

## MOLECULAR BIOLOGY OF THE *APTERONOTUS* NMDA RECEPTOR NR1 SUBUNIT

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### Summary

The complete sequences and expression patterns of the NR1 (aptNR1) subunit of the *N*-methyl-D-aspartate (NMDA) receptor and its alternative splice isoforms have been determined for the weakly electric fish *Apteronotus leptorhynchus*. The deduced amino acid sequence of aptNR1 is approximately 88% identical to the NR1 sequences of other vertebrate. Two of the three alternative splice cassettes previously described for mammalian NR1s, N1 and C1, are present in aptNR1, but the third cassette, C2, is not found. In addition, two teleost-specific splice cassettes occur on the N-terminal side of the C1 sequence. The cellular patterns of aptNR1 expression, including the patterns of N1 and C1 splicing, have been mapped using the *in situ* hybridization technique. High levels of aptNR1 mRNA were detected throughout the central nervous system including most neurons of the electrosensory

system, with the highest levels in electrosensory lateral line lobe pyramidal cells. Expression of the N1 splice isoform was higher in more caudal regions of the brain, and expression of the C1 splice isoform was higher in more rostral regions. The N1 splice isoform was present in almost all NR1-positive cells, in contrast to the C1 splice isoform which was restricted to a subset of NR1-positive cells. These results demonstrate that the NR1 subunit of the NMDA receptor is evolutionarily conserved across species and that regulation of alternative RNA splicing modulates the properties of NR1 in different neurons of the central nervous system of *A. leptorhynchus*.

Key words: electrosensory system, NMDA receptor, NR1 subunit, alternative RNA splicing, *Apteronotus leptorhynchus*, NR1 amino acid sequence homology.

### Introduction

Glutamate is the principle excitatory neurotransmitter of the teleost central nervous system (CNS), as it is for other vertebrates. The properties of glutamatergic synaptic transmission are determined by the kinetics of glutamate release from the presynaptic terminal and by the kinetics, ion selectivity and conductances of the glutamate receptors on the postsynaptic membrane. We have begun an investigation of the molecular mechanisms that control glutamate synaptic transmission within the electrosensory system of the electric fish *Apteronotus leptorhynchus*. We describe here some molecular properties of the NR1 subunit of the *N*-methyl-D-aspartate (NMDA) class of postsynaptic glutamate receptors in this system and compare these with the properties of the mammalian NR1 subunit.

Studies of mammalian neurons have shown that postsynaptic membranes contain a variety of glutamate receptors which are broadly classified into two families, ionotropic and metabotropic. The ionotropic receptors form cation channels that are activated directly by the binding of glutamate, while the metabotropic receptors regulate ion

channels indirectly through the trimeric G-protein intracellular signaling pathways. The ionotropic receptors are subclassified as AMPA ( $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionate), kainate or NMDA types depending on their responses to specific agonists. Members of each receptor group differ in kinetics, voltage-dependencies and regulation, and the properties of the synaptic response therefore depend on which specific receptor types are assembled at the postsynaptic site. For example, NMDA receptors typically produce slower, longer-lasting responses than AMPA/kainate receptors; therefore, synapses containing NMDA receptors produce longer-lasting excitatory postsynaptic currents (EPSCs) than synapses containing only AMPA/kainate types (Hestrin et al., 1990; Nicoll et al., 1990). In fact, many glutamate synapses contain both NMDA and non-NMDA receptors, and synaptic responses typically reflect the properties of both receptor types (Forsythe and Westbrook, 1988; Lester et al., 1990).

NMDA receptors display three unique properties critical to the control of excitatory synaptic transmission. First, activation

of the NMDA receptor depends on the simultaneous binding of two agonists, glutamate released from synaptic vesicles and glycine from the extrasynaptic fluids (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988). Thus, glycine levels can act to modulate NMDA receptor responses. Second, NMDA receptors depend on a depolarized membrane potential for activation due to blockage of the ion pore by  $Mg^{2+}$  from the extrasynaptic space (MacDonald et al., 1982; Mayer et al., 1984; Nowak et al., 1984). This voltage-dependence for activation allows the glutamate synapse to function as a cellular coincidence detector where full synaptic response occurs only when there is coincident depolarization through the activation of adjacent synaptic sites. In this way, the synapse becomes dependent on the simultaneous activity of more than one synapse. Third, the NMDA receptor is highly permeable to  $Ca^{2+}$ , which activates intracellular signals that can modify synaptic function. These unique properties of the NMDA receptor have been implicated in a number of brain processes including neuronal differentiation, synaptic plasticity and neurotoxicity.

