New insights into the not-so-new NR3 subunits of NMDA receptor: Localization, structure and function

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Nonstandard Abbreviations: NMDA, N-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoazolepropionic acid; kainite, 2-carboxy-3-carboxymethyl-4-isopropylpyrrolidine; ATD, amino-terminal domain; CTD, cytoplasmic terminal domain; S1S2, ligand binding domain; RT-PCR, reverse transcription polymerase chain reaction; PSD, postsynaptic density; HEK, Human embryonic kidney; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DCS, D-cycloserine; ACPC, 1-aminocyclopropane-1-carboxylic acid; ACBC, 1-aminocyclobutane-1-carboxylic acid; SCAM, substituted cysteine accessibility method; 5,7-DCKA, 5,7-dichlorokynurenic acid; SP, signal peptide; aa, amino acids; bp, base pairs.
ABSTRACT

The NR3 subunits (NR3A and NR3B) are new players in a well-established field of N-methyl-D-aspartate (NMDA) receptor, previously involving the NR1 and NR2 subunits. Their incorporation into conventional NMDA receptors forms glutamate-activated NR1/NR2/NR3 triheteromers, while the omission of the glutamate-binding NR2 subunits results in excitatory glycine-activated NR1/NR3 diheteromers. These NR3-containing NMDA receptors exhibit several differences in receptor properties compared to the conventional NR1/NR2 receptors. This review highlights the major landmarks which have been achieved in the past decade or so involving NR3 subunit research in four key areas: the spatiotemporal mapping of NR3 protein, the structural elucidation of NR3 domains, pharmacological characterization of NR3-containing receptors and the successful generation of NR3 knockout/transgenic animals. It is expected that further characterization of their functional roles coupled with the identification of endogenous and exogenous ligands will eventually advance the understanding of the basic pharmacology as well as the complex role of NMDA receptors in higher brain functions and neurological disorders. (157 words)
Introduction

The \( N \)-methyl-\( D \)-aspartate receptor (NMDA) receptor is a subtype of the ionotropic glutamate (iGlu) receptors, a family of ligand-gated ion channels that mediates the vast majority of excitatory neurotransmission in the mammalian central nervous system. Named after the agonist originally used to differentiate this receptor from two other iGlu receptor members (\( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor and the 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate) receptor, the NMDA receptor is unlike the other subtypes in three important ways. Firstly, it requires both L-glutamate (agonist) and glycine (co-agonist) for maximum activation (Johnson and Ascher, 1987; Kleckner and Dingledine, 1988; Dalkara et al., 1992). Secondly, the unique presence of a magnesium (\( \text{Mg}^{2+} \)) channel blockade brings about a voltage-dependence property absent in other iGlu receptor members (Nowak et al., 1984; Mayer et al., 1984; Kupper et al., 1998). Thirdly, the NMDA receptor exhibits relatively higher permeability to calcium ion (\( \text{Ca}^{2+} \)) than AMPA and kainate receptors which links it to a variety of neuro-physiological processes including neuro-development and cognition, as well as neuro-pathological conditions such as bipolar disorder, Parkinson’s disease and stroke (for review on the NMDA receptor, see the following reviews: Dingledine et al., 1999; Mayer and Armstrong, 2004).

The NMDA receptor consists of three subunits: glycine-binding NR1, glutamate-binding NR2 and the most recently discovered glycine-binding NR3 (Dingledine et al., 1999) (Fig. 1A). Each NMDA receptor subunit includes (i) an amino-terminal domain (ATD), (ii) three transmembrane domains (M1, M3, M4) with a pore channel-forming re-entrant loop, (iii) a bi-lobed agonist binding domain formed by the remaining distal segment after the ATD (termed as S1 domain) and the large extracellular loop linking M3 and M4 (termed as S2 domain), and (iv) the cytoplasmic terminal domain (CTD) (Dingledine et al., 1999; Mayer and Armstrong, 2004) (Fig. 1B). In the heteromeric NMDA receptor complex, NR1 is the
essential subunit that co-assembles with various combinations of NR2 and/or NR3 subunits (Ciabarra et al., 1995; Sucher et al., 1995; Al-Hallaq et al., 2002; Schuler et al., 2008).

The successful cloning of the third subunit of NMDA receptor, NR3, 15 years ago has taken the complexity of NMDA receptors to a new level. A broad overview on NR3 subunits first appeared in 2008 (Cavara and Hollmann, 2008). This minireview focuses primarily on the NR3 localization, structural and functional understanding gained through immunocytochemistry, structural biology and electrophysiology in the recent years.

**NR3 subunits**

**NR3A subunit.** There are currently two known subtypes of the NR3 subunit and these are known as NR3A and NR3B. NR3A was discovered in 1995 by two independent groups (Ciabarra et al., 1995; Sucher et al., 1995) and was initially named $\chi$-1 (chi-1, GenBank accession no. L34938) and NMDAR-L (NMDAR-like, GenBank accession no. U29873), respectively. NR3A has 27% similarity to NMDA receptor subunits and 23% similarity to other non-NMDA receptor proteins. Despite this low homology, NR3A was grouped under the NMDA receptor because the CTD and the region upstream of M1 are structurally related to other NMDA receptor subunit (Ciabarra et al., 1995). A long splice variant of NR3A with a 60 bp insertion in the C-terminus has also been identified in the rat (Sun et al., 1998; Sasaki et al., 2002). This splice variant was named NR3A-2 (NR3A-long; GenBank accession numbers AF061945 and AF073379.1). The original shorter NR3A subunit was named NR3A-1 (NR3A-short) (Fig. 1A). However, there appears to be no such equivalent within the human adult and fetal brain (Eriksson et al., 2002) and the 60 bp insert was not found within the human gene sequence of NR3A (Andersson et al., 2001). When present, this insertion results in the inclusion of a 20 amino acid cassette that contains two...
potential phosphorylation sites, one for protein kinase C and protein kinase A, and the other for calmodulin dependent protein kinase II (Sun et al., 1998).

