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Glutamate receptors of *Drosophila melanogaster*

Primary structure of a putative NMDA receptor protein expressed in the head of the adult fly

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The NMDA subtype of ionotropic glutamate receptors has been implicated in the activity-dependent modification of synaptic efficacy in the mammalian brain Here we describe a cDNA isolated from *Drosophila melanogaster* which encodes a putative invertebrate NMDA receptor protein (DNMDAR-I). The deduced amino acid sequence of DNMDAR-I displays 46% amino acid identity to the rat NMDAR1 polypeptide and shows significant homology (16–23%) to other vertebrate and invertebrate glutamate receptor proteins. The DNMDAR-I gene maps to position 83AB of chromosome 3R and is highly expressed in the head of adult flies Our data indicate that the NMDA subtype of glutamate receptors evolved early during phylogeny and suggest the existence of activity-dependent synaptic plasticity in the insect brain.

Glutamate receptor; NMDA receptor homologue; *Drosophila melanogaster*

1. INTRODUCTION

Glutamate is the major excitatory neurotransmitter in the vertebrate central nervous system. This amino acid activates a diverse set of postsynaptic receptors which have been implicated in neuronal development, synaptic plasticity, memory formation and excitotoxicity [1–4]. The glutamate receptors are currently classified into metabotropic and ionotropic subtypes [5]. The ionotropic receptors harbour an intrinsic cation channel and are named after their selective agonists, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate, and *N*-methyl-D-aspartate (NMDA) [5]. NMDA receptors are highly permeable to Ca^{2+} ions and display a voltage-dependent block by Mg^{2+} ions [6]. These properties provide the molecular substrate for activity-dependent adaptation mechanisms including long-term potentiation and heterosynaptic depression [6]. The elucidation of the structure and function of NMDA receptors, therefore, constitutes a major goal in the rapidly expanding field of molecular neurobiology.

Expression cloning and homology screening strategies have allowed the isolation of cDNAs encoding several mammalian AMPA, kainate and NMDA receptor proteins (reviewed in [7]). More recently, glutamate receptor cDNAs have also been cloned from invertebrates [8–10]. In insects and crustaceans, glutamate serves as the major excitatory transmitter at the neuromuscular junction, but is also used in the central nervous system [11–13]. For a better understanding of the role of glutamate receptors in synaptogenesis and plasticity, we have started a systematic screening for glutamate receptors of *Drosophila melanogaster*. This organism is particularly suited for the genetic dissection of developmental and behavioural mechanisms and easily amenable to transgenic approaches. Previous work from our laboratory has lead to the identification of cDNAs encoding a kainate receptor protein (DGluR-I) expressed in the central nervous system [10] and a novel ionotropic glutamate receptor subtype (DGluR-II) selectively expressed in developing muscle [8]. Here we report the isolation of a cDNA encoding a *Drosophila* receptor protein (DNMDAR-I) that displays a high sequence identity to the vertebrate NMDAR1 receptor subunit [14]. DNMDAR-I transcripts are abundant in the head of the adult fly.

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Abbreviations. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; GluR, glutamate receptor, NMDA, *N*-methyl-D-aspartate, PCR, polymerase chain reaction.

The first two authors have contributed equally to this work.

2. MATERIALS AND METHODS

2.1. *Molecular cloning of DMDAR-I cDNAs*

A genomic DNMDAR-I fragment, designated gDNMDAR-I, was isolated by PCR-mediated DNA amplification [15] using two degenerate oligonucleotide primers 5'- CAA GAA TCC ATC GA(G,T,A) TT(T,C) TC(G,T,A) AA(G,A) CC(C,G) TT -3' (upstream primer, sense orientation) and 5'- CAA GAA TTC AAG GCG GC(C,T,G) AG(G,A) TT(G,T) GT(G,A) TA -3' (downstream primer, antisense orientation) designed after the peptide sequences I(D,E)FSKPF (residues 550 to 556 in DGluR-I [10], residues 527 to 533 in NMDAR1 [14]) and YTANLAAF (residues 696 to 703 in DGluR-I [10]; residues 647 to 654 in NMDAR1 [14]). Amplification reactions contained, in a total volume of 100 μl, 1 μg of genomic *Drosophila* DNA, 2.25 U Taq DNA polymerase (Northumbria Biologicals Limited), 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 3% dimethylsulfoxide, and 0.2 mM each of dATP, dCTP, dGTP, dTTP. After denaturation at 94°C for 3 min, 35 cycles (94°C, 30 s; 55°C, 20 s; 72°C, 20 s) were carried out using a Perkin-Elmer Cetus thermal cycler. This resulted in the amplification of gDNMDAR-I (≈ 600 bp) along with a number of additional fragments.

