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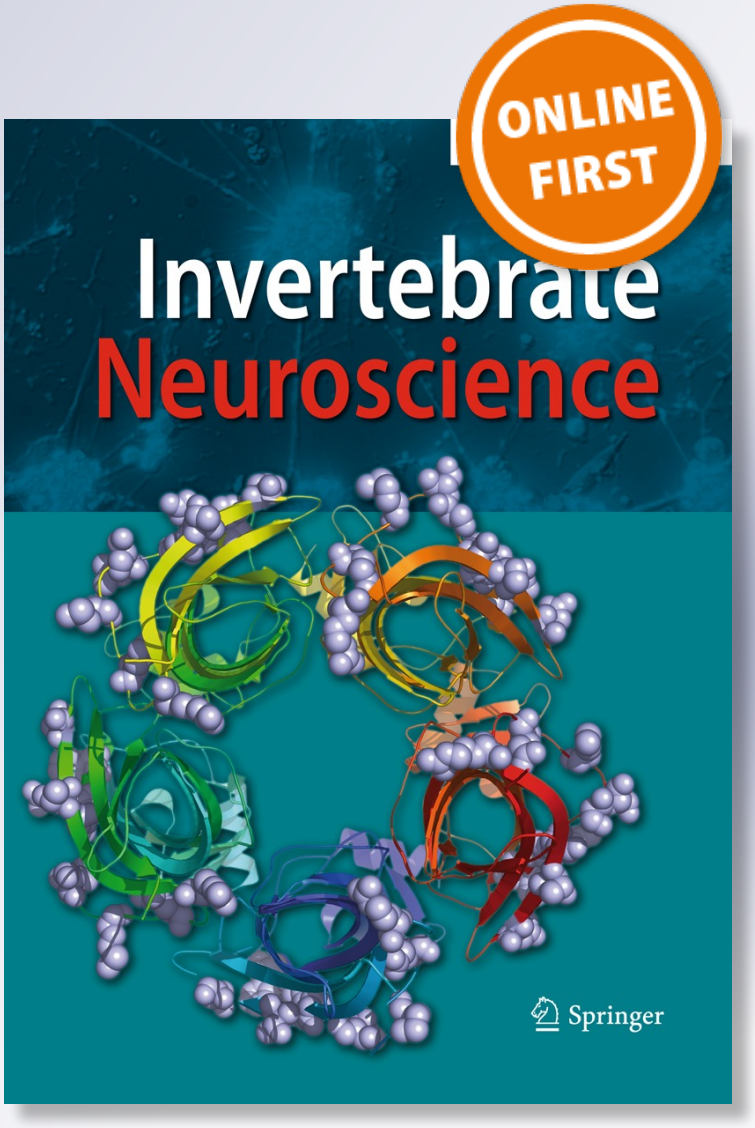

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ORIGINAL PAPER

NMDA receptor expression and C terminus structure in the rotifer *Brachionus plicatilis* and long-term potentiation across the Metazoa

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Abstract The C termini of *N*-methyl-D-aspartate (NMDA) receptor NR2 subunits are thought to play a major role in the molecular establishment of memory across the Bilateria, via the phenomenon known as long-term potentiation (LTP). Despite their long history of use as models in the study of memory, the expression and structure of the NR2 subunit in the Lophotrochozoa has remained uncategorized. Here, we report the phylogenic relationships of NR subunits across the Bilateria, and the cloning and in situ analysis of expression of NMDA NR1 and NR2 subunits in the monogont rotifer *Brachionus plicatilis*. RNA in situ hybridization suggests expression of NMDA receptor subunits in *B. plicatilis* is neural, consistent with expression observed in other species, and ours is the first report confirming NR2 expression in the lophotrochozoan clade. However, the single NR2 subunit identified in *B. plicatilis* was found to lack the long C terminal domain found in vertebrates, which is believed to modulate LTP. Further investigation revealed that mollusc and annelid NR2 subunits possess long intracellular C terminal domains. As data from molluscs (and particularly *Aplysia californica*) are the basis for much of our understanding of LTP, understanding how these diverse lophotrochozoan C termini function in vivo will have many implications for how we consider the evolution of the molecular control of learning and memory across the Metazoa as a whole and interpret the results of experiments into this vital component of cognition.

Keywords Rotifer · NMDA receptors · Learning · Long-term potentiation · Lophotrochozoa

Introduction

N-methyl-D-aspartate (NMDA) receptors, members of the ionotropic glutamate receptor family, were originally identified in the 1970s and first cloned in 1991 (Moriyoshi et al. 1991). The unique functionality of NMDA receptors makes them ideal candidates for regulating long-term potentiation (LTP), a still mysterious process believed to underlie learning and memory.

The molecular mechanism controlling LTP is believed to be governed by a variety of molecules; with NMDA receptors the best studied and perhaps the most important part of this process (Izquierdo 1991; Malenka and Bear 2004). NMDA receptors possess a unique ability to act as a clearing point for a variety of molecular inputs and respond accordingly (Bliss and Collingridge 1993; Kandel 2001; Glanzman 2010) and have been the subject of a variety of experimentation in many different model organisms (Martin et al. 1997; Casadio et al. 1999; Ha et al. 2006), as it is generally believed that the underlying mechanisms of memory could be conserved across the Metazoa (Glanzman 2010).

Molluscs such as the California Sea Slug, *A. californica,* or the Great Pond Snail, *Lymnaea stagnalis,* are common

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models for behaviour and learning (Glanzman 2007; Kemenes and Benjamin 2009) as the large neurons and identified networks in these species make them excellent tools for research (Kandel 1976; Ito et al. 1999; Kandel 2001), and findings from these studies are often applied more generally without consideration as to the differential molecular componentry found across the Metazoa. Understanding how the molecular role and operation of NMDA receptors may differ across the animal kingdom is therefore vital, both in interpreting the results of such previous experimentation and in revealing how molecular memory and learning is conserved, or differs, across metazoan life.

In recent years, it has become more apparent that invertebrates (or at least the mollusc models studied in the laboratory) share many, if not all, of the postsynaptic mechanisms of memory observed in vertebrates (Antonov et al. 2003; Glanzman 2007; Glanzman 2010), including NMDA receptor-based LTP. NMDA receptors and several of the protein kinases involved in learning in vertebrates have been shown to operate in operant conditioning in *L. stagnalis* (Rosenegger and Lukowiak 2010). Furthermore, NMDA receptors have been shown to regulate olfactory learning in *Drosophila melanogaster* (Xia et al. 2005). NMDA antagonists impair memory reactivation in molluscs (Solntseva and Nikitin 2008) and crustaceans (Pedreira et al. 2002) and are thus unequivocally involved in memory in protostome clades.