**NR3B subunit.** The NR3B subunit was initially discovered in 1995 and named γ-2 (Forcina et al., 1995; Sevarino et al., 1996) but its complete characterization was published by other groups (Nishi et al., 2001; Chatterton et al., 2002; Matsuda et al., 2003). NR3B is also the most similar to NR3A with a score of 47% similarity in amino acid sequence but has only 17-21% similarity to NR1 and NR2. There is greater similarity between NR3 and NR1, than NR2 (Andersson et al., 2001). The mouse homolog of NR3B has 1,003 residues (Nishi et al., 2001) (GenBank accession number NM_130455) while the rat homolog is one residue shorter (Chatterton et al., 2002) (GenBank accession number NM_133308).

The genomic sequence of NR3B coding region has also been the subject of study. A null-allele in human NR3B caused by a 4 bp insertion (insCGTT) leads to truncation of the NR3B polypeptide within the ATD (Niemann et al., 2008). In the same study, a genotypic screening involving 2,128 individuals from a total of 42 populations worldwide was performed in order to evaluate the frequency of this null-allele. This null-allele was found to occur at a frequency of 10% in human.

Spatio-temporal expression of NR3 subunits

**Developmental expression of NMDA receptor subunits in the rodent.** The temporal expression profile of different NMDA receptor subunits may need to be precisely controlled during development to coincide with critical periods (Monyer et al., 1994; Hatten, 1999; Medina et al., 2001). Indeed, their expression in the brain has been shown to change throughout the postnatal development stage (Mori and Mishina, 1995). There is a progressive developmental change from predominantly NR1/NR2B receptors to NR1/NR2A receptors in thalamic and cortical neurons during the early critical
period (Barth and Malenka, 2001; Liu et al., 2004). This is accompanied by the detection of shorter excitatory postsynaptic currents (EPSC) and a decrease in sensitivity to ifenprodil, a specific NR1/NR2B antagonist (Williams et al., 1993; Barth and Malenka, 2001). These developmental changes do not appear to only affect NR2A and NR2B but also NR2C and NR2D as well. The total expression of NR2B and NR2D in rat brain is high at birth but decreases to adulthood (Laurie et al., 1997) and electrophysiological data from other studies also reflect this trend in the cortex and hippocampus (Kirson et al., 1999; Barth and Malenka, 2001). Conversely, the expression of NR2A and NR2C progressively increases during postnatal development (Laurie et al., 1997). NR1 subunit protein expression profile shows a similar trend of increase. A study of NR1 protein levels in five different regions of rat brain showed that in all five regions (olfactory, hippocampus, cortex, midbrain and cerebellum), there was a large increase in NR1 within the first 3 weeks of postnatal development (Luo et al., 1996) (Fig. 2). This trend was also seen in the NR1 mRNA profile examined in other studies (Pujic et al., 1993; Riva et al., 1994).

An interesting trend is also observed by comparing between the NR3 subfamily. During early postnatal stage and in newborn pups, NR3A protein level is high in many rodent brain regions whereas NR3B mRNA and protein levels are almost non-detectable (Matsuda et al., 2002; Wong et al., 2002) (Fig. 2). From P7 to P21, there is an obvious dip in the protein levels of NR3A, which remain low into adulthood (Wong et al., 2002). This prominent downregulation occurs just prior to the onset of the critical period of plasticity and has been shown to be physiologically significant in an animal model (Perez-Otano et al., 2006; Roberts et al., 2009). During this time, NR3B protein expression starts to increase and remains high in adult rodents (Matsuda et al., 2002; Fukaya et al., 2005; Prithviraj and Inglis, 2008). In the suprachiasmatic nucleus (SCN) of the hypothalamus, it has been shown that both NR3A and NR3B mRNAs decline around the time of eye opening in the rat (Bendová et al., 2009).

In motoneurons of adult rodents, Fukaya and colleagues (2005) discovered a reciprocal relationship between NR3B and NR2A mRNA expression. The decline of NR2A mRNA levels in
motoneurons was complemented with the rise of NR3B around the second postnatal week 14 (P14). Thereafter, NR3B continues to increase until maximal at P21 and adulthood whereas NR2A continues to decline even to adulthood (Fukaya et al., 2005). It is noteworthy that to-date, there is a lack of data related to protein levels of NR3 (and NR1) in aged mammals (Fig. 2).

**Spatial distribution of NMDA receptor subunits in the rodent.** Although temporal expression of each subunit is important, another essential level of regulation is seen spatially (a) across different brain regions; (b) among different cell types and indeed, even (c) within different microdomains and subcellular compartments of the cell.

**Across brain regions.** The expression of certain subunits may, at different stages of development, predominate in certain brain regions. Within the first postnatal week, the dominance of NR2B in granule cells becomes gradually replaced by NR2C, thus establishing NR2C as the predominant NR2 subunit in the adult cerebellum (Farrant et al., 1994; Laurie et al., 1997; Hatten, 1999). Interestingly, NR2B protein is readily detected in the forebrain and also the striatum, but is very low in the adult cerebellum (Laurie et al., 1997; Goebel and Poosch, 1999). This might possibly explain why the cerebellum is much less sensitive to NMDA as compared to other cerebral regions (Stone, 1993). With respect to NR2D, a strong level of expression is detected in the thalamus, midbrain and brainstem, an expression pattern that contrasts with the more promiscuous expression of NR2A subunits in various regions of the brain (Farrant et al., 1994; Dunah et al., 1996; Laurie et al., 1997). The expression of NR1 is likewise very widespread and is commonly found in all parts of the brain (Moriyoshi et al., 1991; Monyer et al., 1994). Such a ubiquitous pattern of expression is indeed important because NR1 is the obligatory subunit for NMDA receptors (Dingledine et al., 1999).