The NMDA receptor is a tetrameric protein complex containing two different types of subunit, NR1 and NR2 (Hollmann and Heinemann, 1994; Laube et al., 1998). The functional receptor is assembled from two NR1 and two NR2 subunits (Fig. 1A). There are multiple subtypes of both NR1 and NR2. In rodents, eight different NR1 receptor subtypes are generated from a single gene through the utilization of alternative RNA splicing (Anantharam et al., 1992; Durand et al., 1993; Hollmann et al., 1993; Sugihara et al., 1992). In contrast, the four different NR2 subunits (NR2A/2B/2C/2D) are encoded by four separate genes (Ikeda et al., 1992; Ishii et al., 1993; Kutsuwada et al., 1992; Meguro et al., 1992; Monyer et al., 1992). Studies in which different NR1 and NR2 subunits are co-expressed *in vitro* have established that different combinations of NR1 and NR2 can form functional receptor complexes, but that their properties will vary depending on the subunits in the complex (Ishii et al., 1993; Meguro et al., 1992; Monyer et al., 1992; Vicini et al., 1998).

The molecular complexity of the NMDA receptor that results from the selective assembly of different NR1 and NR2 subunits suggests that glutamate synaptic responses can be effected by the selective expression of the different receptor subunits within neurons. This idea has been supported by studies mapping NR1 and NR2 gene expression in rodent brain, which have revealed a complex mosaic of NR1 and NR2 expression within different neuronal populations (Buller et al., 1994; Dunah et al., 1996; Ishii et al., 1993; Johnson et al., 1996; Landwehrmeyer et al., 1995; Laurie et al., 1995; Laurie and Seeburg, 1994; Monyer et al., 1994; Paupard et al., 1997; Petralia et al., 1994a; Standaert et al., 1994, 1996). Our studies of the *Apteronotus* NMDA receptor investigated whether different subunits are selectively expressed in the neurons of the electrosensory system and how the pattern of receptor expression affects synaptic transmission.

At the level of protein secondary structure, both the NR1 and NR2 subunits are thought to form structures similar to

those proposed for non-NMDA receptors, as illustrated in Fig. 1B (Hollmann et al., 1994; Wo and Oswald, 1994). Four structural domains are defined within this structure: (i) the amino-terminal region forms the extracellular surface of the receptor; (ii) two segments, S1 which lies just N-terminal to the first transmembrane segment and S2 which lies between the third and fourth transmembrane segments, form the ligand-binding pocket (O'Hara et al., 1993; Stern-Bach et al., 1994); (iii) the transmembrane core of the receptor is formed by three helices TM1, TM3 and TM4 and a pore-forming P region; (iv) the carboxy-terminal intracellular domain is the site of intracellular protein-protein interactions and also contains the sites for protein kinase modulation of the receptor.

#### Amino acid sequence of the teleost NR1 subunit

We have recently determined the cDNA and deduced amino acid sequences of the aptNR1, the NR1 subunit of teleost *Apteronotus leptorhynchus* (Bottai et al., 1998). Fig. 2 illustrates this sequence and indicates sequence homologies to orthologous receptors from frog, duck, rat and human. The aptNR1 amino acid sequence, excluding the alternatively spliced segments, is approximately 88% identical to the other vertebrate NR1s. Sequence homologies are most pronounced in the carboxyl half of the protein, where only eight amino acid positions are divergent (homology more than 98%) in the segment stretching between aptNR1 amino acid 486 and the site of carboxy-terminal alternative splicing. This highly conserved segment includes the three proposed transmembrane segments, the pore segment and the ligand-binding domains. Included in the highly conserved region is segment C0, which occurs immediately following the final transmembrane helix TM4 (Fig. 1B). C0 contains one of the two calmodulin-binding sites in NR1; the other is present in segment C1, as discussed below. Calmodulin binding to C0 inhibits NMDA receptors and is proposed to cause the use-dependent desensitization of NMDA EPSCs mediated by  $Ca^{2+}$  influx (Ehlers et al., 1996; Zhang et al., 1998). The very high sequence conservation of these critical functional domains of aptNR1 suggests that the pharmacology and functional properties associated with mammalian NR1 subunits are likely to be conserved to a large extent in aptNR1. Future studies will test this hypothesis directly by electrophysiological analysis of recombinant fish NMDA receptor subunits expressed in cultured animal cells.

