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RESEARCH ARTICLE

Identification and characterization of the NMDA receptor and its role in regulating reproduction in the cockroach *Diploptera punctata*

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ABSTRACT

The NMDA receptor (NMDAR) plays important roles in excitatory neurotransmission and in the regulation of reproduction in mammals. NMDAR in insects comprises two subunits, NR1 and NR2. In this study, we identified two NR1 paralogs and eleven NR2 alternatively spliced variants in the cockroach *Diploptera punctata*. This is the first report of NR1 paralogs in insects. The tissue distributions and expression profiles of *DpNR1A*, *DpNR1B* and *DpNR2* in different tissues were also investigated. Previous studies have demonstrated NMDA-stimulated biosynthesis of juvenile hormone (JH) in the corpora allata through the influx of extracellular Ca^{2+} in *Diploptera punctata*. However, our data show that the transcript levels of *DpNR1A*, *DpNR1B* and *DpNR2* were low in the corpora allata. MK-801, a high-affinity antagonist of NMDAR, did not show any effect on JH biosynthesis *in vitro*. In addition, neither partial knockdown of *DpNR2* nor *in vivo* treatment with a physiologically relevant dose of MK-801 resulted in any significant change in JH biosynthesis or basal oocyte growth. Injection of animals with a high dose of MK-801 (30 µg per animal per injection), which paralyzed the animals for 4–5 h, resulted in a significant decrease in JH biosynthesis on days 4 and 5. However, the reproductive events during the first gonadotrophic cycle in female *D. punctata* were unaffected. Thus, NMDAR does not appear to play important roles in the regulation of JH biosynthesis or mediate reproduction of female *D. punctata*.

KEY WORDS: NMDA receptor, JH biosynthesis, MK-801, Reproduction, *D. punctata*

INTRODUCTION

L-glutamate (Glu), a major excitatory amino acid transmitter, mediates diverse physiological functions in the vertebrate nervous system (Mahesh and Brann, 2005). The Glu receptors (GluRs) have been classified into three major subtypes: the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor, N-methyl-D-aspartate (NMDA) receptor and kainate receptor (Madden, 2002). The NMDA receptors (NMDARs) are distinguished from other ionotropic receptors by their unique properties, including selective agonists and antagonists, high Ca^{2+} permeability and voltage-dependent Mg^{2+} blockade (McBain and Mayer, 1994). The unique properties of NMDAR allow it to play key roles in excitatory neurotransmission and important neurological processes, including learning, memory and behavior (Mussig et al., 2010; Newcomer and Krystal, 2001; Xia et al., 2005).

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NMDAR in vertebrates is composed of two subunits, NR1 and NR2, and in some cases NR3 subunits (Madden, 2002). The NR1 subunit is essential for the basic channel activity of NMDAR, whereas the NR2 subunit contributes to enhance and modulate the receptor function (Sydow et al., 1996). Although much is known about the function of NMDAR in vertebrates, little information is available on NMDARs in insects. Thus far, two subunits, NR1 and NR2, have been identified in insects. The NR1 and NR2 subunits were previously reported to be distributed throughout the brain of *Drosophila melanogaster* and *Apis mellifera* (Wu et al., 2007; Xia et al., 2005; Zannat et al., 2006).

The importance of NMDAR in the regulation of reproduction of mammals is well known. NMDAR mediates reproduction through the regulation of pulse and surge gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) secretion (Maffucci et al., 2009; Mahesh and Brann, 2005). In insects, juvenile hormones (JHs) are key regulators of growth, development, metamorphosis, aging, caste differentiation and reproduction (Goodman and Granger, 2005; Hartfelder, 2000). As a result of the importance of JH in physiological processes, its biosynthesis is tightly regulated by many factors, including neuropeptides (allatostatins, allatotropins) (Stay and Tobe, 2007) and neurotransmitters (octopamine, dopamine and glutamate) (Granger et al., 1996; Pszczolkowski et al., 1999; Thompson et al., 1990). Chiang et al. (2002) demonstrated that JH biosynthesis by corpora allata (CA) of the cockroach, *Diploptera punctata*, is stimulated by an NMDA-induced influx of Ca^{2+} ions and this elevation was significantly reduced by NMDAR antagonists including Mg^{2+}, MK-801 or conantokin T. Furthermore, a *Drosophila* larval mutant for *NMDAR1* showed reduced mRNA levels of the gene encoding JH acid methyltransferase (JHAMT), a key regulatory enzyme of JH biosynthesis in this species (Huang et al., 2011). These studies suggest that NMDAR plays a role in the regulation of JH biosynthesis.