A *Drosophila* λ ZAP head library was screened with the ^{32}P-labelled gDNMDAR-I fragment; hybridization was performed at 57°C as described [8]. Two classes of partial cDNA clones, among them N3.4A and N7A, were thus isolated and subcloned into a Bluescript vector for DNA sequence analysis [16]. Clone N7A contained the entire 3'-untranslated region of the DNMDAR-I cDNA and coding sequence for the C-terminal half of the DNMDAR-I protein up to an *Eco*RI recognition site of the cDNA. A restriction fragment of clone N3.4A that extended clone N7A in 5'-direction by ≈ 900 bp was radioactively labelled and used to isolate, from a λgt11 *Drosophila* head cDNA library, clone N13C. This clone contained both coding sequence for the N-terminal half of DNMDAR-I and 148 bp of 5'-untranslated sequence. The full-length DNMDAR-I cDNA was constructed from clones N7A and N13C using standard procedures. Sequences were analysed using the HUSAR program package of the Deutsches Krebsforschungszentrum, Heidelberg.

2.2. *Northern blot analysis*

Poly(A)$^+$RNA was isolated from *Drosophila* heads and whole fly preparations at different developmental stages according to Cathala et al. [17], enriched by oligo(dT) chromatography (Fast Track mRNA Isolation Kit, Invitrogen), and separated in a 1% agarose gel containing 6.3% formaldehyde (10 μg per lane). After transfer onto Hybond-N membrane by capillary blotting, the filter was stained with methylene blue and hybridized to the ^{32}P-labelled DNMDAR-I clone N7A (specific activity ≈ 1.5×10^9 cpm/μg) as described [8].

2.3. *In situ hybridization to polytene chromosomes*

Hybridization of a digoxygenin-labelled DNMDAR-I probe to salivary gland polytene chromosomes was performed as described in Sawruk et al. [18].

2.4. *Expression of DNMDAR-I in* Xenopus *oocytes*

Stage VI *Xenopus* oocytes were injected with DNMDAR-I cRNA generated by in vitro transcription of the full-length DNMDAR-I cDNA cloned in pBluescript II SK(-) (Stratagene), and agonist-induced membrane currents were recorded under voltage clamp conditions as described previously [19,20].

3. RESULTS

3.1. *Molecular cloning of DNMDAR-I cDNAs*

A genomic fragment, designated gDNMDAR-I (Fig. 1A), was isolated by PCR-mediated DNA amplification. Degenerate oligonucleotide primers for this reaction were designed from conserved regions of amino acid sequence homology between rat NMDAR1 [14] and *Drosophila* DGluRI [10]. Several cDNA clones were isolated from *Drosophila* head cDNA libraries using this gDNMDAR-I PCR-fragment as initial hybridization probe. The full-length DNMDAR-I cDNA covered by the overlapping clones N7A and N13C (Fig. 1A), has a size of 4099 bp and contains an open reading frame of 2991 bp that encodes a 997 amino acid protein (Fig. 1B). The 3' untranslated region of 960 bp harbours a polyadenylation recognition signal and several 'ATTTA' motifs, the latter of which are believed to enhance mRNA instability [21]. Polyadenylate stretches are found in several clones downstream of the last nucleotide shown in Fig. 1 (not shown).

3.2. *The primary structures of DNMDAR-I and NMDAR1 are closely related*

The deduced primary structure of the DNMDAR-I protein displays features characteristic of ionotropic glutamate receptor subunits including: a signal peptide (amino acids −26 to −1 in Fig. 1B), a large hydrophilic N-terminal region, and four putative membrane spanning segments in the C-terminal half of the polypeptide. Several potential sites for N-linked glycosylation are found in the putative extracellular N-terminal region, and potential phosphorylation sites for protein kinases are located in the putative intracellular loop between the third and fourth transmembrane spanning region (Fig. 1B and not shown).