Among the two types of NR3 subunits, NR3A has been shown to be more widespread than NR3B. Regions with the highest levels of NR3A were spinal cord, thalamus, hypothalamus, brainstem,
CA1 of hippocampus, amygdala and certain parts of the cortex (Ciabarra et al., 1995; Sucher et al., 1995; Wong et al., 2002). On the other hand, NR3B expression was initially shown to be restricted to a few prominent regions such as the spinal cord, brain stem, cerebellum and hippocampus (Nishi et al., 2001; Matsuda et al., 2002; Bendel et al., 2005). Recently, Wee and colleagues (2008) reported a comprehensive study to systematically compare NR3B protein and NR1 localization across major brain regions. The study employed immunolabeling with specific NR3B antibody on rat brain slices revealed that there is a more wide-spread distribution of NR3B protein in the forebrain (hippocampus, cerebral cortex, caudoputamen, and nucleus accumbens), cerebellum, and lumbar sections of the spinal cord. Interestingly, when co-stained with NR1 protein, the distribution of NR3B protein appears to be as ubiquitous as NR1. The detection of NR3B in wider brain regions has been demonstrated by others showing the presence of NR3B protein (Tang et al., 2004) and mRNA (Andersson et al., 2001; Nishi et al., 2001; Bendel et al., 2005) in these several of these regions previously. At the ultrastructural level, NR3A was shown to associate specifically with asymmetrical synapses and localized to postsynaptic membranes (Wong et al., 2002). However, information on the ultrastructural localization of NR3B subunit is currently lacking.

**Among different cell types.** The different cell types present within a particular brain region often have a specific role to play in neuronal circuitry. These cells may carry out specialized functions. Therefore, it is not surprising that certain cell types will require specific configurations of NMDA receptors that are different from those present within neighbouring cells. Using single-cell reverse transcription polymerase chain reaction (RT-PCR), majority of rat locus coeruleus neurons were found to express high amounts of NR2C mRNA but low levels of NR2A and NR2B mRNA (Allgaier et al., 2001). Contrary to other preceding studies, these NR2C-rich cells produced larger currents (Monyer et al., 1992; Allgaier et al., 2001) than cells which were less enriched with NR2C. In the dorsal striatum (also known as caudate putamen), there is a much clearer correlation between subunit type and network circuitry. The
striatal projection neurons, which send far-reaching projections to other regions of the brain, have been shown to predominantly express NR1, NR2A and NR2B proteins but are completely lacking in NR2D. On the other hand, striatal interneurons express the NR2D subunit but interestingly, these neurons almost completely lack the NR2C subunit (Standaert et al., 1996; Standaert et al., 1999).

NR3A protein spatial distribution in the mammalian brain has been reported to parallel that of NR1, NR2A and NR2B, with some notable exceptions (Petralia et al., 1994a and 1994b; Wong et al., 2002). NR3A protein is heavily expressed in all areas of the cerebral cortex with the highest level in layer V, in portions of the amygdaloid nuclei and selective cell layers and nuclei of the hippocampal formation, thalamus, hypothalamus, brainstem and spinal cord. Other regions of the brain that showed significant levels of NR3A protein expressions are the central auditory pathways and pons. NR3A expression is detected in the cerebellar cortex, in which only weak signal was detected in the previous in situ studies by using riboprobes (Ciabarra et al., 1995; Sucher et al., 1995). At an ultrastructural level, NR3A protein is detected specifically localized to postsynaptic membranes in both the cerebral cortex and thalamus, with virtually no labeling in the presynaptic termini of the cerebral cortex (Wong et al., 2002).

NR3B protein was found to be expressed in all the substructures of the hippocampus (CA1, CA3, dentate gyrus), the various layers of the cerebral cortex, projection neurons and interneurons of the striatum, different cell types of the cerebellum and motor neurons of the spinal cord (Wee et al., 2008). In the cerebral cortex, NR3B immunoreactivity was detected in all the layers. Of note, NR3B expression was detected prominently in layer II. This pattern of expression resembles that of NR1 and NR2A/B (Petralia et al., 1994a and 1994b) which may suggest a possible role for NR3B in olfaction (Zatorre et al., 1992). In hippocampus, NR3B staining is not confined to any one cell type. In fact, NR3B is detected in pyramidal cells in CA1 and CA3, as well as the non-pyramidal cells in the alveus, stratum oriens, stratum radiatum and stratum lucidum. In the dentate gyrus, the hilar perforant path-associated cells were immunoreactive for NR3B. In the striatum, it was shown that NR3B protein is expressed in the medium spiny projection
neurons. NR3B is also expressed in all the cholinergic, somatostatin- and parvalbumin-type of interneurons within striatum. In cerebellum, NR3B staining was observed in the large Purkinje cells. This pattern of expression contrasts with that of NR3A, in which NR3A was more highly expressed in granule cells (Wong et al., 2002). NR3B staining is detected within the large multipolar cholineacetyltransferase (ChAT)-positive neurons in the ventral horn (characteristics of motor neurons) and smaller multipolar neurons in the dorsal horn of spinal cord. These observations raised the question whether NR3B could be involved in the processing of nociceptive information. Nonetheless, there is lack of information on the ultrastructural localization of NR3B protein at the synapses currently. Coupling of whole-cell patching of key regions in brain slices and spinal cord sections with by single-cell RT-PCR (reverse transcription polymerase chain reaction) has allowed scientists to correlate expression of subunits to their functional behavior. Such approach would further accelerate the understanding on the physiological roles of NR3-containing NMDA receptors.

Synaptic and extrasynaptic microdomains. A finer layer of regulation is seen at the subcellular level when NMDA receptors with different subunit compositions are targeted to different locations within the neuron. Among the various subcellular organelles and structures within the neuron, the postsynaptic density (PSD) is one of the most important structures for neurotransmission. A recent study employing pre-embedding immunogold labeling showed that NR3A was more enriched in the perisynaptic membrane domains, with lower frequencies in the synaptic and extrasynaptic domains (Perez-Otano et al., 2006). This contrasts with other NMDA receptor subunits (NR1 and NR2A) which tend to be denser at the centre of the PSD and decrease outwards towards the peripheral of the PSD (Fig. 3). The perisynaptic enrichment and accumulation of NR3A is the result of an activity-dependent removal of NR3A from the synapse, possibly through lateral diffusion followed by endocytosis (Perez-Otano et al., 2006). Indeed, sites adjacent to the PSD edge has been shown to be stable sites of endocytic zones (Blanpied et al., 2002; Racz et al., 2004). Upon mobilization of NR3A-containing NMDA receptors to sites adjacent to the PSD,
PACSIN1/syndapin1 ensures further endocytic removal of NR3A-containing receptors, thus leaving a higher enrichment of NR1 and NR2A subunits at the synapse in the mature rodent (van Zundert et al., 2004; Perez-Otano et al., 2006). Based on these findings, it appears that the perisynaptic enrichment of NR3A reflects a passive reservoir for receptors transiting outwards from the synapse, rather than an active and functional pool ready for activation. However, it is certain that the selective removal of NR3A-containing receptors from the synaptic pool will have functional implications for the remaining synaptic receptors that are “NR3A-lacking”. These receptors would be expected to display high Mg\(^{2+}\) sensitivity, brief open times and high Ca\(^{2+}\) permeability (Perez-Otano et al., 2006). Currently, it is not known whether NR3B is preferentially localized within any particular compartment or whether it undergoes a similar mechanism of endocytosis in developing or mature neurons.