As a high-affinity antagonist of NMDARs, MK-801 has been used to study the function of NMDAR in both vertebrates and invertebrates (Rawls et al., 2009; Sircar et al., 1987; Troncoso and Maldonado, 2002). In addition to the inhibitory effect of MK-801 in NMDA-stimulated JH biosynthesis in *D. punctata*, MK-801 was found to influence ovarian development and vitellogenesis in the flesh fly *Neobellieria bullata* and the locust *Schistocerca gregaria* (Begum et al., 2004; Chiang et al., 2002). In *S. gregaria*, the inhibition of vitellogenesis was overcome by treatment with JH. A later study on the butterfly *Bicyclus anynana* and the cricket *Gryllus bimaculatus* showed that MK-801 affects JH biosynthesis *in vitro* and JH titres in both species, and subsequently regulates insect reproduction (Geister et al., 2008).

Diploptera punctata is a well-known model for studying the physiology of JH biosynthesis and regulation; in this animal, JH

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biosynthesis is high and stable, and the reproductive events correlate very well with rates of JH production (see review by Marchal et al., 2013a). In this study, we chose *D. punctata* as our model to determine the role of NMDAR in the regulation of JH biosynthesis and reproduction. We identified the genes encoding the subunits of NMDAR in *D. punctata*, and examined the expression of NMDAR in several tissues. In addition, we investigated the roles of NMDAR in the regulation of JH biosynthesis, vitellogenesis and oocyte growth *in vivo* using RNA interference (RNAi) and MK-801.

RESULTS

Identification of DpNR1 subunits

Two *DpNR1* were identified with open reading frames of 2871 bp (*DpNR1A*) and 2703 bp (*DpNR1B*). This was accomplished employing a degenerate PCR approach with adult brain cDNA. 5′-RACE and 3′-RACE experiments were performed to complete the sequences. The two *DpNR1* sequences were deposited in NCBI GenBank (accession numbers KJ747198 and KJ747199). Unlike the alternative splicing variants of NR1 genes in other insects, the differences between the two variants in *D. punctata* are distributed throughout the whole gene (Fig. 1) and may be the result of gene duplication. A phylogenetic tree of NR1 was constructed by maximum-likelihood methods (Fig. 2). Generally, the sequences of NR1 were grouped based on insect orders, and the two DpNR1s (Blattodea) cluster together with confidence. However, the relationship between orders is not well resolved, as indicated by low bootstrap values at deeper nodes. The lack of power to resolve these nodes could be explained by insufficient sampling among hemimetabolous insects.

Identification of DpNR2 subunits

DpNR2 undergoes alternative splicing, generating 11 different transcripts. Full-length cDNAs for all 11 variants have been isolated (GenBank accession numbers KJ747200 to KJ747210). In the 11 transcripts, there are two 5′ untranslated region, two insertions, and four different 3′ ends (Fig. 3A). The sequence of insertions, deletions and different 3′ ends are shown in Fig. 3B. All 11 *DpNR2* variants contain the domain structure, four hydrophobic regions (TM1–TM4) and two ligand binding domains. Amino acid sequence comparisons between DpNR2A-1 and NR2 subunits from *D. melanogaster*, *A. mellifera* and *A. aegypti* were determined (supplementary material Table S1). DpNR2A-1 shares the highest similarity with NR2 subunits from *A. mellifera*.

Expression of *DpNR1A*, *DpNR1B* and *DpNR2*

Previous studies of NMDAR in insects have focused on its function in brain (Wu et al., 2007; Xia et al., 2005; Zannat et al., 2006). In the present study, we were interested in assessing the role of NMDAR in other tissues. We therefore determined the localization and relative abundance of *DpNR1A*, *DpNR1B* and *DpNR2* mRNA in day 4 adult male and female cockroaches using q-RT-PCR (Fig. 4). Specific q-RT-PCR primers for each *DpNR1* variant were designed to determine whether transcripts for the different *DpNR1* paralogs are present in the same tissue. Q-RT-PCR primers for *DpNR2* are located in the conserved region of *DpNR2* common to all putative splice variants. In mated female cockroaches, *DpNR1A*, *DpNR1B* and *DpNR2* were highly expressed in brain, followed by nerve cord. A previous study has shown that NMDA stimulated JH biosynthesis by the CA of *Diploptera* (Chiang et al., 2002). However, the