#### Alternative RNA splicing generates NR1 subunit isoforms

Although there appears to be only a single gene encoding the NR1 subunit in all vertebrate species analyzed to date, a variety of different subtypes of the NR1 subunit are produced by alternative RNA splicing at one site in the extracellular amino-terminal domain and at two sites in the intracellular carboxy-terminal domain (Anantharam et al., 1992; Bottai et al., 1998; Durand, 1993; Hollmann et al., 1993; Sugihara et al., 1992). In Fig. 1B, the types of alternative splice segment found in aptNR1 are compared with the those in the mammalian NR1.

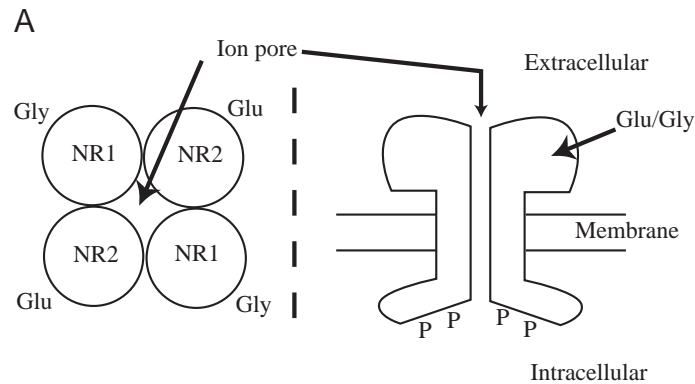
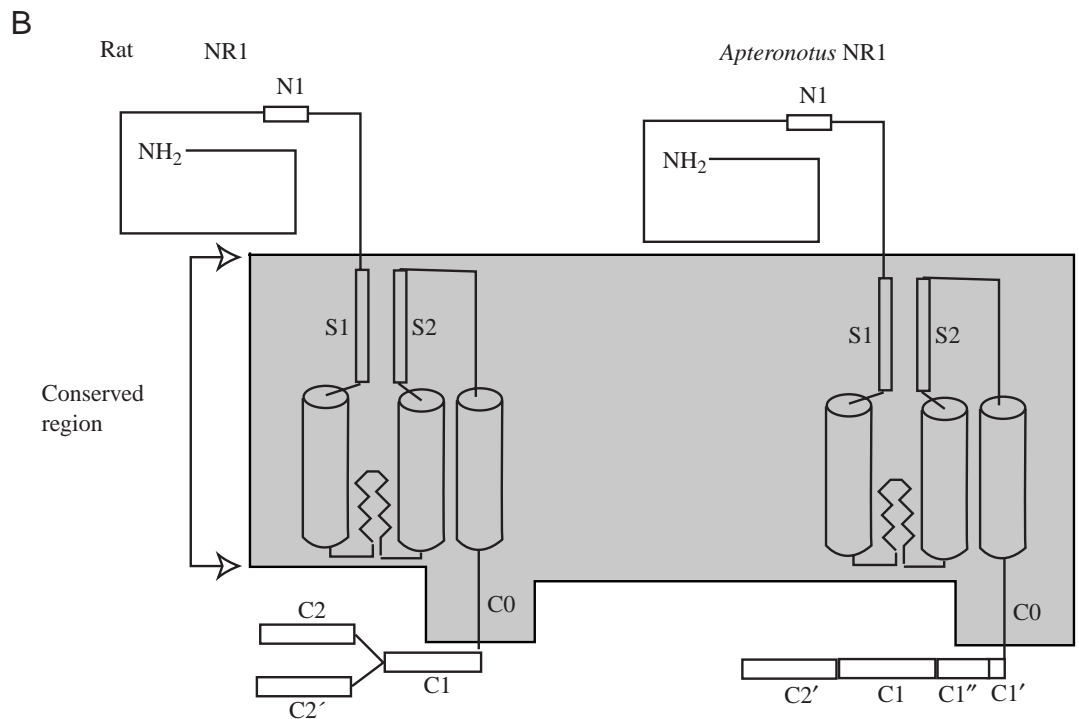


Fig. 1. Structure of the *N*-methyl-D-aspartate (NMDA) receptor complex. (A) The NMDA receptor contains four subunits, two NR1 and two NR2 subunits. The ion conduction pore lies in the center of the complex. Glu, glutamate; Gly, glycine; P, phosphate. (B) The proposed secondary structures for NR1 from rat and *Apteronotus leptorhynchus*. The shaded area includes the regions in which the amino acid sequences are nearly identical. The boxed segments labeled N1, C1, C1', C1'', C2 and C2' indicate the positions of alternatively spliced cassettes. The boxed segments S1 and S2 indicate the ligand-binding domains. Segment C0 is highly conserved.