The amino acid sequence identity between the deduced *Drosophila* DNMDAR-I and the rat NMDAR1 proteins is remarkable (Fig. 2). Nearly 46% of all amino acids are conserved between DNMDAR-I and the NMDAR1 receptor subunit from rat (Table I). The amino acid conservation is particularly high in the C-terminal halves (64%) which include the previously defined 'core' region of GluR proteins (see [8]). By contrast, the DNMDAR-I subunit is only distantly related to the mammalian NMDA receptor NR2/ε subunit family (23–25% identity to NR2A-C and ε4; see [22,23]) and the mammalian AMPA receptor subunits GluR1 to GluR4 (21–23%; see [24,25]). Similarly, a low homology

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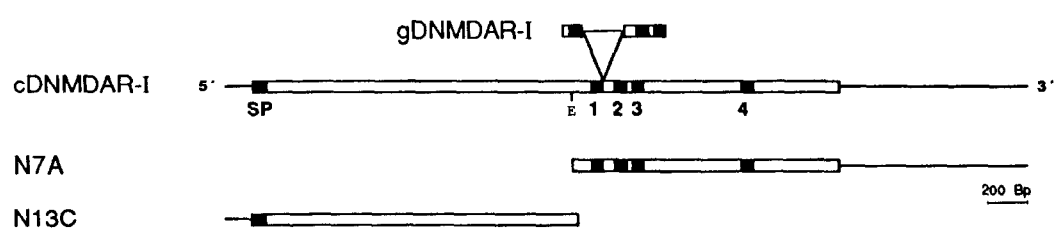
Fig. 1. (A) Schematic description of DNMDAR-I cDNA clones N7A and N13C. The coding region is symbolized by an open box. The signal peptide (SP), the putative transmembrane segments (1–4) and the recognition site for *Eco*RI (E) are indicated. The genomic PCR fragment (gDNMDAR-I) containing intronic sequence (thin line), is shown above the cDNA. (B) Nucleotide and deduced amino acid sequences of the DNMDAR-I cDNA. Bp, basepairs. The transmembrane segments are boxed; the signal peptide, the polyadenylation signal (AATAA) and several mRNA instability signals (ATTTA, see [21]) are underlined. Black squares mark protein kinase C consensus phosphorylation sites, and circled residues indicate potential sites of N-glycosylation. The DNMDAR-I sequence data will appear in the Genebank/EMBL Database under accession number X71790.



B

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 121 TCCCCCAGAAACTATAGACTTCCGCATCATGGCTATGGCTGAGTTTGTTTTTTGCCGGCCATTGTTTGGGCTCGCCATTGTTCTCCTGGTGGCGCCAATTGATGCCGCCCAGAGGCACAC
 -26                                M A M A E F V F C R P L F G L A I V L L V A P I D A A Q R H T

 241 TGCCTCGGATAATCCCTCGACGTACAACATTGGCGGCGTACTGAGCAATTCCGACAGCGAGGAGCACTTTAGCACCACAATAAAGCACCTCAACTTCGATCAGCAGTATGTGCCGCGGAA
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 361 GGTCACCTACTACGACAAAACCATCCGCATGGACAAGAATCCCATCAAGACGGTCTTCAATGTGTGCGACAAGCTCATCGAGAATCGGGTGTACGCCGTAGTCGTTTCGCATGAGCAAAC
  46 V T Y Y D K T I R M D K N P I K T V F N V C D K L I E N R V Y A V V V S H E Q T

 481 CTCAGGTGATTTGTCGCCGGCGGCCGTGAGTTATACAAGCGGATTCTATTCAATTCCCGTCATTGGCATCTCCTCGCGGGACGCGGCCTTCTCCGACAAGAACATCCACGTCTCCTTCCT
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 601 GCGGACGGTGCCGCCGTACTACCACCAGGCGGATGTCTGGCTGGAGATGCTCAGCCACTTTGCCTACACGAAGGTAATCATCATCCACAGCTCCGACACGGATGGCCGGGCCATCCTGGG
 126 R T V P P Y Y H Q A D V W L E M L S H F A Y T K V I I I H S S D T D G R A I L G