**NR3 Ligand Binding Domain**

**Ligand recognition at chemical level.** Glycine binds to the ligand binding sites (S1S2) of NR3A and NR3B subunits, (Figs. 1B and 1C). The ligand recognition exhibited by NR3 subunits is in contrast to NR2 but resembles that of NR1 (Chatterton et al., 2002). Using a soluble NR3A ligand binding domain recombinant protein (NR3A S1S2) expressed in *Escherichia* coli, Yao and Mayer (2006) provided evidence that NR3A S1S2 has very high affinity for glycine (apparent $K_D = 40$ nM). This is 650-fold lower than the glycine $K_D$ for NR1 (26 µM) (Furukawa and Gouaux, 2003). More significantly, the same study revealed that $L$-glutamate and NMDA bind with $K_D$ values $\geq 10$ mM. NR3A subunit shows absolute selectivity for glycine over glutamate. These biochemical data corroborated with functional electrophysiological observations that showed glycine alone is sufficient to activate heterologous diheteromeric NR1/NR3 and triheteromeric NR1/NR3A/NR3B receptors (Chatterton et al., 2002; Smothers and Woodward, 2007) (Fig. 4). Since both ligand binding domains (S1S2) of NR1 and NR3A
show similar preference for full-agonists, could NR3A S1S2 bind NR1 selective competitive antagonists as well? First-generation glycine site antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and kynurenic acid bind with indistinguishable affinities to NR3A S1S2 and NR1 S1S2 recombinant proteins. In contrast, the more potent NR1 competitive antagonists like CGP78608, 5,7-DCKA, L689560 and L701324 have significantly lower affinities to NR3A S1S2 (Yao and Mayer, 2006).

Like NR1 S1S2, NR3A S1S2 displays selectivity for stereoisomer D-serine over L-serine (K_D 0.6 vs 158 µM, respectively) (see Table 1 in Yao and Mayer, 2006). Known NR1 partial agonists, example D-cycloserine (DCS), 1-aminocyclopropane-1-carboxylic acid (ACPC) and 1-aminocyclobutane-1-carboxylic acid (ACBC) are able to displace [3H]glycine from NR3A S1S2. Interestingly, the affinities of these NR1 subunit partial agonists exhibit considerable differences between NR1 S1S2 and NR3A S1S2. The rank order of affinity for NR1 is ACPC > DCS > ACBC, whereas for NR3A, the rank order is ACBC > ACPC > DCS (Yao and Mayer, 2006).

**Ligand binding at atomic resolution.** Recently, high-resolution crystal structures (approximately 1.45 – 1.62 Å) have been obtained for the ligand binding domains of NR3A and NR3B complexed with glycine, D-serine and ACPC (Yao et al., 2008). These achievements followed and built upon much effort from the crystallographic successes in solving the glutamate binding domains (S1 connected to S2 via the “GT” linker) of AMPA, kainate and NR1/NR2-subunits of NMDA receptors at atomic resolutions in bacteria expression system (Fig. 5; Chen and Gouaux, 1997; Armstrong et al., 1998; Armstrong and Gouaux, 2000; Mayer et al., 2001; Hogner et al., 2002; Armstrong et al., 2003; Furukawa and Gouaux, 2003; Jin et al., 2003; Furukawa et al., 2005; Inanobe et al., 2005; Sobolevsky et al., 2009). As expected, the glycine binding domain of each NR3A and NR3B subunit adopts a similar clamshell structure formed from two globular domains (S1 and S2) conserved within NR3 family and homologous to NR1 subunit. Upon binding the agonist glycine, NR3A and NR3B S1S2 domains undergo
similar domain closure, $22.2^\circ$ vs $23.4^\circ$ respectively, as observed in the NR1-glycine complex ($25.4^\circ$; Fig. 5A, 5B and 5C). Notwithstanding, such agonist-induced domain closure of NR3 S1S2 has (i) the largest loop 1 among all other glutamate receptors with a 12- to 13- residues $\alpha$-helix inserted between $\beta$-strands 3 and 4, (Fig. 5A and 5B), (ii) three different key amino acids coordinating glycine binding as compared to NR1 which resulted in the recruitment of three water molecules to maintain structural integrity in NR3-glycine complex instead of five water molecules in NR1-glycine complex, and (iii) no ACPC-dependent conformational switch in helix F and $\beta$-strand 14 for the NR3A-ACPC complex in contrast to the different conformations observed in the NR1-ACPC complex (Yao et al., 2008). These differences between NR1 and NR3 ligand binding sites might explain why NR3 has 650-fold higher affinity for glycine than NR1 (Yao and Mayer, 2006; Yao et al., 2008).

Pharmacological Characterization of NR3-containing NMDA receptors

An interesting property of the NR3 subunits is the ability to bind glycine rather than glutamate, a property that is very much like NR1. This has important implications because, like NR2 subunits, NR3 subunits are not able to be surface expressed in the absence of NR1 (Ciabarra et al., 1995; Sucher et al., 1995; Nishi et al., 2001; Perez-Otano et al., 2001; Matsuda et al., 2002 and 2003). Therefore, a novel type of NMDA receptors composed of NR1 and NR3 subunits would only require glycine, and not glutamate for activation. These channels are only mildly responsive to $\text{Mg}^{2+}$, MK-801 and memantine, all of which are common channel blockers for conventional NMDA receptors and are proposed to act on NR2 subunits (Fig. 4; Chatterton et al., 2002; Smothers and Woodward, 2007).