The fish NR1 contains splice cassettes corresponding to two of the three mammalian cassettes, N1 in the amino-terminal domain and C1 in the carboxy-terminal domain. In addition, two carboxy-terminal domain splice cassettes C1' and C1'' are present in aptNR1 but have not been detected in mammals. The C2 splice cassette appears to be an evolutionary development of mammalian NR1 that is absent from both the fish and frog sequences (Soloviev et al., 1996).

#### Expression of NMDA receptors in *Apteronotus* central nervous system

The relative levels of NR1 subunit gene expression provide an estimate of overall NMDA receptor synthesis because NR1 is an obligate component for all functional NMDA receptors. We have used both RNase protection and *in situ* hybridization assays to determine the levels of NR1 expression in the CNS of *Apteronotus* (Bottai et al., 1997). Similar to the results in

mammalian systems (Laurie et al., 1995; Moriyoshi et al., 1991; Petralia et al., 1994b), NMDA receptors are expressed in most regions of the fish brain, with the highest levels in forebrain and hypothalamus. One striking difference is the very low level of expression in fish cerebellar Purkinje cells, which express high levels of NR1 in mammals (Laurie et al., 1995; Petralia et al., 1994b; Watanabe et al., 1994). However, the functional significance of this difference is unclear because, despite high levels of NR1 expression, mammalian Purkinje cells do not express functional NMDA receptors (Farrant and Cull-Candy, 1991; Krupa and Crepel, 1990; Perkel et al., 1990).

Consistent with the findings that NMDA receptor mediated synaptic responses are involved in several levels of electrosensory processing (Bastian, 1993; Berman et al., 1997; Dye et al., 1989; Heiligenberg et al., 1996; Maler and Monaghan, 1991), many cells of the electrosensory system exhibit robust expression of NR1 (Bottai et al., 1997). In the

electrosensory lateral line lobe (ELL), the pyramidal cells express high levels of NR1, higher than any other cell type in the brain. When compared across the four segments of the ELL, pyramidal cells of the central medial segment (CMS) express significantly higher levels (134%) than those of the lateral segment (LS), which may relate to the tuning of CMS pyramidal cells to lower-frequency inputs than those in the LS (Shumway, 1989a,b). The very high level of NR1 expression in ELL pyramidal cells is probably required to maintain NMDA receptors in the very extended apical dendritic structures, which are rich in glutamate synapses (Berman et al., 1997; Maler and Monaghan, 1991).

Moderate levels of NR1 expression were detected in most of the interneurons in the ELL (Bottai et al., 1997). Granule cells, ovoid cells, polymorphic cells and neurons of the ventral molecular layer all express NR1. As expected, spherical cells, which receive gap-junction synaptic inputs from T-type electroreceptor afferents, show no evidence of NR1 expression. Moderate NR1 expression was detected in most cells at higher levels of the electrosensory system, including the torus semicircularis, nucleus praeminentialis and optic tectum. In the medullary pacemaker nucleus, both pacemaker and relay cells express moderate levels of NR1.

The results described above indicate that NMDA receptors are present in neurons that are involved with most levels of electrosensory processing. In particular, the very high level of NMDA receptor expression in ELL pyramidal cells provides an ideal system for detailed molecular and physiological investigations of the contribution of NMDA receptors to synaptic transmission during sensory processing.