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 166 R F Q T T S Q T Y Y D D V D V R A T V E L I V E F E P K L E S F T E H L I D M K

 841 GACCGCCCAGTCCAGGGTCTACCTCATGTACGCGAGCACGGAAGACGCTCAAGTAATCTTCCGCGATGCCGGGGAGTACAACATGACCGGTGAGGGTCACGTGTGGATTGTAACGGAGCA
 206 T A Q S R V Y L M Y A S T E D A Q V I F R D A G E Y (N) M T G E G H V W I V T E Q

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 246 A L F S N N T P D G V L G L Q L E H A H S D K G H I R D S V Y Y V L A S A I K E M

1081 GATTTCCAACGAGACCATTGCCGAGGCTCCCAAGGATTGCGGCGATTCGGCCGTTAACTGGGAATCGGGCAAGCGACTGTTTCAGTACCTCAAAAGCCGTAATATCACCGGCGAAACGGG
 286 I S (N) E T I A E A P K D C G D S A V N W E S G K R L F Q Y L K S R (N) I T G E T G

1201 TCAGGTGGCCTTCGATGACAATGGTGACCGAATCTACGCCGGCTACGATGTGATCAACATTCGGGAGCAGCAGAAGAAGCACGTGGTTGGAAAGTTCAGCTACGACAGCATGCGGGCCAA
 326 Q V A F D D N G D R I Y A G Y D V I N I R E Q Q K K H V V G K F S Y D S M R A K

1321 AATGCGGATGAGGATCAACGACAGCGAGATCATTTGGCCGGGAAAGCAGCGCCGCAAGCCCGAGGGCATCATGATTCCCACCCACCTGAGGCTGCTCACCATCGAGGAGAAGCCCTTCGT
 366 M R M R I (N) D S E I I W P G K Q R R K P E G I M I P T H L R L L T I E E K P F V

1441 CTACGTGCGGCGCATGGGCGACGATGAGTTCCGCTGTGAGCCGGATGAGCGTCCGTGTCCGCTGTTCAACAACTCGGATGCCACGGCCAACGAGTTCTGCTGCCGCGGCTACTGCATCGA
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2041 CAGCGGGATCGGCGAGGGCACGCCCCGGAGCTTCTCCGCCCGGGTGCTGGGCATGGTCTGGGCCGGATTTGCTATGATCATCGTTGCCTCGTACACCGCCAACCTGGCCGCCTTCCTCGT
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3001 TGGCCAGAGTCCGAAGGTGATGGCTGCCAACCAGCCGGGAATGCCCATGCCCATGCTGGGCAAGACACGGCCCCAACAGAGTGTCCTGCCACCTCGCTACTCGCCCGGATACACCAGCGA
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3121 CGTGTCGCATCTGGTCGTCTAAGTTAACATTTGCCAAAGCAGCTAGAACTTAGAATCAAGTATTATCATTGCTCAGCCCATGGCGATAGCCAGCTAAAGAGAATTTAACTGTAGCAAAT
 966 V S H L V V *