In vivo, functioning NMDA receptors are formed from two NR1 and two NR2 subunits, which form a heterotetrameric, nonselective cation channel. The operation of this channel is regulated by a variety of mechanisms (Traynelis et al. 2010). In addition to these two subunit types, vertebrates also possess the ability to regulate NMDA receptor function through the production of an inhibitory NR3 subunit (Low and Wee 2010). Both NR1 and NR2 subunits consist of an extracellular N terminus, with a ligand binding site, three transmembrane regions (M1, M3 and M4) and the M2 region, which forms the channel pore. NR1 and NR2 subunits both possess an intracellular C terminus, although the structure and length of this varies. NR2 subunits, while superficially similar in structure to NR1 subunits, can vary far more in their sequence from species to species, especially at their C terminus (Ryan et al. 2008). To date, no NR2 subunit expression data have been presented in the Lophotrochozoa (which comprises phyla such as molluscs, annelids, nemerteans, brachiopods, bryozoans, rotifers and platyhelminthes), despite inferences being made as to their role in LTP in many studies (e.g. (Grey et al. 2009). Deletion of the C terminus of NR2 subunits in mice results in the same phenotype as null mutants for the NR2 allele in question, indicating that the role of the intracellular portion of the NR2 subunit is crucial for correct NMDA receptor functionality in this species (Sprengel et al. 1998). Furthermore, NMDA function in LTP, memory and behaviour may be intimately tied to C terminal structure (Liu et al. 2004; 2007; Barria and Malinow 2005; Chen and Roche 2007).

NR1 subunit expression has been catalogued in molluscs (Ha et al. 2006) and annelids (Grey et al. 2009), where they have been shown to be expressed in central ganglia, motor neurons and mechanosensory cells. NR2 subunits have been tentatively identified by automated software in some recent genome and transcriptome projects (Moroz et al. 2006), and an NR2 subunit has previously been putatively identified in *A. californica* and reported to GenBank (EU327683.1); however, expression data for NR2 subunits in the Lophotrochozoa have yet to be reported.

The Lophotrochozoa remain undersampled in terms of genetic sequence information, and as a result, an understanding of the sequence and structure of the C terminal end of NR2 subunits has been lacking. These structures are vitally important for the functionality of these molecules (Ryan et al. 2008) and for our interpretation of the results of testing LTP using lophotrochozoan models. We thus present the first confirmation of neural expression of the NR2 gene in the Lophotrochozoa, alongside a consideration of the C terminal structure of this gene in *B. plicatilis* compared to that possessed by other species. These data provide a vital starting point for assessing the functionality of the NR2 gene in the rotifer *B. plicatilis* and in the lophotrochozoan superphylum as a whole.

Materials and methods

Rotifer culture and gene identification

Brachionus plicatilis Nevada were identified and cultured as described in (Smith et al. 2010). RNA was extracted and transformed into cDNA, which was normalized by Evrogen JSC (Russia) and used to create a *B. plicatilis* transcriptome by 454 pyrosequencing. These sequence data were assembled using CAP3 (Huang and Madan 1999), and known NMDA NR1 and NR2 sequences (*Mus musculus,* NR1 NP_032195.1 and NR2A NP_032196.2) were used to putatively identify contigs within our transcriptome using tBlastn (Altschul et al. 1990). Reciprocal Blastx against the NCBI nr database was used as initial confirmation of identity, and two contigs identified as likely NR1 and NR2 subunit sequences were chosen for further investigation.

Sequence confirmation

As contig length was initially insufficient, 3′ RACE was used to amplify additional sequence for each of these

contigs. RACE products were cloned into pBluescript KS+ (Stratagene) and sequenced by the Otago Sequencing Unit, and consensus transcriptome and RACE-derived sequence used to make probes for RNA in situ hybridization, as described below. More recently, sequence has been confirmed by Blastn against a draft version of the *B. plicatilis* genome. Cloned sequence has been entered into the NCBI database (Accession Numbers: NR1: KC626073 NR2: KC626074).

RNA in situ hybridization

Rotifer were prepared for in situ hybridization using the protocol described previously in (Smith et al. 2010). Digoxigenin (DIG)-labelled RNA probes were prepared using the protocol described in (Osborne and Dearden 2005) using the primers NR1for 5′ TGCTAGGGTTCTT GGAATGG 3′ and NR1rev 5′ AGCCATGGATTTCCCTT TTT 3′; and NR2for 5′ TGACCAATCCCTACGGCTAC 3′ and NR2rev 5′ CCGGCAAACAGGAGAATAAA 3′ to recover a portion of each subunit. PCR products were cloned into pBluescript KS+ (Stratagene) for transformation into DH5α *Escherichia coli*, sequencing and subsequent probe construction. In situ hybridization was then performed as described in (Smith et al. 2010), and rotifer were mounted in 70 % glycerol and examined on an Olympus BX61 microscope.

Phylogenetic analysis

Brachionus plicatilis consensus sequences were translated into predicted protein sequence using Expasy (Gasteiger et al. 2003) and aligned using MAFFT 6 under the G-INS-I model (Katoh et al. 2002) to homologues from other organisms within the NCBI nr database, and this alignment can be seen in Supplementary Fig. 1, presented using Jalview (Clamp et al. 2004). Phylogenetic analysis was performed on the well-conserved intra-membrane ligand-gated ion channel portion of these proteins, the alignment of which can be seen in Supplementary Fig. 2. Gaps were excluded from this region for the purposes of phylogenetic analysis, resulting in a final total of 328 informative residues. A maximum likelihood analysis was performed using MEGA5 (Tamura et al. 2011) using the WAG model (Whelan and Goldman 2001), 1,000 bootstrap replicates and all other default prior settings. Bayesian phylogenetic analysis was performed with MrBayes v3.2.1 x64 software (Huelsenbeck and Ronquist 2001) using the WAG model (Whelan and Goldman 2001) of amino acid substitution after initial identification using mixed models. The Monte Carlo Markov Chain search was run over 1,000,000 generations, trees were sampled every 1,000 generations, and the first 25 % of trees thus gathered were discarded as 'burn-in'.

Protein motif and intronic identification

Protein motifs were identified using PROSITE (Sigrist et al. 2010) on the MyHits database (Hulo et al. 2008) and absence confirmed by manual search where necessary. Intron/exon structure and domain locations for known NR2 genes were downloaded from HomoloGene (http://www.ncbi.nlm.nih.gov/homologene/) and Ensembl (Flicek et al. 2012) and visualized using Fancygene (Rambaldi and Ciccarelli 2009).