#### Alternative RNA splicing in the amino-terminal domain of aptNR1

As shown in Fig. 1B, alternative RNA splicing produces two isoforms of NR1 which differ in the presence or absence of the N1 segment in the amino-terminal domain. The sites and sequences of N1, a segment of 21 amino acid residues, are highly conserved among species (Fig. 2). The effects of N1 on NMDA receptor function have been investigated by expression of recombinant receptors in cultured animal cells and *Xenopus laevis* oocytes. These studies indicate that the presence of N1 in NR1 results in receptors that display higher current amplitudes (Hollmann et al., 1993; Zheng et al., 1994), reduced affinity for glycine (Durand et al., 1993; Hollmann et al., 1993; Nakanishi et al., 1992), resistance to proton inhibition (Traynelis et al., 1995) and are not potentiated by polyamines such as spermine (Durand et al., 1993). Recently, two elements,  $Zn^{2+}$  released into the synaptic cleft and intracellular tyrosine kinases of the src family, have been shown to cooperate in the regulation of NMDA receptor function (Zheng et al., 1998). NMDA currents are suppressed by low concentrations of extracellular  $Zn^{2+}$ ; however, this suppression is removed by tyrosine kinase phosphorylation of the receptor NR2 subunits. This mechanism could, in principle, allow intracellular control of NMDA receptor currents through second messengers that control

tyrosine kinase activities. Interestingly, NMDA receptors that contain NR1 subunits with the N1 insert are relatively resistant to inhibition by low concentrations of extracellular  $Zn^{2+}$  and are therefore not as dependent on activation by the tyrosine kinase pathway (Traynelis et al., 1998). Thus, the control of N1 alternative splicing provides two functionally distinct NMDA receptor populations at the synapse, N1- receptors, which require activation of the tyrosine kinase signaling system for full activity, and N1+ receptors, which are constitutively active. The presence of these two functionally distinct NMDA receptor types could provide very different synaptic responses that may have important consequences for synaptic transmission within functional neuronal networks.

We have used the RNase protection assay to determine the percentage of NR1 subunits that contain N1 in different regions of the *Apteronotus* CNS (Bottai et al., 1998). In general, NMDA receptors in the forebrain contain lower levels of the N1+ NR1 subunit (8%) than more caudal regions such as the brainstem (43%). This result may indicate that NMDA responses in the brainstem are generally less subject to modulation by intracellular tyrosine kinase mechanisms than are those in the forebrain, where synaptic plasticity is a more relevant feature.

At the cellular level, we have detected N1 transcripts in most neurons that express the NR1 gene. For example, ELL pyramidal cells and granule cells contain both N1+ and N1- receptors. One exception were the pyramidal cells of the optic tectum, which contained only a very low level of N1+ mRNA. Possibly, tyrosine kinase modulation is particularly important for the control of NMDA responses in these cells. Overall, our results indicate that most neurons in the CNS of *Apteronotus* express both N1+ and N1- receptor types, but that the levels of N1+ are reduced in more rostral regions. An important question that remains is to determine the distribution of N1+ and N1- receptors among the different synaptic populations in this system.

#### Alternative RNA splicing in the carboxy-terminal domain of aptNR1

In mammals, a complex pattern of alternative RNA splicing occurs within the carboxy-terminal domain of NR1 (Fig. 1B).

Fig. 2. Sequence comparison of the NR1 proteins. The *Apteronotus leptorhynchus* NR1 protein sequence (aptNR1) is shown and compared with NR1 sequences of *Xenopus laevis* (Soloviev et al., 1996), duck (Kurosawa et al., 1994), rat (Moriyoshi et al., 1991; Anantharam et al., 1992) and human (Foldes et al., 1993; Karp et al., 1993; Planells-Cases et al., 1993). Residues that are identical to the aptNR1 sequence are omitted in the other sequences. Positions of deletions are indicated by dashes (-). The amino acid positions are indicated on the right. TM1, TM3 and TM4 indicate the putative transmembrane segments, and P indicates the proposed pore segment. Glycosylation (filled squares), protein kinase C (filled circles) and protein kinase A (asterisk) are indicated above the aptNR1 sequence. N1, C1, C1' and C1'' indicate the positions of the alternatively spliced cassettes. SS indicates the signal sequence.