NR1/NR3 receptors expressed in oocytes. In oocytes, NR1/NR3 receptors exhibit novel pharmacological and functional properties (Ciabarra et al., 1995; Sucher et al., 1995; Wada et al., 2006; Madry et al., 2007; Awobuluyi et al., 2007). Early attempts to elicit agonist-activated currents by
glutamate or NMDA on NR1/NR3A receptors expressed in *Xenopus* oocytes generated either no robust currents (Sucher et al., 1995) or diminutive currents (<20 nA) (Ciabarra et al., 1995). Chatterton and colleagues (2002) subsequently revealed that the co-agonist glycine alone (10 µM) is sufficient to produce up to several microamperes when applied to NR1/NR3A and NR1/NR3B receptors. In the same study, the reported EC\(_{50}\) values for glycine activation of NR1/NR3A and NR1/NR3B receptors were approximately 1 µM and 5 µM, respectively. Recently, similar glycine EC\(_{50}\) (6.5 ± 1.2 µM) was described for NR1/NR3A receptors in oocytes by Madry and colleagues (2007, 2008). Both studies reported rapid desensitization of glycine-evoked currents at glycine concentrations 3 µM and higher. Another distinctive feature of NR1/NR3 receptors in oocytes is that glycine binding to NR3 alone is able to activate NR1/NR3 receptors (Awobuluyi et al., 2007). This suggests that there is a direct role, in addition to the hypothesized modulatory role, of NR3 subunit in NR1/NR3 receptor activation which is supported by higher affinity of glycine for the NR3A S1S2 domain (Yao and Mayer, 2006; Awobuluyi et al., 2007).

Using SCAM (substituted cysteine accessibility method), Wada and colleagues (2006) showed that the N-site residue of NR1 but not NR3 subunits forms the tip of the channel pore loop. The M3 segments of both NR1 and NR3A appeared to form a narrow constriction in the outer vestibule of the channel, thus preventing the influx of sulfhydryl-specific agents (MTSEA and MTSET) applied externally (Wada et al., 2006). The most internal reactive residue in each M3 segment is the threonine in the conserved SYTANLAFF motif which appears to be symmetrically aligned.

The excitatory glycine sites on both NR1 and NR3 S1S2 segments display differential effects towards other agonists as well as antagonists. D-serine has a greater potency than glycine as a co-agonist in NR1/NR2 receptors (Kemp and Leeson, 1993; Schell et al., 1995). D-serine evokes only diminutive/ no detectable inward current from NR1/NR3A receptors but small steady-state inward currents in NR1/NR3B receptors heterologously expressed in oocytes (Chatterton et al., 2002). Interestingly, Yao and Mayer (2006) demonstrated D-serine binds to the soluble recombinant NR3A S1S2 protein with ~11-
fold higher affinity (0.6 µM) than the soluble recombinant NR1 S1S2 protein (7.0 µM) (Furukawa and Gouaux, 2003). Based on the observation that D-serine inhibits glycine-induced currents from wild-type NR1/NR3B receptors and Asp732 in NR1 is a critical amino acid coordinating glycine binding (Williams et al., 1996), Awobuluyi and colleagues (2007) showed that D-serine has a relatively higher affinity but lower efficacy for NR3 than NR1 as revealed by glycine-induced currents in the absence and presence of D-serine on NR1(D732N)/NR3B receptors expressed in oocytes.

5,7-dichlorokynurenic acid (5,7-DCKA) is a non-competitive antagonist at the NR1 glycine binding site. On NR1/NR3B receptors, 5,7-DCKA exhibits a U-shape glycine-induced current profile. In the presence of low glycine concentrations and absence of current decay, 5,7-DCKA inhibited NR1/NR3A and NR1/NR3B receptors. At higher glycine concentrations where there is rapid current decay, applying 5,7-DCKA not only relieved NR1/NR3B receptor current decay but also potentiated NR1/NR3B steady-state currents. NR1/NR3A receptors also display similar inhibitor-mediated potentiation of steady-state currents (Awobuluyi et al., 2007). In contrast, strychnine, an antagonist of inhibitory, chloride-permeable glycine receptors (Curtis et al., 1967) showed minimal inhibitory effects on NR1/NR3B receptors (Chatterton et al., 2002). Other NR1-specific glycine site antagonists (MDL-29951 and L-689560) did not inhibit but strongly potentiated glycine-induced currents of NR1/NR3A receptors (Madry et al., 2007). The divalent cation Zn$^{2+}$ can potentiate NR1/NR3A receptor alone (178 µM as compared to 6.5 µM for glycine) and can cause a supra-linear potentiation of NR1/NR3A receptors in the presence of MDL-29951. Thus, Zn$^{2+}$ can act as both a weak agonist and a potent positive modulator at NR1/NR3 receptors (Madry et al., 2008). Yamakura and colleagues (2005) reported that NR3B subunit did not alter isoflurane and nitrous oxide sensitivities of NMDA receptors. Taken together, antagonists of or mutations within the glycine-binding site of the NR1 subunit produced large NR1/NR3 receptor currents while mutations within the NR3-binding site resulted in a severe reduction of glycine currents.
(Awobuluyi et al., 2007). Heterologously expressed NR1/NR3 receptors in oocytes provided evidence for differential roles of the NR1 and NR3 subunits in excitatory glycine receptor function.