Results

Sequence and structural comparison

The *B. plicatilis* NR2 homologue possesses a markedly shorter C terminus than that found in *A. californica* and vertebrate models, as can be seen diagrammatically in Fig. 1a, with a length more akin to that found in a variety of sequenced ecdysozoans (for example, arthropods and nematodes). An alignment of these regions can be seen in Supplementary Fig. 1 beginning at position 1260, along with the sequences of a variety of other species. It should be noted that the full N terminus sequences of *B. plicatilis* NR subunits are as yet unresolved.

As can be seen in Fig. 1b, many of the binding sites commonly found on other NR2 subunit 3′ domains are found in the C terminus of the *B. plicatilis* NR2 homologue, including the terminal PDZ domain that is a noted feature of these molecules. The functionality of these motifs in many cases has not been subject to experimental validation, and those shown are the result of automated annotation, which may result in overprediction of these sites. A variety of effectors have been shown to act on this domain in some species, including MAGUK PSD-95 proteins, which act through the PDZ domains found at the C termini of NR2 subunits (Husi and Grant 2001; Furukawa et al. 2005; Ryan et al. 2008). A form of N-myristoylation has been noted as playing a role in providing an alternative, C terminus-located membrane-anchoring site in some organisms, after first being observed in the chicken (Lopez-Colome et al. 2004; Goto et al. 2009; Flores-Soto et al. 2012). Other effectors include CaMKII (Leonard et al. 1999; Barria and Malinow 2005), Fyn, which promotes tyrosine kinase activity (Tezuka et al. 1999), alpha-actinin (Wyszynski et al. 1997) and many others (Ryan et al. 2008; Traynelis et al. 2010). While many predicted binding sites for these molecules are observed in our data, an absence of predicted N-myristoylation sites is observed within the *B. plicatilis* C terminal region. We were also unable to identify a predicted binding site for CaMKII, which we believe must exist in molluscs due to previous evidence (Wan et al. 2010) and the

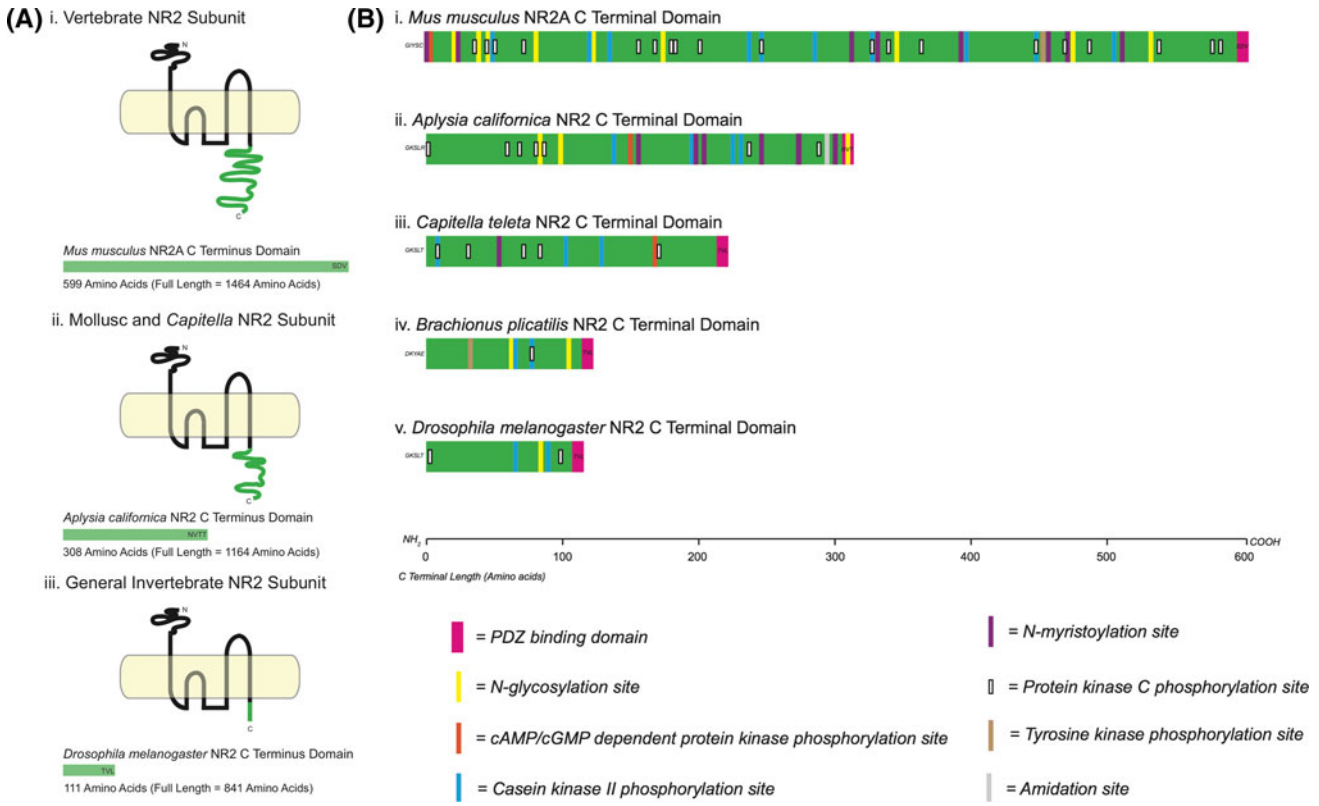

Fig. 1 **a** Stylized NMDA NR2 subunits (after Ryan et al. 2008). (*i*) Vertebrate NR2 subunits, for example, *Mus musculus*, (*ii*) Mollusc and annelid NR2 subunits (for example, *A. californica*) and (*iii*) Ecdysozoans and rotifer NR2 subunits (for example, *B. plicatilis*). Note the alternate forms of the NMDA receptor NR2 subunit in the Lophotrochozoa, with annelid and mollusc forms possessing a significantly longer C terminus than those of other organisms. The longer C termini allow more space for the binding of molecules that may help regulate the involvement of NMDA receptors in LTP and memory. **b** NMDA NR2 subunit C Termini: predicted protein interactions as proposed by MyHits PROSITE software (Hulo et al. 2008, Sigrist et al. 2010). *Note*: these are automated predictions and in many cases are not experimentally validated. C termini length to scale, measured from the end of the M4 transmembrane domain. Protein-binding sites and interactions as indicated

known importance of CaMKII in LTP in mammals (Barria and Malinow 2005).

Size and structure of exons and introns vary widely within NR2 genes, from 16 exons in molluscs and annelids, and 14 in chordates to 10 in *D. melanogaster* and 9 in the putative sole *Hydra magnipapillata* NMDA receptor subunit, as can be seen in Supplementary Fig. 3. This variability in exon number is coupled to extreme locus size in chordates, with the NMDA NR2A and B subunit loci spanning nearly 500 kb in this clade.