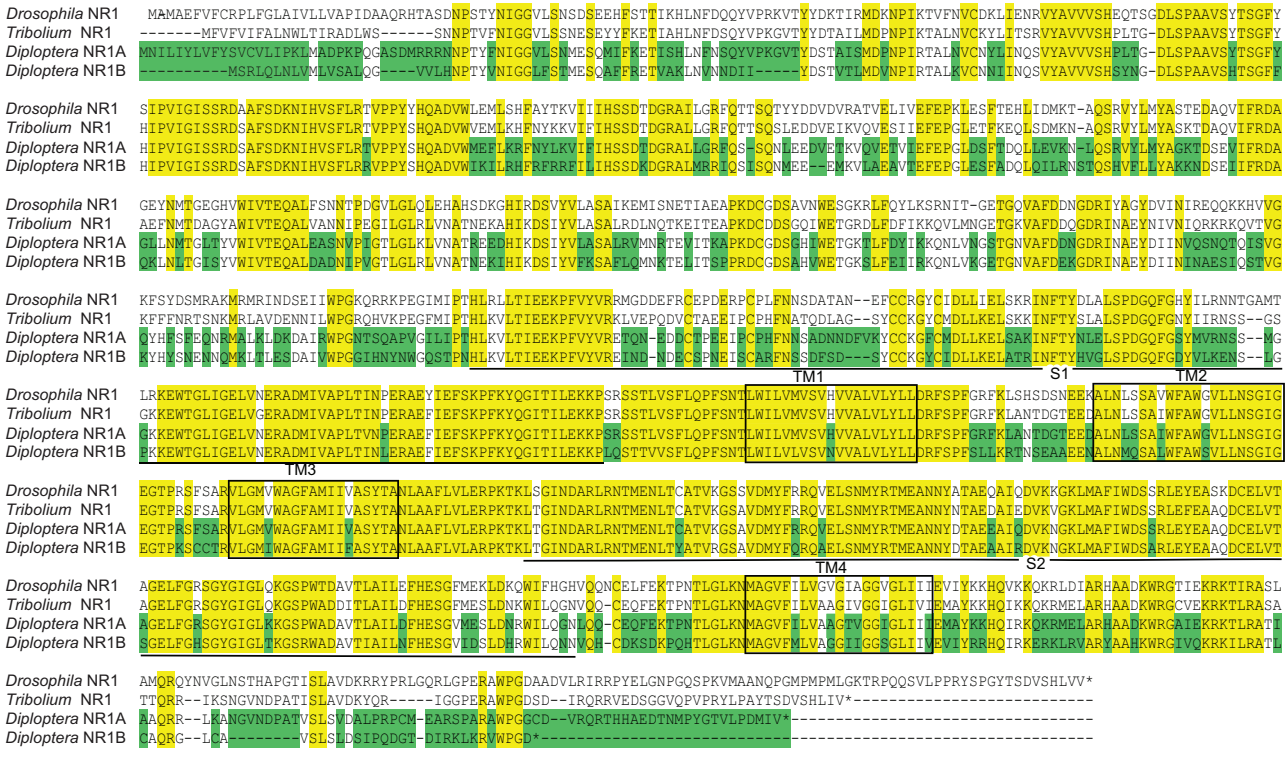

Fig. 1. Amino acid sequence alignment of the two *Diploptera* NR1 subunits and homologous receptors from *D. melanogaster* and *T. castaneum*. *Diploptera* NR1A and NR1B aligned with *D. melanogaster* DNR1 (GenBank acc. no. NP_730940.1) and *T. castaneum* TNR1 (GenBank acc. no. XP_969654.1, predicted sequence). Conservatively substituted residues are highlighted in yellow, and the different residues between *DpNR1A* and *DpNR1B* in green. Three putative hydrophobic transmembrane regions (TM1, TM3, and TM4) and one hydrophobic pore-forming segment (TM2) are highlighted in the boxes. Agonist-binding domain (S1 and S2 domains) are underlined.