**NR1/NR3 receptors expressed in HEK293 cells.** Surface triheteromeric NR1/NR2A/NR3A receptors were detected but not diheteromeric NR1/NR3A receptors in transiently transfected HEK293 cells (Perez-Otano et al., 2001). This is in stark contrast to that reported in *Xenopus* oocyte expression system (Ciabarra et al., 1995; Sucher et al., 1995; Wada et al., 2006; Awobuluyi et al., 2007; Madry et al., 2007 and 2008). Outside-outside patches pulled from HEK293 cells cotransfected with NR1/NR2A/NR3A cDNAs revealed a low conductance state (28 pS) in the presence of glutamate and glycine that occurs only when NR3A was cotransfected with other NMDA subunits. Similarly, NR3B cannot substitute for NR2 since coexpression of NR1 and NR3B in HEK293 cells did not yield electrophysiologically functional channels when activated with 1 mM glutamate and 10 µM glycine (Nishi et al., 2001). Cotransfection of NR1, NR2A and NR3B in HEK293 cells resulted in robust whole-cell currents but significantly lower in amplitude and calcium permeability as compared with NR1/NR2A receptors (Nishi et al., 2001; Matsuda et al., 2002). Increasing the amount of NR3B cDNA with respect to NR1 and NR2A correspondingly reduced the current amplitudes (Nishi et al., 2001). This is consistent with that reported in *Xenopus* oocyte expression system (Yamakura et al., 2005) that NR3 subunits exert a negative interfering effect in NMDA receptors (Ciabarra et al., 1995; Sucher et al., 1995; Das et al., 1998; Chatterton et al., 2002; Matsuda et al., 2002 and 2003; Yamakura et al., 2005).

In hippocampal neurons, Matsuda and colleagues (2003) overexpressed NR3B via viral infection to investigate the functional contribution of NR3B subunit to native NMDA receptors. A reduced shift in reversal potential was detected in NR3B overexpressed neurons (16.1 mV) versus 27.2 mV (in native neurons). However, same hippocampal neurons expressing NR3B did not show a response to glycine (10 µM) when applied rapidly (3 – 4 ms) in the presence of tetrodotoxin and picrotoxin (Matsuda et al., 2003).
NR3-containing triheteromeric NMDA receptors. The co-transfection of NR1, NR3A and NR3B cDNAs into HEK293 cells produces triheteromeric NR1/NR3A/NR3B receptors yielding glycine-activated currents with novel functional and pharmacological properties (Pina-Crespo and Heinemann, 2004; Talantova et al., 2007; Smothers and Woodward, 2007). The glycine-activated currents of NR1/NR3A/NR3B receptors resemble that observed in oocytes expressing NR1/NR3A, i.e. currents undergo rapid activation followed by rapid desensitization to steady-state levels (Chatterton et al., 2002; Yamakura et al., 2005). The desensitization of activated NR1/NR3A/NR3B receptors is not affected by cyclothiazide. Glycine transporter antagonists (ALX-1393 1-10 µM), agonists (L-alanine, β-alanine, taurine and acamprosate) and antagonists (strychnine, picrotoxin) of strychnine-sensitive glycine-activated channels did not affect the glycine-activated currents of NR1/NR3A/NR3B receptors in HEK293 cells (Smothers and Woodward, 2007). Partial agonist, D-serine and antagonists (AP5, MK-801, memantine, magnesium) of conventional NMDA receptors did not affect the glycine-activated currents of NR1/NR3A/NR3B receptors – effects corroborating with that of diheteromeric NR1/NR3B receptors expressed in oocytes (Chatterton et al., 2002).

The functional implications of NR3 subunits

NR1/NR3 receptors have been expressed in oocytes with very small, but detectable glycine-activated currents (Chatterton et al., 2002). In human embryonic kidney (HEK 293) cells however, NR1/NR3A or NR1/NR3B receptor combination does not express well on the surface. A combination of NR1/NR3A/NR3B instead was found to be necessary in HEK 293 to form functional glycine-activated receptors (Pina-Crespo and Heinemann, 2004; Smothers and Woodward, 2007). Another type of triheteromeric NMDA receptor comprises of NR1/NR2/NR3 and requires both glutamate as agonist and glycine as co-agonist (Perez-Otano et al., 2001; Matsuda et al., 2003; Karadottir et al., 2005; Tong et al.,
NR1/NR3 receptors were first characterized in heterologous cells, and there has yet to be evidence proving the existence of these glycine receptors in vivo. Attempts to detect these receptors in transgenic mice expressing NR3A were not successful because glycine application alone did not elicit detectable currents (Tong et al., 2008). This result might indicate that if glycine receptors are indeed present endogenously, then only a small population of these contains NR3A. There are a few lines of evidence however suggesting the existence of endogenous NR1/NR2/NR3 type of receptors. Firstly, transfection of NR3B into hippocampal neurons results in the incorporation of NR3B into conventional NMDA receptors (Matsuda et al., 2003). Secondly, neurons from NR3A knockout mice display increased current amplitudes when stimulated with NMDA (Tong et al., 2008). Thirdly, neurons from NR3A transgenic mice were less sensitive to Mg$^{2+}$ and less permeable to Ca$^{2+}$ when stimulated with NMDA and glycine (Das et al., 1998; Tong et al., 2008). However, Ulbrich and Isacoff (2008) described an exclusion rule on the formation of triheteromeric NR1/NR2/NR3 receptors. This group utilized a single-molecule fluorescence colocalization method that focuses selectively on the plasma membrane and simultaneously determines the subunit composition of hundreds of individual protein complexes within an optical patch on an oocyte. NR3 was found to exist exclusively in either diheteromeric forms consisting of NR1/NR3 or triheteromeric forms consisting of NR1/NR3A/NR3B receptors on the surface of oocytes (Ulbrich and Isacoff, 2008). The latter follows a fixed stoichiometry of two NR1 subunits with one NR3A and one NR3B. Given the inconsistent NR3-containing NMDA receptor expression precedence reported in oocyte and HEK293 cell line, such exclusion rule and fixed stoichiometry of NR3 assembly with other NMDA receptor subunits have yet to be verified conclusively in mammalian cell lines and neurons.
NR3 knockout/transgenic mice

To study the physiological roles of NR3A and NR3B subunits, genetic ablation of their respective genes in mouse have successfully led to the generation of NR3A knockout (NR3A-/-) and NR3B knockout (NR3B-/-) mice (Das et al., 1998; Niemann et al., 2007). Phenotypically, both knockout mice are fertile and grow to adulthood without apparent behavioural abnormalities.