Phylogenetic analysis

The results of phylogenetic comparison of our sequences with that of known NMDA subunits can be seen in Fig. 2. Both *B. plicatilis* NR1 and *B. plicatilis* NR2 subunit sequences are found within monophyletic protostome clades, with excellent posterior probability/bootstrap support supporting these nodes (1/85 and 1/98, respectively). While a monophyletic lophotrochozoan clade is not recovered, likely due to the fast-evolving sequences of the likes of *Caenorhabditis elegans* drawing these sequences towards the base of their clades by long-branch attraction, it is clear that the homology of these *B. plicatilis* NR1 and NR2 subunits to known examples has been established.

Only one apparent NMDA receptor subunit gene could be found in the genome of *H. magnipapillata*, which phylogenetic analysis places as more akin to the NR1 clade. This corroborates earlier investigations in this regard (Pierobon et al. 2004; Scappaticci et al. 2004) and may reflect the subunit complement of the cnidarian: bilaterian common ancestor. The sea anemone *Nematostella vectensis* possesses a number of NMDA receptor subunit genes (data not shown), but these all show similar affinity to the NR1 subunit and may represent recent independent duplications at this locus.

An apparent NR3 subunit can be found within the acorn worm *Saccoglossus kowalevskii* genome, suggesting that the full diversity of NMDA subunit genes emerged prior to the whole genome duplications that occurred on the vertebrate lineage.

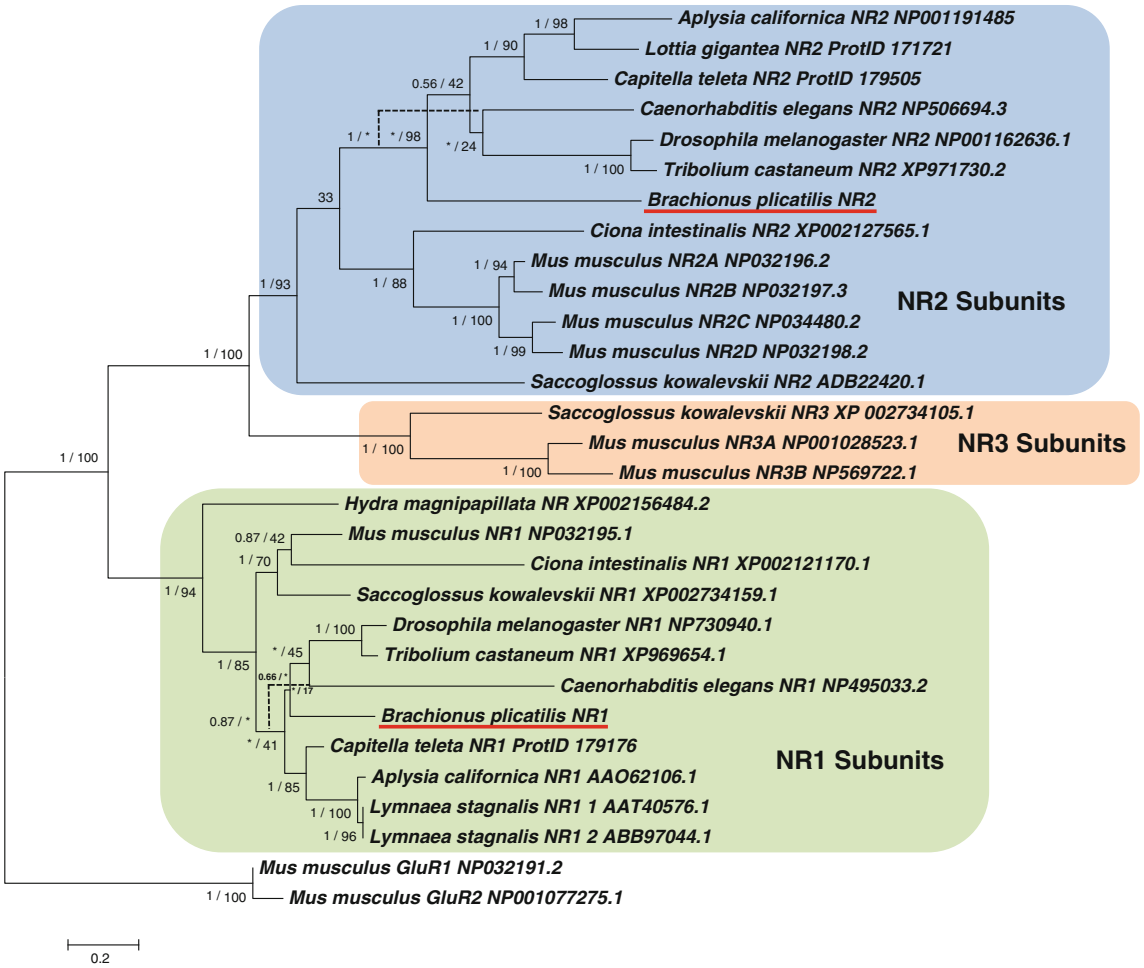

Fig. 2 Phylogenetic analysis of *B. plicatilis* NMDA subunit protein sequence. Phylogenetic tree, produced using maximum likelihood (Tamura et al. 2011) and Bayesian (Huelsenbeck and Ronquist 2001) approaches of NMDA NR1, NR2 and NR3 subunit amino acid sequences rooted with *Mus musculus* GluA1 and GluA2 AMPA receptor subunits. Phylogenetic analysis was performed on the well-conserved intra-membrane ligand-gated ion channel portion of these proteins, the alignment of which can be seen in Supplementary Fig. 2. Gaps were excluded from this region for the purposes of phylogenetic analysis, resulting in a final total of 328 informative residues. At *nodes*, first number represents Bayesian prior probability, and second represents bootstrap percentage recovery (of 1,000 replicates). *Dotted lines* represent the architecture of the Bayesian tree when it differs from the maximum likelihood tree shown as *solid lines*. This occurs twice, in the NR1 clade where *C. elegans* NR1 is the sister group to all other protostome NR1 subunits under Bayesian analysis and in the NR2 clade where *C. elegans* NR2 is the sister group to all other protostome NR2 subunits under Bayesian analysis. *Scale bar* represents number of substitutions per site at given distance. Protein sequences used for phylogenetic analysis and alignment can be found in Supplementary File 1

NMDA receptor subunit expression

By in situ hybridization analysis, mRNA from the NMDA receptor NR1 subunit gene was found expressed in a set of cells at the anterior region of developing embryos and adult rotifer (Fig. 3). These cells lie in regions around the mastax that likely represent the cerebral ganglion, with putative NMDA expressing cells observed from a early phase of development (a, b) persisting dorsally (d), through to adulthood (e). Expression was evident along the centre line of rotifer near to hatching (c), implying a role throughout the central nervous system in developing individuals, although this is not evident in adults (e). No staining is evident in the control (f). Key features of rotifer anatomy can be seen labelled in (g). The pattern of expression seen in (a–e) implies localization of NMDA receptor transcripts within the central nervous system of the rotifer.

