laevis* NR1 (*Xen*NR1) subunit, as every functional NMDA receptor complex is dependent on the presence of the obligatory NR1 protein (Paoletti and Neyton, 2007).

When investigating the splice pattern of *Xen*NR1 we identified alternatively spliced N1, C1, and C3 cassettes but no C2 cassette. The six *Xen*NR1 isoforms that can be generated by combining these splice cassettes were then heterologously coexpressed with rat NR2B (rNR2B) in oocytes and functionally characterized. The resulting functional receptor complexes showed properties very similar to those known from mammalian NMDA receptors. We then coexpressed all *Xen*NR1 isoforms together with the *X. laevis* NR2B subunit (*Xen*NR2B) to investigate NMDA receptor complexes naturally occuring in *X. laevis*. Even in such native *Xenopus* NMDA receptors we found many properties to be identical to those known from native rat NMDA receptos. However, we also observed that the combination of *Xen*NR1 with *Xen*NR2B instead of rNR2B significantly altered the EC_{50} value for NMDA as well as the agonist-induced current amplitudes for several subunit combinations. Furthermore, the modulatory effect of Zn^{2+} ions on *Xenopus* NMDA receptors was different as compared to mammalian receptors. When *Xen*NR1 "a" splice variants were combined with *Xen*NR2B, Zn^{2+} mediated a strong potentiation of agonist-induced currents, lasting for several minutes. This Zn^{2+}-mediated effect presents a novel NMDA receptor modulation that is not present in mammals and might well serve as a mechanism for short-term potentiation (STP) in the central nervous system (CNS) of *X. laevis*. These data indicate that NMDA receptors of amphibians and mammals indeed do have some differences in their molecular and functional properties, a finding that should be kept in mind during future studies on *X. laevis*.

Results

Identification of three different alternatively spliced exons of the X. laevis NR1 subunit

Two regions are known where alternative splicing takes place in NMDA receptor NR1 subunits: the extracellularly located N-terminal domain (NTD) and the intracellular C-terminal domain (CTD; Fig. 1A). We therefore set out to investigate if the *Xen*NR1 subunit is also alternatively spliced within these regions. For that purpose we isolated total RNA from *X. laevis* brain, specifically transcribed mRNA molecules into cDNA, and then performed specific PCRs using primers flanking the putatively alternatively spliced exons of *Xen*NR1 (Fig. 1A).

The analysis of the NTD-encoding region of *Xen*NR1 using the primer combination P1/P2 (Fig. 1A) yielded two specific bands which were 455 bp and 518 bp in length (Fig. 1B). These PCR products were isolated and analyzed by sequencing. We confirmed that both fragments indeed were derived from *Xen*NR1, and the longer one contained an alternatively spliced 63 bp-long N1 cassette (518 bp). The sequence of this N1 cassette exactly matched the previously published sequence for this exon (GenBank acc. no. X94081).

We then tried to amplify the CTD-encoding region of *Xen*NR1. We expected to find several cDNA fragments of different length which should contain the various putative *Xenopus* C1, C1′, C1″, C2, or C3 cassettes. However, when using the primer combination P3/P4 only a single band of 615 bp was obtained (Fig. 1C). This PCR product corresponded to the *Xen*NR1-4 splice variant, an isoform that had already been described in the literature (Schmidt et al., 2006; Soloviev et al., 1996). However, previous experiments in our laboratory had indicated that the amplification of alternatively spliced C-termini of NR1 subunits in general may be problematic, especially when large cDNA fragments had to be amplified (Paarmann and Hollmann, unpublished observations on rat NR1 subunits). To increase the chances to identify other *Xen*NR1 fragments than those coding for *Xen*NR1-4 splice variants, we decided to amplify smaller fragments by using the primers P5/P6 (Fig. 1A). Following this strategy yielded three different PCR products (Fig. 1D). The most prominent fragment was 163 bp in length and encoded the *Xen*NR1-4 variant, but the two remaining bands indicated the presence of additional splice variants. We therefore isolated these PCR products, analyzed them by sequencing and found them both to originate from *Xen*NR1. However, each fragment contained an additional sequence between the C0 and C2′ cassettes (Fig. 1A). In the case of the shorter fragment (250 bp) an 87 bp-long sequence was inserted coding for five amino acids until a stop-codon followed, effectively producing a C-terminally truncated *Xen*NR1 isoform when translated (Fig. 2). A similarly truncated splice variant also occurs in avian NR1 after insertion of the C3 cassette (Zarain-Herzberg et al., 2005). In contrast to the newly identified *Xenopus* sequence, the avian C3 cassette encodes six amino acids,