NR3A-/- mice. There are no discernible changes in the expression of NR1, NR2A and NR2B proteins. Na⁺ current density and kainate-evoked current density are similar whereas the NMDA-induced current density is ~3-fold higher than wild-type mice. This increased NMDA-induced response is consistent with the hypothesized negative regulatory role of NR3-containing NMDA receptors (Ciabarra et al., 1995; Sucher et al., 1995). Single channel analyses of outside-out patches expressing NR1, NR2A and NR3A from oocytes as well as from wild-type P8 cerebrocortical neurons revealed two conductance states: a small conductance and a large conductance which showed no transitions between them. In contrast, the smaller conductance is not observed in outside-out patches from NR3A-/- mice. It is important to note that NR3A-/- mice shows an increase in the number of dendritic spines as well as modification of spine morphology in early postnatal cerebrocortical neurons of layer IV and V where the spine heads appear enlarged and spine necks elongated (Das et al., 1998). Such difference becomes less robust in the adult NR3A-/- mice.

NR3A-Tg mice. In order to further understand the physiological role of NR3A subunit, Tong and colleagues (2008) generated a NR3A transgenic (NR3A-Tg) mouse. By taking advantage of the weak expression of NR3A protein in newborn hippocampus (Wong et al., 2002), the group compared glycine-evoked inward currents between wild-type and NR3A-Tg cultured (DIV14) hippocampal neurons. Their findings demonstrated that the overexpression of NR3A protein in NR3A-Tg neurons did not elicit detectable excitatory inward currents when evoked by glycine in the presence of strychnine (Tong et al.,
2008). This was not due to lack of surface and total NR1 proteins as their expression levels (including those of NR2A and NR2B) were unaltered in the postsynaptic density fractions of cerebrocortical neurons cultured from NR3A-/- P7 and NR3A-Tg adult mice (Nakanishi et al., 2009). These results implied that overexpression of NR3A protein does not produce functional NR1/NR3 channels within the sensitivity of patch-clamp methods. Nonetheless, compared to wild-type newborn mice, NR3A-/- mice displayed significantly larger ratio of the amplitudes of the NMDA receptor-mediated component to AMPA receptor-mediated component of the excitatory postsynaptic current. Lastly, NR3A-/- neurons displayed greater sensitivity to damage by NMDA application than wild-type neurons. Overexpression of NR3A conferred greater resistance to NMDA-mediated neurotoxicity (Nakanishi et al., 2009). Both the NR3A-/- and NR3A-Tg studies show that NR3A subunit can modulate NMDA receptor-mediated synaptic transmission in mouse hippocampal and cerebrocortical neurons.

In order to understand the significance of the prominent downregulation of NR3A during the postnatal period, Roberts and colleagues (2009) generated a new breed of transgenic mice that extended the expression of NR3A beyond the natural developmental window in postnatal forebrain neurons. The effects of prolonging NR3A expression were manifold and included deficits in long-term potentiation (LTP), hypofunction of synaptic NMDA receptors, impaired maturation at Schaffer collateral-CA1 synapses and even deficits in memory consolidation. As an additional proof-of-principal, the genetic deletion of endogenous NR3A induced a premature onset of LTP compared to wild type mice. These findings not only highlight the critical importance of the developmental dip in NR3A protein, but also indicate the role of NR3A in maintaining synapses in a refractory state for LTP and structural plasticity.

**NR3B-/- mice.** Almost a decade after NR3A-/- mice was reported, Niemann and colleagues (2007) has in recent years created a mouse with genetic ablation of NR3B. Consistent with the higher expression
of NR3B in somatic motoneurons, NR3B<sup>−/−</sup> mice showed moderate but significant impairment in motor learning/coordination and decreased activity in their home cages. NR3B<sup>−/−</sup> mice also displayed increased social behaviour since they show more social interaction with familiar cage mates in their home cages but moderately increased anxiety-like behavior with decreased social interaction in a novel environment. Such altered social behaviour is consistent with the negative regulatory role of NR3B on NMDA receptor function (Niemann et al., 2007).

**Outlook**

There have been a few major events in the short history of the study of NR3 subunits of NMDA receptor: the cloning of the first cDNA encoding NR3A in 1995 by two independent groups (Ciabarra et al., 1995; Sucher et al., 1995), the creation of NR3A knockout and transgenic animals and NR3B knockout mice (Das et al., 1998; Niemann et al., 2007; Tong et al., 2008), the pharmacological characterization of NR3-containing receptors (Chatterton et al., 2002; Awobuluyi et al. 2007) and the unraveling of the atomic structures of both the agonist recognition domains of NR3A and NR3B (Yao et al., 2008). The first two endeavours stimulated more interest in the roles of NR3 subunits while pharmacological characterization efforts will greatly expand our knowledge on the effect of existing agonists and antagonists on NR3-containing receptors. Finally, the structural data will undoubtedly provide a foundation for rationale compound design on NR3-selective and NR3-specific agonists and antagonists. Any success in this area will fill the long-overdue vacuum that is currently seen in the field of NMDA receptor, where a lack of useful pharmacological tools has slowed down efforts to dissect NR3 subunit-containing NMDA receptor functions in synaptic transmission.

On a different note, much progress has been made in the past 5 years to lay the foundations to solve the structures of the ATD of ionototropic glutamate receptors (Perin-Dureau et al., 2002; Wong et al.,
2005; Ng et al., 2007; Han et al., 2008; Ng et al., 2008). In 2009, the first ATD structures of all three families of ionotropic glutamate receptors, namely AMPA receptor iGluR2 (Jin et al., 2009), kainate receptor iGluR6 (Kumar et al., 2009) and NMDA receptor NR2B (Karakas et al., 2009) were reported. It would not be long before similar endeavours based on the success gained in these studies would be applied to solving the ATD structures of NR3 subunits and thus, augment the study of modulator- (positive and negative) binding, ATD-mediated receptor desensitization and oligomerization of NR3-containing NMDA receptors. The recent success in solving the architecture and atomic structure of intact iGluR2 (exclude CTD) paves the way to also solve various intact heteromeric NR1/NR2/NR3 NMDA receptors (Sobolevsky et al., 2009).