Figure 4 shows the expression of NR2 subunit mRNA in *B. plicatilis.* Early expression is diffuse (a). The anterior dorsal expression mirrors that seen in Fig. 3 in later stage embryos and adults (a, b, d, e), suggesting co-expression in the nervous system with the NR1 subunit. No expression is observed, however, in the central body mass or tail of the rotifer, and expression is more restricted to the cerebral ganglion than that seen for NR1 subunit mRNA. Expression is particularly marked in two cells, situated symmetrically

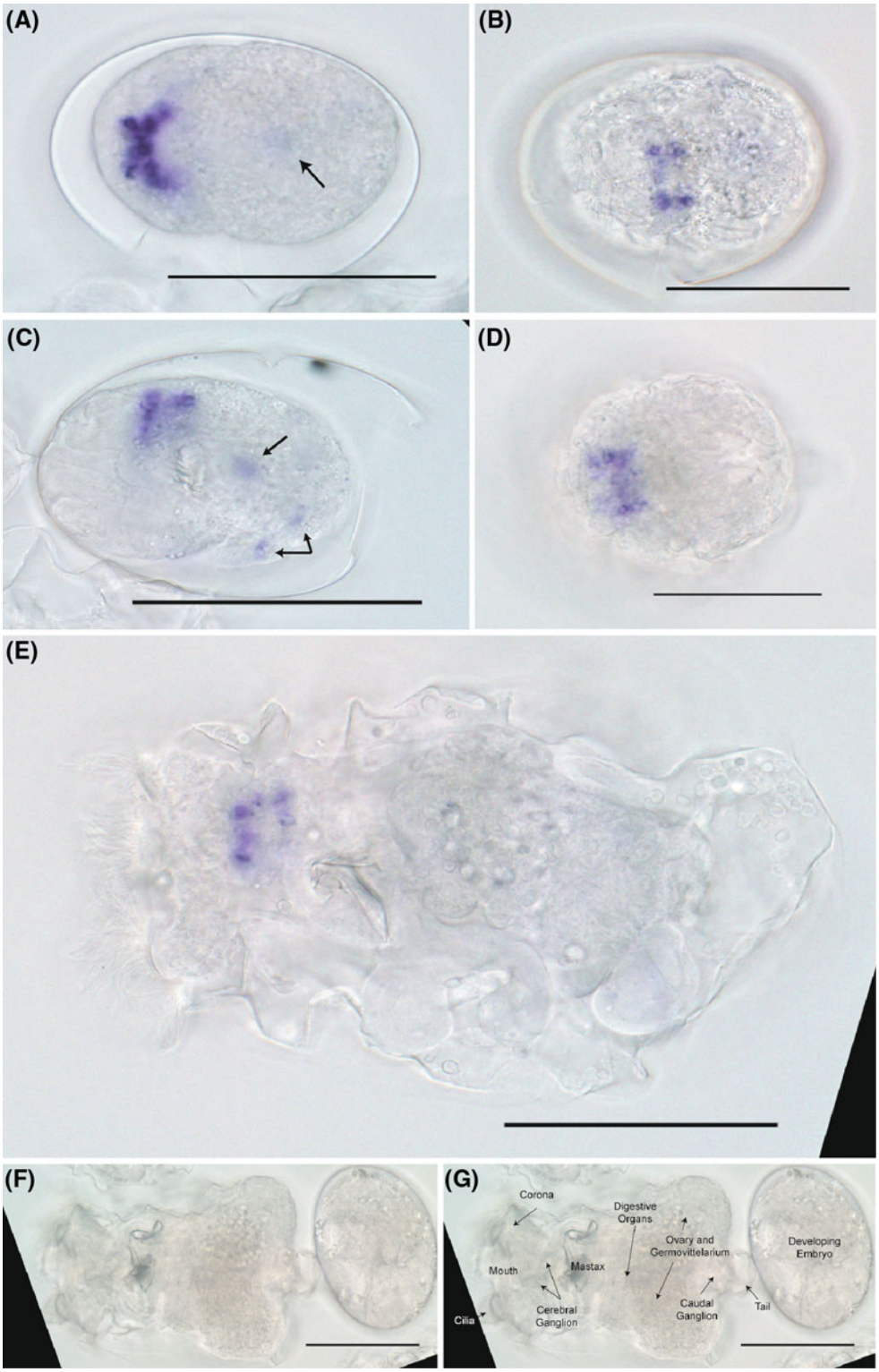

Fig. 3 Expression of NMDA NR1 subunits in *B. plicatilis*, NMDA receptor NR1 subunit mRNA expression in the rotifer *B. plicatilis,* as observed via DIG-labelled probe in situ hybridization. Rotifer are arranged with anterior at left unless otherwise noted. **a** Neural expression is observed in a 'cross' consisting of five cells in early embryo development in the anterior. Note also faint expression in the centre of developing embryo, indicated with arrow. **b** As rotifer grows, the 'cross' of cells continues to express NR1 subunit mRNA. **c** Lateral view, showing expression in the dorsal anterior, in the centre of the embryo (*single arrow*) and at the base of the 'foot' of the rotifer (indicated by *double arrow*). **d** Cross-section through the transverse plane of the rotifer, looking down the centre line, showing expression is localized at the dorsal side of the body. **e** Adult rotifer, with expression in similar areas to B, however, expression at the most anterior portion of the 'cross' is reduced, resulting in a posterior-facing 'U' of cells expressing mRNA. **f** Sense control. **g** Rotifer anatomy, with key features labelled. *Dark regions* in corners of **e**, **f** and **g** are the result of image rotation. *Scale bar* indicates 100 μm

opposite to one another anterior to the mastax, although broader expression is seen mirroring that of NR1 expression (a, e). No staining is evident in controls at early stages, with very faint background staining visible in the ovary of some adult controls (c, lower image, at far right).