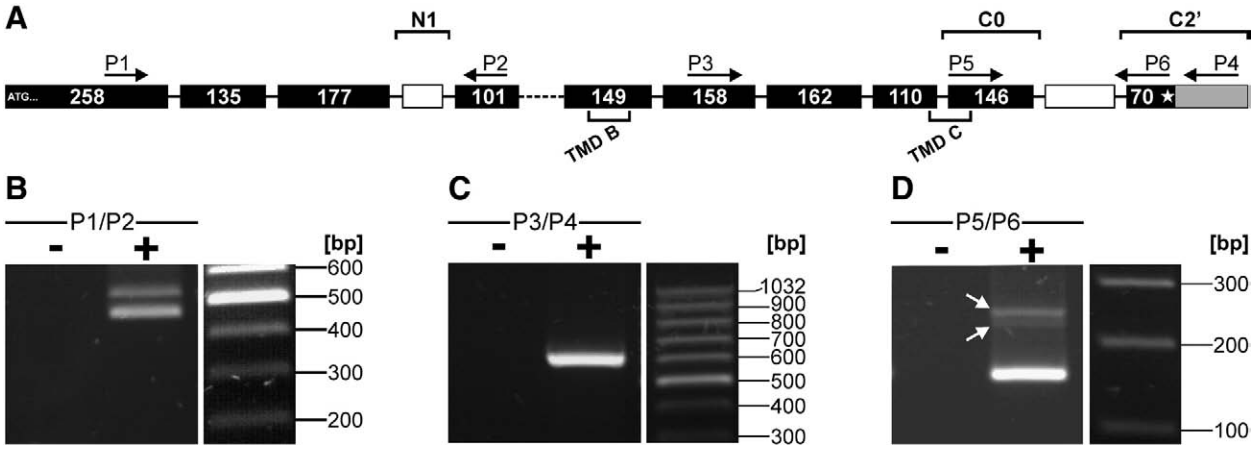

Fig. 1. Alternative splicing of NMDA receptor NR1 subunits. (A) Schematic drawing of the putative gene structure of *Xen*NR1. Black boxes represent sequences which encode parts of the NR1 protein and are not alternatively spliced while white boxes correspond to alternatively spliced exons. Grey regions represent sequences belonging to untranslated regions (UTR) of the NR1-encoding mRNA. Numbers within the boxes indicate the length of the exon in nucleotides. "ATG" shows the position of the start codon and a star (★) represents a stop-codon. Horizontal lines connecting the boxes represent intron sequences, and the dashed line in the middle of the drawing means that a total of 9 putative exons has been left out at this position. Exons are drawn to scale, introns are not. Above, names of the most important splice cassettes are indicated in bold letters. As the gene structure of the *X. laevis* NR1 gene has not been published, yet, the scheme is based on the NR1 gene structure of the closely related frog *Xenopus tropicalis*. That frog is likely to share its NR1 gene structure with *X. laevis*. Arrows above the drawing indicate positions of oligonucleotides. The positions of the transmembrane domains (TMDs) B and C are marked below for better orientation. (B–D) Representative agarose gels showing amplified *Xen*NR1 splice variant-encoding cDNA fragments from *X. laevis* brain cDNA (+) and the corresponding negative controls (−). Primer combinations used are noted above the gel. Length of marker bands are indicated in base pairs (bp). White arrows were used to mark faint bands (D).