3241 TGATTGTTATCGCTGAATGGAATGGCCAGGACCAAAGAAAAAAGGACGATTAGCGTAATCTCACACAACATTTATGTCATTTTTGCATTGTTCCGTCGTTGGTCCTGTTATATCAGAGGT
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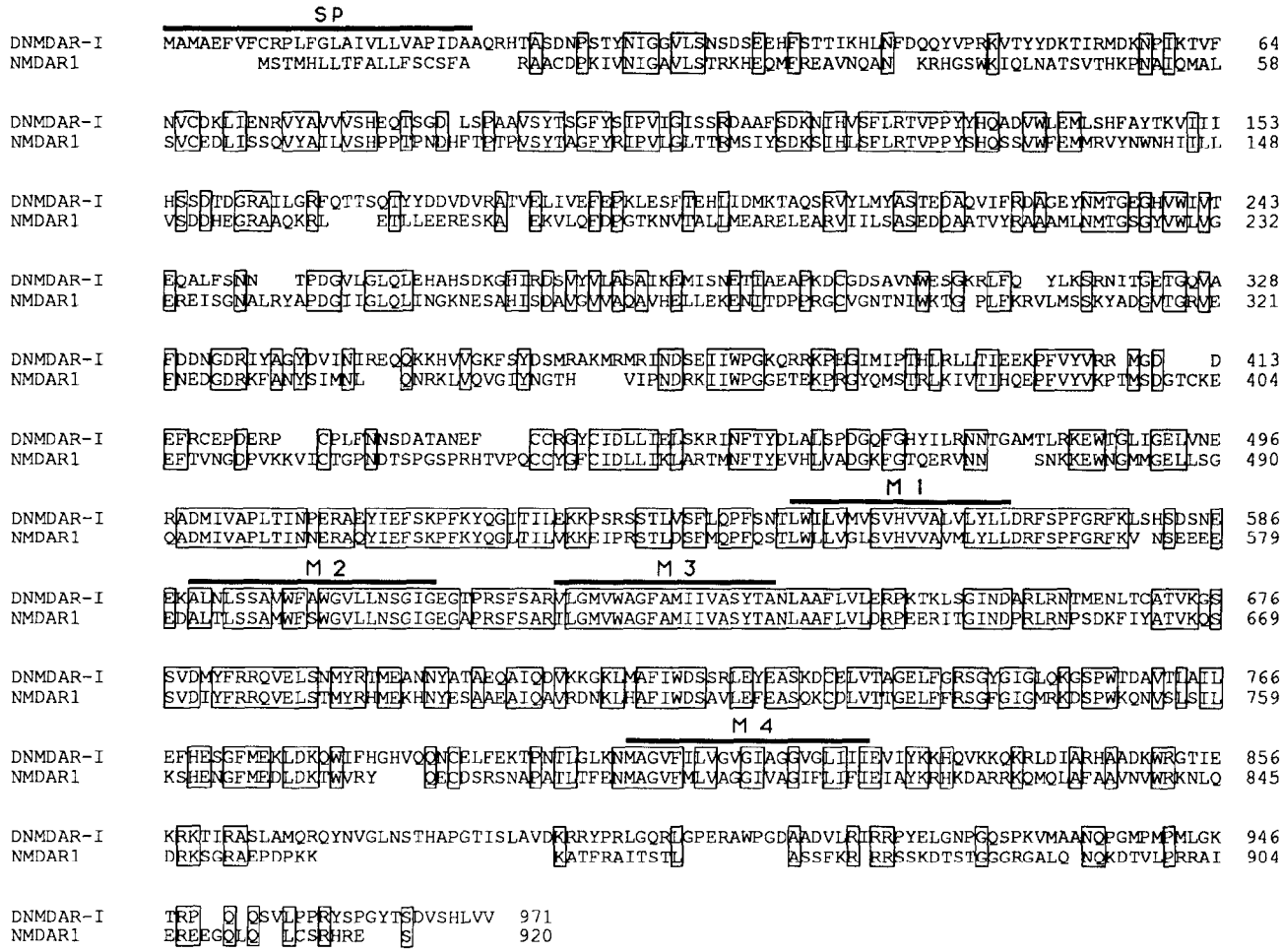

Fig. 2. Comparison of the primary structures of DNMDAR-I with the rat NMDAR1 subunit. Gaps were introduced to optimize the alignment; protein sequences are numbered starting with their predicted mature N-termini. The putative signal sequence (SP) and the putative membrane-spanning segments (M1 through M4) are denoted by bars. Conserved amino acid residues are boxed.

is also found to the rat kainate receptor and kainate binding proteins GluR5–7 [26–28] as well as KA-1 and -2 [29,30] (see Table I). The *Drosophila* subunits DGluR-I [10] and DGluR-II [8] and a putative glutamate receptor subunit from *Lymnaea stagnalis* [9] are 16–23% identical to DNMDAR-I (Table I).