Discussion

The data presented here represent the first confirmation of expression of an NR2 subunit in the Lophotrochozoa and the first investigation of NMDA receptors within the

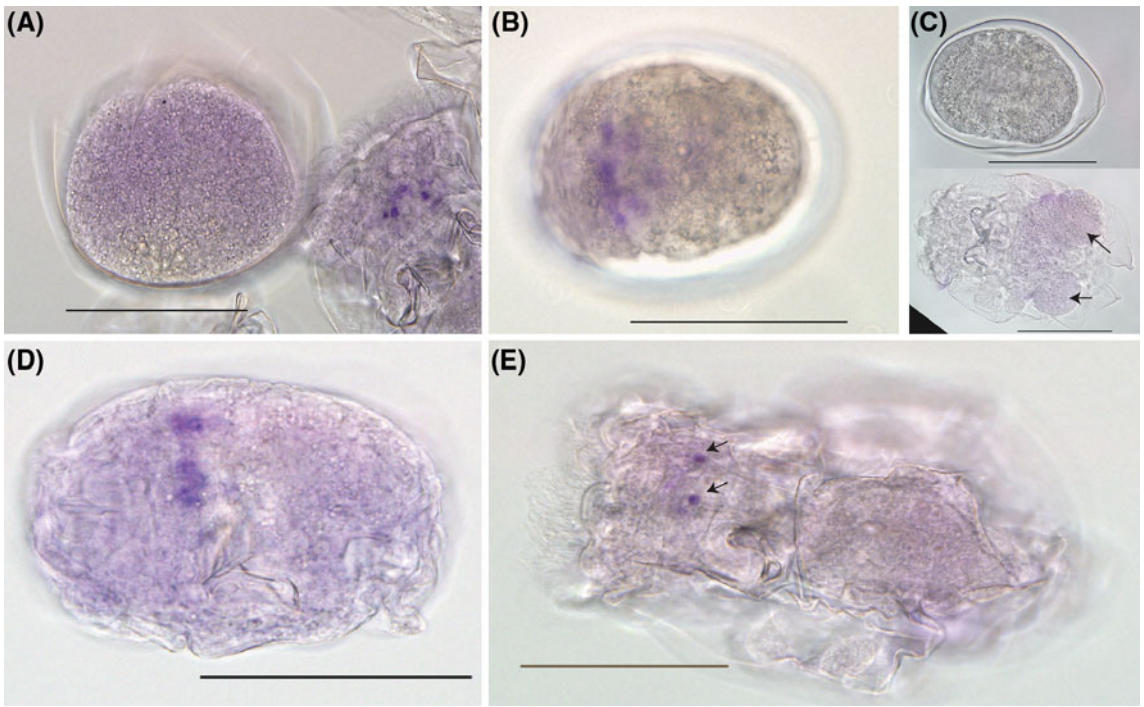

Fig. 4 Expression of NMDA NR2 subunits in *B. plicatilis*, as determined by DIG-labelled RNA in situ hybridization. Anterior is positioned to the far left and posterior to the right, unless otherwise noted. **a** An early embryo, with diffuse staining present throughout the majority of its tissue. Also visible at *right* is an adult head, at a 45° angle to the horizontal, with expression delineating a clear posteriorly facing 'U' reminiscent of that seen in Fig. 3e, in the presumptive cerebral ganglion. **b** An intermediate stage embryo, with RNA expression confined to a symmetrical cell set, in the same location as the presumed ganglion, immediately anterior to the developing mastax. **c** Sense controls, with no staining evident in observed embryos, and very diffuse nonspecific staining observed in the ovaries of some adults as shown (indicated with *arrows*). **d** Lateral view, showing expression in the dorsal anterior of the developing embryo immediately proceeding hatching. **e** RNA expression in an adult, with clear expression in a symmetrical set of cells, the inferred cerebral ganglion. *Note*: particular expression in two cells, as indicated with *arrows*, symmetrically arranged around the midline forward of the mastax (also visible in **a**). In all cases, *scale bars* indicate 100 µm, and *dark regions* in the corner of **c** are the result of image rotation

Rotifera. While expression broadly correlates with that expected from that seen in the Ecdysozoa, the differing C termini structures observed in the Lophotrochozoa raise questions relating to the functionality of this molecule in vivo and the comparability of the results of functional testing in this superphyla with the Deuterostomia.

NR2 subunit C terminus structure and function in memory in the Lophotrochozoa

Figure 1 shows the length of a variety of C termini, along with the binding motifs predicted along their length. The length of the C terminal domain of *A. californica* and vertebrates far outstrips that of ecdysozoan model organisms, for example, *D. melanogaster,* and other lophotrochozoans. The C termini of genomically sequenced species such as *Schistosoma mansoni* and the gastropod *Lottia gigantea* tend towards the intermediate point of this spectrum, approximately the length of the C terminus found in the polychaete *Capitella teleta* and shown here.

Perhaps the most parsimonious explanation for the short C termini observed in rotifer, other lophotrochozoans and in ecdysozoan models is, at present, independent loss of sequence at the C terminus of the NR2 subunit in these organisms. The independent evolution of long C termini in the Lophotrochozoa and Deuterostomia is unlikely, given the conservation of motifs of known functionality (e.g. Glanzman 2010) seen between these species (Fig. 1b), although it is possible that the automated prediction methods used here have overpredicted the occurrence of these motifs in these domains, making them appear more similar than they are.

It has not escaped our attention, however, that vertebrate NR2 C termini lie entirely on the last exon of this gene, as can be seen in Supplementary Fig. 3, and this exon appears to have been formed by the fusion of the multiple exons on which this domain is found in nonvertebrates, as postulated in Ryan et al. 2008, with capture of the intronic region found between these (data not shown), which could explain the extended length of this domain in chordates. As yet, the

state of the *A. californica* genome is insufficient to tell whether this has also occurred in this species. The increasing accessibility of such data sets will, however, allow the rigorous testing of this hypothesis, as more lophotrochozoan genome sequences become publicly available. Even so, the choice of *A. californica* as a model for research into LTP seems serendipitous, given the relative conservation at the C terminus these species show compared to other protostomes in general and gastropods in particular.

NMDA phylogeny

The glutamate-gated receptor family predates the split between plants and animals (Chiu et al. 1999), and NMDA-like receptor subunits have been observed in cnidarians (Pierobon et al. 2004; Scappaticci et al. 2004) but have, however, correlated more closely with NR1 subunits. Our data support the conjecture that the NR2 subunit appeared before the advent of the last common ancestor of protostomes and deuterostomes, despite the paucity of previous lophotrochozoan evidence in this regard. Furthermore, the NR3 subunit appears to have arisen earlier in the Deuterostomia than previously thought, although a conserved inhibitory role is impossible to speculate on without functional testing.

Expression

In rotifer, neurons are arranged around the cerebral ganglion in a bilaterally symmetrical manner, in a distinct pattern that varies from species to species (Kotikova 1998). NR1 and NR2 mRNA expression is observed symmetrically in the central ganglion of *B. plicatilis*, with faint NR1 expression also seen elsewhere in the body.