Two alternative splice cassettes, C1 and C2, can be present. The carboxyl terminus of NR1 is encoded by segment C2, or in the absence of C2 by a segment termed C2'. There are significant differences in this pattern for the aptNR1 gene. We have been unable to identify a C2 segment in fish mRNA, which suggests that fish lack the C2 cassette. Thus, all fish NR1 proteins would contain the C2' sequence at their carboxyl terminus. Because C2', but not C2, contains a PDZ recognition motif, this means that all fish NR1 subunits can interact directly with the PSD95 class of synaptic organizational proteins (Sheng and Kim, 1996). In contrast, because a majority of mammalian NR1 proteins include the C2 cassette, most mammalian NMDA receptor complexes interact with PSD95 proteins through the NR2 subunits. This difference may have significance for the organization of postsynaptic NMDA receptors within different organisms.

cDNA cloning and polymerase chain reaction (PCR) analysis have revealed the presence of two novel splice cassettes in the fish NR1 mRNA, which we have termed C1' and C1'' (Fig. 1B) (Bottai et al., 1998). These two segments, which can be present just before the C1 segment, are 19 and nine amino acid residues in length, respectively. The potential functional significance of C1' and C1'' is not known.

The fish C1 sequence differs from the mammalian sequence at seven of 37 residues (Fig. 2); however, these substitutions are highly conservative, maintaining charge and relative hydrophilicity at all positions except for a single serine→alanine substitution. Most importantly, the four protein kinase recognition sites and the high-affinity calmodulin-binding site identified in the mammalian C1 are maintained in the fish sequence. The functional significance of the C1 calmodulin-binding site is unknown; however, it has been shown that it is not required for Ca<sup>2+</sup>/calmodulin inhibition, which is mediated by the low-affinity calmodulin site in C0 discussed above. To date, most of the evidence suggests that calmodulin and protein kinase interactions with C1 may be important for the control of the subcellular targeting of the NMDA receptor complex (Ehlers et al., 1996, 1998; Liu et al., 1994).

RNase protection studies indicate that in *Apteronotus* only 9% of brain NR1 mRNAs include the C1 segment and that the relative levels of NR1 C1+ mRNA vary among different brain regions, with highest levels in the hypothalamus (24%), forebrain (13%) and ELL (12%). Interestingly, the C1+ splice form is not present in all neurons that express NR1, but rather displays a markedly heterogeneous pattern of expression. A good example is the ELL, where the C1+ form of NR1 mRNA is strongly expressed by pyramidal cells, but is not detectable in granule cells. Thus, NMDA receptors in pyramidal cell synapses are subject to regulation involving the C1 segment, while those in granule cells are not. This result suggests that, in ELL pyramidal cells, NMDA receptors undergo intracellular protein-protein interactions and/or protein phosphorylation involving the C1 segment and that these processes may play a special role in the targeting and/or control of ELL pyramidal cell NMDA currents.

## Discussion

We have begun a molecular analysis of NMDA receptors by cloning the NR1 subunit from *Apteronotus leptorhynchus* cDNAs. The deduced amino acid sequence of aptNR1 is highly similar to that of other vertebrate NR1 subunits, suggesting that the fundamental properties of NMDA receptors are conserved across vertebrate species. The central role for NMDA-receptor-mediated neurotransmission in electrosensory processing is strongly supported by our finding that most electrosensory neurons express the NR1 subunit of the NMDA receptor. In the ELL, a particularly important role for NMDA receptors is suggested by the very high level of NMDA receptor gene expression in both pyramidal and granule cells. The amount of NR1 subunit mRNA in ELL pyramidal cells exceeds that detected in all other *Apteronotus leptorhynchus* CNS cell types.

Control of NMDA-receptor-mediated postsynaptic responses is determined by the selective expression of different receptor subunits in different neuronal types. For the NR1 subunit, different subtypes are generated through alternative RNA splicing of mRNAs transcribed from a single NR1 gene. We find that two of the three alternative splice cassettes (N1 and C1) previously described in mammals are present in the fish NR1 mRNAs. The third cassette (C2) was not detected in aptNR1 mRNA, although two novel sequences (C1' and C1'') were detected. These results indicate that the control of NMDA receptor responses through alternative splicing of N1 and C1 predates the divergence of teleost and mammalian lineages, which suggests that the control of these two forms of NR1 is an important aspect of NMDA receptor function. A dramatic example of the control of the C1 alternative RNA splice cassette was found in neurons of the ELL, where C1 expression is restricted to pyramidal cells and excluded from granule cells. Thus, glutamate inputs to pyramidal cells, but not to granule cells, will be influenced through intracellular interactions such as protein kinase activities that are known to act on the C1 sequence. The very high level of NR1 expression in ELL pyramidal cells will assist in future studies determining the specific role of C1-mediated activities on NMDA receptor function and the contribution of this form of regulation to the control of electrosensory signal processing.

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