3.3. *Developmental expression of DNMDAR-I*

The accumulation of DNMDAR-I mRNA during *Drosophila* development was analyzed by Northern blot analysis (Fig. 3). At early embryonic stages, DNMDAR-I transcripts could not be detected; a prominent 5-kb mRNA appeared only in late embryos. During different larval stages, the level of DNMDAR-I transcripts was low. After some increase during early pupal development, strong DNMDAR-I gene expression was seen in late pupae. Transcript levels comparable to those seen in late embryos were found when the poly(A)$^+$ RNA from adult flies was analyzed. Poly(A)$^+$ RNA extracted from adult heads, however, gave a

Table I

Amino acid sequences identities (in %) between DNMDAR-I and different glutamate receptor proteins from rat, *Drosophila* and *Lymnaea*

	NMDAR1	GluR1 to -4	GluR5 to -7	KA-1/KA-2	DGluR-I	DGluR-II	Lymnaea
DNMDAR-I	46	21–23	22	19/22	19	16	23

Pairwise comparison of mature glutamate receptor subunits was performed employing the algorithm of Needleman and Wunsch [38]. Sequences were taken from the following references: rat GluR1 to -4 [24,25]; rat GluR5 to -7 [26–28]; rat KA-1 and -2 [29,30]; *Drosophila* DGluR-I and -II [8,10], *Lymnaea stagnalis* glutamate receptor [9].

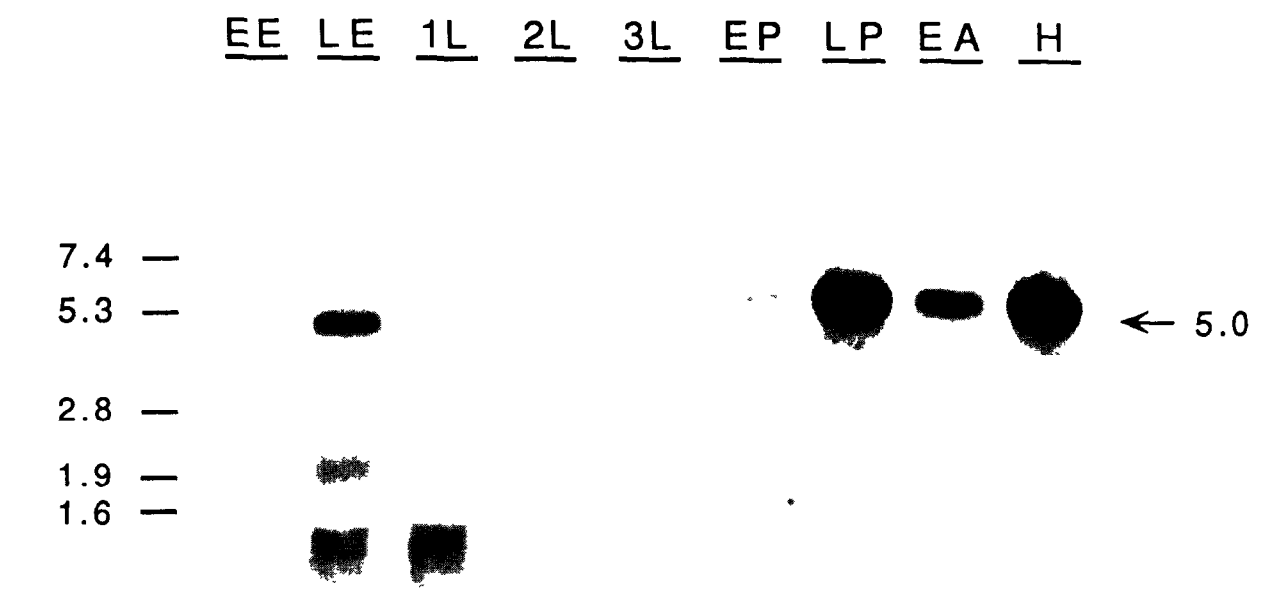

Fig. 3. Northern blot analysis of poly(A)$^+$RNA (10 μg per lane), isolated from various *Drosophila* developmental stages and *Drosophila* heads: EE, early embryo, 0–4 h; LE, late embryo, 4–22 h; 1L, first instar larva; 2L, second instar larva; 3L, third instar larva; EP, early pupa, LP, late pupa; EA, early adult fly, 1–2 days old; H, mRNA from *Drosophila* heads. The ^{32}P-labelled clone N7A was used as a hybridization probe. RNA size markers (in kb) are given on the left. An arrow indicates the 5-kb DNMDAR-I transcript.