Clearly, much still remains to be learned about the ultrastructural localization of NR3B subunit in the central nervous system. Given the power of in vitro/in vivo imaging coupled to electrophysiology techniques, and taking into consideration the ability today to generate brain region-specific knock-out/transgenic mice, the NMDA receptor field can expect a surge in new findings concerning the putative physiological roles of NR3 proteins.

In summary, much momentum has been gained in research involving this ‘last’ member of the NMDA receptor family. The future outlook appears promising since several major landmarks have been achieved in the past decade or so. These landmarks have been detailed in this review and include the spatiotemporal mapping of NR3 protein expression, the pharmacological characterization of NR3-containing receptors, the three-dimensional structural elucidation of strategic domains and the generation of NR3 knockout/transgenic animals. Certainly, the field of glutamate receptors is beginning to pay more attention to this not-so-new subunit; the newest player in an already established field of NMDA receptor. Nonetheless, many more possibilities lie ahead in areas that have yet to be explored and particular attention should be paid to how NR3 subunits fit into the existing body of knowledge already gained in the field. This includes modulation by drugs at the channel pore, cytoplasmic modifications (putative
phosphorylation, proteolytic cleavage), subunit-mediated NMDA receptor trafficking and linkage studies between NR3 genes and NMDA receptor associated neurological diseases. It is hoped that the opening of these areas will further advance the understanding of the basic pharmacology as well as the complex role of NMDA receptors in higher brain functions and neurological disorders.

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References


Footnotes

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Legend for figures

Fig. 1. Structural domains of NMDA receptor subunits. A, relative subunit coding regions of mature mouse (m), rat (r) and human (h) NR3 subunits to rat NR1 and NR2 are shown schematically (no signal peptide, not drawn to scale). The N-terminal is shown to the left and the C-terminal is at the right. Transmembrane domains are indicated by brown boxes. The reported lengths of mature subunits in literatures are shown in brackets (Hollmann and Heinemann, 1994; Ciabarra et al., 1995; Sucher et al., 1995; Nishi et al., 2001; Chatterton et al., 2002; Matsuda et al., 2002). B, Diagram shows molecular organization of a NR3 subunit comprising the amino-terminal domain (ATD), the two lobes (S1-S2), the three transmembrane domains (M1, M3 and M4), the re-entrant loop (traditionally known as M2) and the intracellular cytoplasmic terminus domain (CTD). The endogenous ligand for NR3 S1-S2 is glycine. Yet to be discovered ligands (?) might bind to ATD. C, Amino acid sequence of rat NR3B and approximate representation of various domains (Chatterton et al., 2002). Different colours represent the amino acid sequences translated from 8 exons of the rat NR3B gene (GenBank accession number AF440691). The white coloured region is not accounted for in the Genbank records of exons for rat NR3B. The signal peptide (SP) is estimated as 17 aa (Chatterton et al., 2002) (boxed, light yellow circles). The start and end of S1 and S2 domains are indicated based on (Yao et al., 2008). * denotes the Q/R/N site determining Ca\(^{2+}\) permeability of the channel. ** denotes the putative YTANLAAF region affecting gating.

Fig. 2. Relative changes in NR1, NR3A and NR3B protein levels across various developmental ages. NR3A expression is relatively higher at younger ages and declines towards adulthood whereas NR3B expression gradually increases over development. NR1 protein does not fluctuate much across different ages but there is an age-related decline. Current studies in aged rodents are few and often focus on
specific brain regions. Densitometric data were extracted from (Wong et al., 2002) for NR1 and NR3A protein expression in rat total brain and (Prithviraj and Inglis, 2008) for NR3B protein expression in rat spinal cord.

**Fig. 3.** A schematic diagram showing the relative distribution of NR3A-containing subunits versus NR2 subunits within a dendritic unit. NR3A-containing NMDA receptors are actively endocytosed at the perisynaptic region into endocytic vesicles. There is a significant population of extrasynaptic NR3A-containing receptors. NR2-containing NMDA receptors have been shown to be present at both synaptic and extrasynaptic sites. The exact subunit compositions of NR3A-containing receptors are still unclear.

**Fig. 4.** A schematic diagram showing the relative differences between a conventional NR1/NR2 NMDA receptor (*left*), a heterotrimeric NR1/NR2/NR3 NMDA receptor (*middle*) and a heterodimeric NR1/NR3 glycine-activated receptor (*right*). Three major differences in the properties of these receptors are: (i) relative sensitivity to Mg\(^{2+}\), (ii) requirement or lack of requirement for glutamate and glycine as ligands, and (iii) relative permeability to Ca\(^{2+}\).

**Fig. 5.** Crystal structures of ligand-bound S1S2 domains of NR3A, NR3B, NR1 and NR2A subunits. Ribbon diagrams for the A, NR3A glycine complex (2RC7.pdb), B, NR3B glycine complex (2RCA.pdb), C, NR1 glycine complex (1PB7.pdb), and D, NR2A glutamate complex (2A5S.pdb) coloured to show the S1 (magenta), S2 (blue) and loop 1 (green) were created using PyMOL (DeLano, 2002). The GT linker that replaces the 1\(^{st}\) and 2\(^{nd}\) transmembrane segments is indicated in black. The ligands are drawn as spacefilled spheres (glycine – yellow, glutamate – orange). The N-terminus is facing the *left* while the C-terminus linking to transmembrane M1 is on the *right*. Note the significant difference in loop 1 of NR3 S1S2 domains and those of NR1 and NR2A (see text for detailed discussion).
Figure 3

Key
- PSD
- NR1/NR2
- NR1/NR3A/NRxx

Synaptic
Perisynaptic
Endocytic vesicles
Extrasynaptic
Figure 4

Key:
- glycine
- glutamate

NR1/NR2x
high Mg$^{2+}$ sensitivity

NR1/NR2x/NR3x
low Mg$^{2+}$ sensitivity

NR1/NR3x
low Mg$^{2+}$ sensitivity

Ca$^{2+}$