NR1 subunits have been found to be expressed in the brain of *D. melanogaster* (Ultsch et al. 1993), and NR1 and NR2 homologues have been observed in *C. elegans,* where they are expressed in a subset of neurons (Brockie et al. 2001). NR1 subunit expression has been catalogued in the leech (Grey et al. 2009), where it has been shown to be expressed in central ganglia, motor neurons and mechanosensory cells. *A. californica* was also found to express NR1 extrasynaptically, in neurites of metacerebral cells (Ha et al. 2006). NR2 subunits have been tentatively identified by automated software in some recent genome and transcriptome projects (Moroz et al. 2006), and an NR2-like sequence has previously been published to GenBank (EU327683.1).

The expression observed in the cerebral ganglion suggests that NR1 and NR2 subunits play a canonical role in the nervous system of rotifer, although functional testing is necessary to confirm this finding. The expression of NR1 subunits in putative motor neurons and mechanosensory cells suggests similar functionality to that found in other organisms, although expression is observed in a more diffuse pattern in these locations.

Conclusion

Our work has shown expression of NMDA receptor subunit mRNA in areas of the rotifer *B. plicatilis* consistent with a canonical role in neurotransmission, the first time neurotransmitter receptor expression has been studied via DIG-labelled ISH in this phylum, further proving the amenability of this species as a tractable model. We have provided a robust phylogeny for these vital signalling components, casting much light on their evolution across the Bilateria, and have also provided the first insight into the structure and expression of NR2 subunits in the Lophotrochozoa as a whole. This work will represent an important starting point for investigation into the evolution of NR2 C termini and for consideration when making hypotheses as to the conservation of mechanisms of LTP and molecular memory across the Metazoa.

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Conflict of interest None.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215(3):403–410

Antonov I, Antonova I, Kandel ER, Hawkins RD (2003) Activity-dependent presynaptic facilitation and hebbian LTP are both required and interact during classical conditioning in *Aplysia*. Neuron 37(1):135–147

Barria A, Malinow R (2005) NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. Neuron 48(2):289–301

Bliss TV, Collingridge GL (1993) A synaptic model of memory: long-term potentiation in the hippocampus. Nature 361(6407): 31–39

Brockie PJ, Madsen DM, Zheng Y, Mellem J, Maricq AV (2001) Differential expression of glutamate receptor subunits in the nervous system of *Caenorhabditis elegans* and their regulation by the homeodomain protein UNC-42. J Neurosci 21(5):1510–1522

Casadio A, Martin KC, Giustetto M, Zhu H, Chen M, Bartsch D, Bailey CH, Kandel ER (1999) A transient, neuron-wide form of CREB-mediated long-term facilitation can be stabilized at specific synapses by local protein synthesis. Cell 99(2):221–237

Chen B, Roche K (2007) Regulation of NMDA receptors by phosphorylation. Neuropharmacol 53(3):362–368

Chiu J, DeSalle R, Lam HM, Meisel L, Coruzzi G (1999) Molecular evolution of glutamate receptors: a primitive signaling mechanism that existed before plants and animals diverged. Mol Biol Evol 16(6):826–838

Clamp M, Cuff J, Searle SM, Barton GJ (2004) The Jalview Java alignment editor. Bioinformatics 20(3):426–427

Flicek P, Amode MR, Barrell D, Beal K, Brent S, Carvalho-Silva D, Clapham P, Coates G, Fairley S, Fitzgerald S, Gil L et al (2012) Ensembl 2012. Nucleic Acids Res 40(D1):D84–D90

Flores-Soto ME, Chaparro-Huerta V, Escoto-Delgadillo M, Vazquez-Valls E, Gonzalez-Castaneda RE, Beas-Zarate C (2012) Structure and function of NMDA-type glutamate receptor subunits. Neurologia 27(5):301–310

Furukawa H, Singh SK, Mancusso R, Gouaux E (2005) Subunit arrangement and function in NMDA receptors. Nature 438(7065):185–192

Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A (2003) ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res 31(13):3784–3788

Glanzman DL (2007) Simple minds: the neurobiology of invertebrate learning and memory. In: North G, Greenspan RJ (eds) Invertebrate neurobiology. Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Glanzman DL (2010) Common mechanisms of synaptic plasticity in vertebrates and invertebrates. Curr Biol 20(1):R31–R36

Goto H, Watanabe K, Araragi N, Kageyama R, Tanaka K, Kuroki Y, Toyoda A, Hattori M, Sakaki Y, Fujiyama A, Fukumaki Y, Shibata H (2009) The identification and functional implications of human-specific "fixed" amino acid substitutions in the glutamate receptor family. BMC Evol Biol 9(1):224

Grey K, Moss B, Burrell B (2009) Molecular identification and expression of the NMDA receptor NR1 subunit in the leech. Invert Neurosci 9(1):11–20

Ha TJ, Kohn AB, Bobkova YV, Moroz LL (2006) Molecular characterization of NMDA-like receptors in *Aplysia* and *Lymnaea*: relevance to memory mechanisms. Biol Bull 210:255–270

Huang X, Madan A (1999) CAP3: a DNA sequence assembly program. Genome Res 9(9):868–877

Huelsenbeck JP, Ronquist F (2001) MRBAYES: bayesian inference of phylogenetic trees. Bioinformatics 17(8):754–755

Hulo N, Bairoch A, Bulliard V, Cerutti L, Cuche BA, de Castro E, Lachaize C, Langendijk-Genevaux PS, Sigrist CJ (2008) The 20 years of prosite. Nucleic Acids Res 36(Database issue): D245–D249

Husi H, Grant S (2001) Isolation of 2000-kDa complexes of N-methyl-D-aspartate receptor and postsynaptic density 95 from mouse brain. J Neurochem 77(1):281–291

Ito E, Kobayashi S, Kojima S, Sadamoto H, Hatakeyama D (1999) Associative learning in the pond snail *Lymnaea stagnalis*. Zoolog Sci 16:711–723

Izquierdo I (1991) Role of NMDA receptors in memory. Trends Pharmacol Sci 12:128–129

Kandel ER (1976) Cellular basis of behavior: an introduction to behavioral neurobiology. W.H. Freeman, San Francisco

Kandel ER (2001) The molecular biology of memory storage: a dialogue between genes and synapses. Science 294:1030–1038

Katoh K, Misawa K, Kuma K, Miyata T (2002) MAFFT: a novel method for rapid multiple sequence alignment based on fast fourier transform. Nucleic Acids Res 30(14):3059–3066