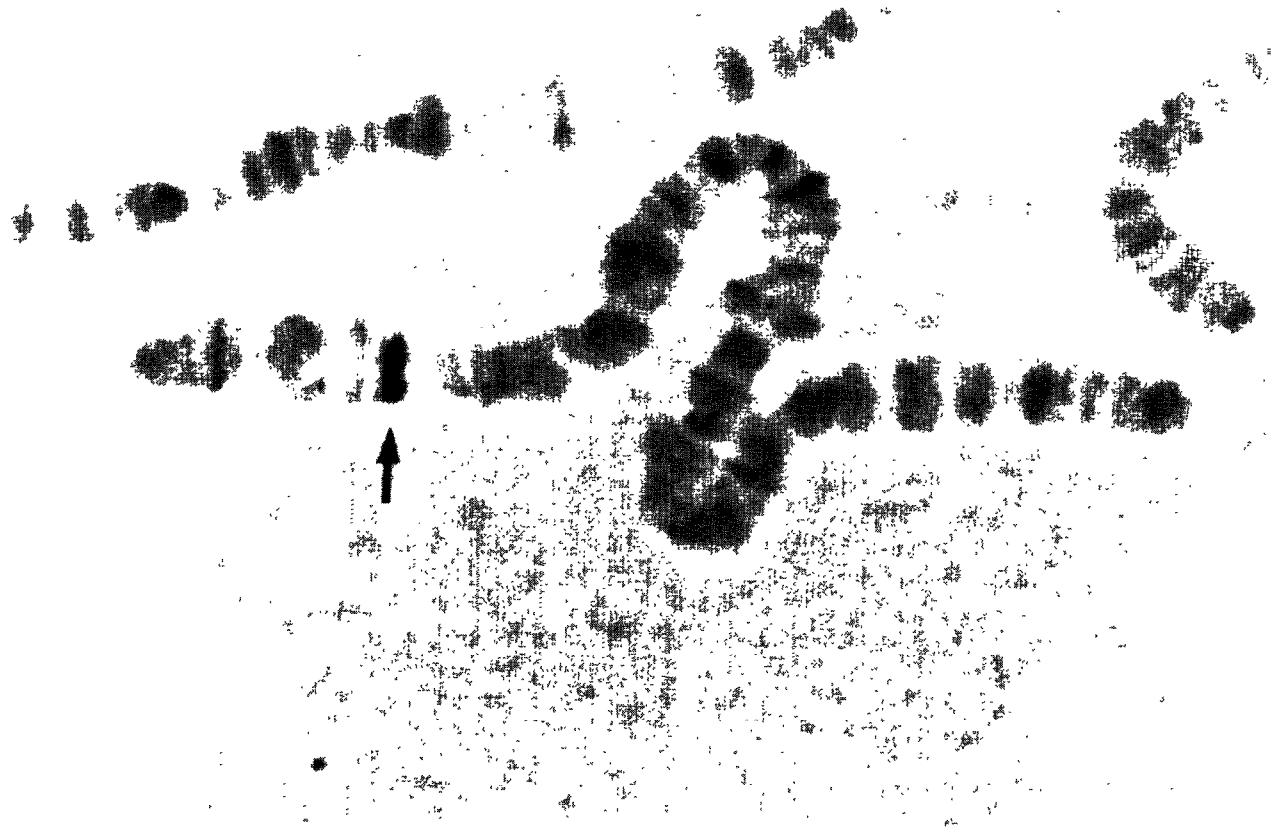

Fig. 4. In situ hybridization to salivary gland polytene chromosomes. The digoxygenin-labelled DNMDAR-I clone N7A generates a hybridization signal at the 83AB region of chromosome 3R (arrow).

much stronger signal. This indicates a preferential localization of DNMDAR-I transcripts in the *Drosophila* head.

3.4. *Cytogenetical localisation of the DNMDAR-I gene*

Polytene chromosomes from salivary glands of Canton S third instar larvae were hybridised with the digoxygenin-labelled cDNA clone N7A (see Fig. 1A) as a probe. A single hybridization signal was observed at position 83AB on the right arm of the third chromosome near the centromer (Fig. 4).

3.5. *Heterologous expression in* Xenopus *oocytes*

Despite numerous attempts to functionally characterize the DNMDAR-I protein by ectopic expression in *Xenopus* oocytes, a small current response (20–40 nA) to the NMDA receptor co-agonist glycine [5,6] was only obtained with two out of ten batches of DNMDAR-I cRNA-injected oocytes (data not shown). This response, though saturable and evoked by low glycine concentrations (EC_{50} = 60 μM), was insensitive to both the NMDA receptor antagonist 7-chlorokynurenate and the inhibitory glycine receptor antagonist strychnine. Moreover, no potentiation was seen upon co-application of glycine with the different glutamate receptor agonists: glutamate, NMDA, aspartate, AMPA, quisqualate, homocysteate (all at 1 mM), and kainate (up to 10 mM). Neither did they produce detectable currents, when applied alone. Thus, additional subunits are likely to be required for the assembly of a functional *Drosophila* NMDA receptor channel.

4. DISCUSSION

The data reported here unravel further molecular diversity of ionotropic glutamate receptors in *Drosophila*. In addition to the previously described neural kainate receptor protein DGluR-I [10] and the muscle-specific subunit DGluR-II [8], a novel type of *Drosophila* glutamate receptor has now been identified. The primary structure of the DNMDAR-I protein is remarkably homologous to the mammalian NMDAR1 polypeptide. In contrast, pairwise comparisons of DNMDAR-I with the rat NR2 (also designated ε; for nomenclature see [31]) class of NMDA receptor subunits as well as with AMPA and kainate receptor proteins disclosed much lower amino acid identities. Therefore, DNMDAR-I can be classified, on a structural basis, as an invertebrate homolog of the mammalian NMDA receptor subunit NMDAR1. Unfortunately, this conclusion could not be confirmed in our electrophysiological studies. However, in a few recording experiments small responses were observed after application of the NMDA receptor co-agonist glycine to DNMDAR-I cRNA-injected *Xenopus* oocytes. We thus assume that *Drosophila* homologs of the ε subunit family of rat NMDA receptors [22,23] may be required for the assembly of functional NMDA-operated ion channels. Importantly, a recent report on the electrophysiological characterization of an arthropod NMDA receptor [32] lends further credit to our assumption that DNMDAR-I is indeed a subunit of a *Drosophila* NMDA receptor.

The DNMDAR-I gene is located on the third *Drosophila* chromosome at position 83AB. Its expression is highly regulated during development, with transcription first being seen in late embryos, i.e. when the larval nervous system is formed [33]. Notably, transcriptional activity of the DNMDAR-I gene is much lower at this stage than that of the neurally expressed DGluR-I [10] and the muscle-specific DGluR-II [8] genes, suggesting that DNMDAR-I corresponds to a comparatively late differentiation marker in *Drosophila* neurogenesis. This view is supported further by the presence of high levels of DNMDAR-I mRNA in late pupae at a stage when the adult central nervous system develops. DNMDAR-I gene expression continues in adulthood, suggesting that synthesis of this receptor protein accompanies the maturation of the nervous system and may be required for adaptation and plasticity processes in the brain of the adult fly. Consistent with this interpretation, transcript levels were highest in poly(A)$^+$RNA preparations from adult heads. Considering the well-documented role of mammalian NMDA receptors in activity-dependent synaptic modulation and a high conservation of the putative channel-forming second transmembrane segment between the DNMDAR-I and the rodent NMDAR1 proteins, we speculate that similar mechanisms of Ca^{2+}-mediated long-term potentiation and depression may operate in both insect and mammalian brains. Thus, genetic approaches in *Drosophila* may help to identify further components of the glutamate-induced learning and memory cascade.

The DNA sequence data presented here imply that the different subtypes of glutamate receptors including those of the NMDA subtype evolved before the separation of *Chordata* and *Arthropoda*. In other words, ionotropic glutamate receptor specialization may have occurred early during phylogeny to allow the formation of different types of excitatory synapses. On the other hand, the different glutamate receptor proteins do not display significant sequence similarity to subunits of other ligand-gated ion channels, including the nicotinic acetylcholine, $GABA_A$ and glycine receptors, in both *Drosophila* [34,35] and mammals [36,37]. This suggests that convergent rather than divergent evolution created similarities in the putative subunit transmembrane topology seen with all synaptic ligand-gated ion channel proteins known.

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