Kemenes G, Benjamin PR (2009) *Lymnaea*. Curr Biol 19(1):R9–R11

Kotikova EA (1998) Catecholaminergic neurons in the brain of rotifers. Hydrobiologia 387:135–140

Leonard A, Lim I, Hemsworth D, Horne M, Hell J (1999) Calcium/calmodulin-dependent protein kinase II is associated with the N-methyl-D-aspartate receptor. Proc Natl Acad Sci USA 96:3239–3244

Liu L, Wong T, Pozza M, Lingenhoehl K, Wang Y, Sheng M, Auberson Y, Wang Y (2004) Role of NMDA receptor subtypes in governing the direction of hippocampal synaptic plasticity. Science 304:1021–1024

Liu Y, Wong T, Aarts M, Rooyakkers A, Liu L, Lai T, Wu D, Lu J, Tymianski M, Craig A, Wang Y (2007) NMDA receptor subunits have differential roles in mediating excitotoxic neuronal death both in vitro and in vivo. J Neurosci 27:2846–2857

Lopez-Colome A, Lee-Rivera I, Zarain-Herzberg A (2004) Novel features of avian NMDA receptors: NR1 splice variant expression profile in the retina. Invest Ophthalmol Vis Sci 45(5), abstract 663

Low CM, Wee KS (2010) New insights into the not-so-new NR3 subunits of N-methyl-D-aspartate receptor: localization, structure, and function. Mol Pharmacol 78(1):1–11

Malenka RC, Bear MF (2004) LTP and LTD: an embarrassment of riches. Neuron 44(1):5–21

Martin KC, Casadio A, Zhu H, Yaping E, Rose JC, Chen M, Bailey CH, Kandel ER (1997) Synapse-specific, long-term facilitation of *Aplysia* sensory to motor synapses: a function for local protein synthesis in memory storage. Cell 91(7):927–938

Moriyoshi K, Masu M, Ishii T, Shigemoto R, Mizuno N, Nakanishi S (1991) Molecular cloning and characterization of the rat NMDA receptor. Nature 354(6348):31–37

Moroz LL, Edwards JR, Puthanveettil SV, Kohn AB, Ha T, Heyland A, Knudsen B, Sahni A, Yu F, Liu L, Jezzini S, Lovell P, Iannucculli W, Chen M, Nguyen T, Sheng H, Shaw R, Kalachikov S, Panchin YV, Farmerie W, Russo JJ, Ju J, Kandel ER (2006) Neuronal transcriptome of *Aplysia*: neuronal compartments and circuitry. Cell 127(7):1453–1467

Osborne P, Dearden PK (2005) Non-radioactive in-situ hybridisation to honeybee embryos and ovaries. Apidologie 36(1):113–118

Pedreira ME, Perez-Cuesta LM, Maldonado H (2002) Reactivation and reconsolidation of long-term memory in the crab *Chasmagnathus*: protein synthesis requirement and mediation by NMDA-type glutamatergic receptors. J Neurosci 22(18):8305–8311

Pierobon P, Sogliano C, Minei R, Tino A, Porcu P, Marino G, Tortiglione C, Concas A (2004) Putative NMDA receptors in *Hydra*: a biochemical and functional study. Eur J Neurosci 20(10):2598–2604

Rambaldi D, Ciccarelli FD (2009) FancyGene: dynamic visualization of gene structures and protein domain architectures on genomic loci. Bioinformatics 25(17):2281–2282

Rosenegger D, Lukowiak K (2010) The participation of NMDA receptors, PKC, and MAPK in the formation of memory following operant conditioning in *Lymnaea*. Mol Brain 3(1):24

Ryan T, Emes R, Grant S, Komiyama N (2008) Evolution of NMDA receptor cytoplasmic interaction domains: implications for organisation of synaptic signalling complexes. BMC Neurosci 9(1):6

Scappaticci AA, Jacques R, Carroll JE, Hufnagel LA, Kass-Simon G (2004) Immunocytochemical evidence for an NMDA1 receptor subunit in dissociated cells of *Hydra vulgaris*. Cell Tissue Res 316(2):263–270

Sigrist CJ, Cerutti L, de Castro E, Langendijk-Genevaux PS, Bulliard V, Bairoch A, Hulo N (2010) prosite, a protein domain database for functional characterization and annotation. Nucleic Acids Res 38(1):161–166

Smith J, Cridge A, Dearden P (2010) Germ cell specification and ovary structure in the rotifer *Brachionus plicatilis*. Evodevo 1(1):5

Solntseva S, Nikitin V (2008) Serotonin and NMDA glutamate receptor antagonists selectively impair the reactivation of associative memory in the common snail. Neurosci Behav Physiol 38(7):687–693

Sprengel R, Suchanek B, Amico C, Brusa R, Burnashev N, Rozov A, Hvalby O, Jensen V, Paulsen O, Andersen P, Kim J, Thompson R, Sun W, Webster L, Grant S, Eilers J, Konnerth A, Li J, McNamara J, Seeburg P (1998) Importance of the intracellular domain of NR2 subunits for NMDA receptor function in vivo. Cell 92:279–289

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 28(10):2731–2739

Tezuka T, Umemori H, Akiyama T, Nakanishi S, Yamamoto T (1999) PSD-95 promotes Fyn-mediated tyrosine phosphorylation of the N-methyl-D-aspartate receptor subunit NR2A. Proc Natl Acad Sci USA 96(2):435–440

Traynelis SF, Wollmuth LP, McBain CJ, Menniti FS, Vance KM, Ogden KK, Hansen KB, Yuan H, Myers SJ, Dingledine R, Sibley D (2010) Glutamate receptor ion channels: structure, regulation, and function. Pharmacol Rev 62(3):405–496

Ultsch A, Schuster CM, Laube B, Betz H, Schmitt B (1993) Glutamate receptors of *Drosophila melanogaster*: primary structure of a putative NMDA receptor protein expressed in the head of the adult fly. FEBS Lett 324(2):171–177

Wan H, Mackay B, Iqbal H, Naskar S, Kemenes G (2010) Delayed intrinsic activation of an NMDA-independent CaM-kinase II in a critical time window is necessary for late consolidation of an associative memory. J Neurosci 30:56–63

Whelan S, Goldman N (2001) A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. Mol Biol Evol 18(5):691–699

Wyszynski M, Lin J, Rao A, Nigh E, Beggs A, Craig A, Sheng M (1997) Competitive binding of alpha-actinin and calmodulin to the NMDA receptor. Nature 385:439–442

Xia S, Miyashita T, Fu T, Lin W, Wu C, Pyzocha L, Lin I, Saitoe M, Tully T, Chiang A (2005) NMDA receptors mediate olfactory learning and memory in *Drosophila*. Curr Biol 15:603–615