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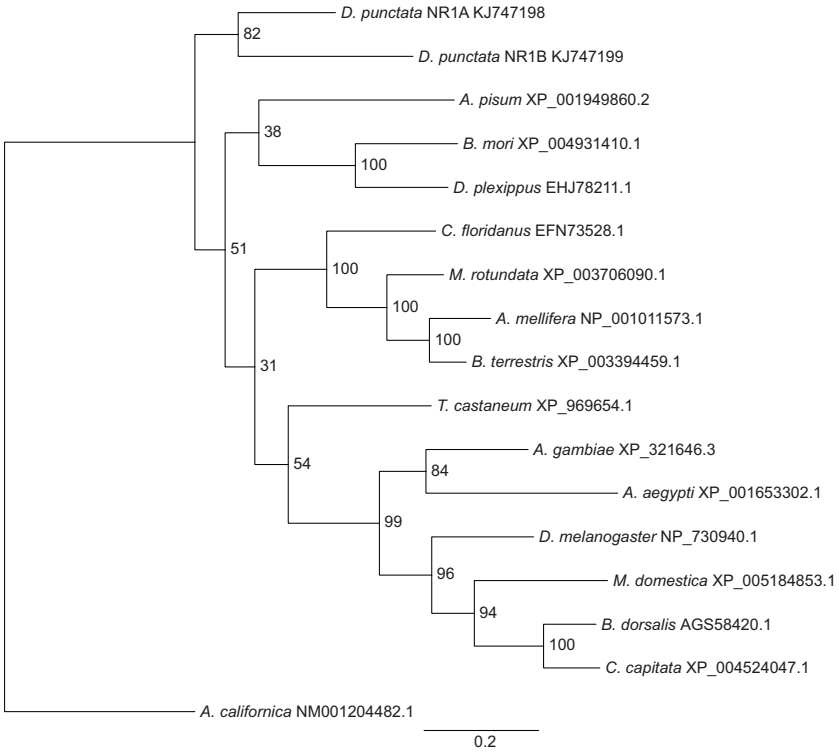
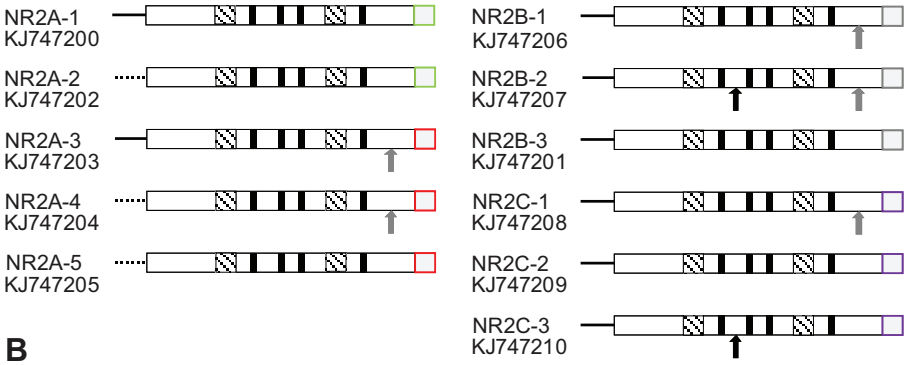


Fig. 2. Phylogram depicting the relationship between the NR1 subunits from *Diploptera* and orthologues of this receptor from other insects. Phylogenetic analysis was conducted in PhyML 3.0 using WAG substitution model with 100 bootstrap replicates. Poorly aligned amino acids were eliminated by eye, resulting in 854 positions. The bar represents 0.2 substitutions per site. The sea slug, *Aplysia californica* was used as outgroup to root the tree.

transcript level of *DpNR1A*, *DpNR1B* and *DpNR2* in the CA was relatively low compared with other tissues. In male cockroaches, the highest transcript levels of *DpNR1A*, *DpNR1B* and *DpNR2* were measured in the brain (Fig. 4). Interestingly, the transcript level of *DpNR2* in testes was very low, whereas levels of mRNA encoding both NR1 isoforms were high.

To further study the function of NMDAR in *D. punctata*, we determined the developmental profile of *DpNR1A*, *DpNR1B* and *DpNR2* in the brain, CA and testes. *DpNR1A* and *DpNR1B* were stably transcribed in the brain of day 0 to day 7 post-emergence mated female *D. punctata*. *DpNR2* mRNA in the brain showed a slight increase on days 2, 3 and 7, but none of the changes was significant (Fig. 5A). In the CA, the transcript levels of *DpNR1B* and *DpNR2* remained very low and did not show any significant change during the first gonadotrophic cycle (Fig. 5B). *DpNR1A* mRNA levels exhibited a significant increase on day 6. In male cockroaches, we studied the expression of *NMDAR* in testes of males of differing ages. The transcript level of *DpNR2* was very low for all ages assayed (Fig. 5C).

A



B

	Sequence
Insertion 1	GATGGTTTCCAGCAAGTG
Insertion 2	AAGAAGAAGAAACGCGTCCCGTCCTGCTGGGTCTCCTGCTTCACACGAAACCAGCCCGCGGGAGAGCAGGCGTCCCAG
C-terminal A1	TTGTTGAATGCAGATGACGTCCTCAAACCGCGTGATATGACAAGAAATCACGTGACATCTACAGAATTCTCCGGCCGTTTT CACAGTGCGGGCCAGTTATACAGGCCTTGCAGGACAGAAATAGCGGAGATGGAAACTGTTCTGTGA
C-terminal A2	CCTTGCAGGACAGAAATAGCGGAGATGGAAACTGTTCTGTGA
C-terminal B	TTGTTGAATGCAGATGACGTCCTCAAACCGCGTGATATGACAAGAAATCACGTGACATCTACAGAATTCTCCGGCCGTTTT CACAGTGCGGGCCAGTTATACAGGTAA
C-terminal C	TATTTATCTGGACAACGAACGTAG

Fig. 3. Schematic representation of the structures of the 11 variants of NR2. (A) The 5′ end untranslated region is indicated with solid or dashed lines, whereas the open reading frames (ORFs) are indicated with boxes. Alternative splicing generated different NR2 variants, including two 5′ untranslated regions, two insertions and four different 3′ ends. The position of insertion 1 is indicated with a black arrow and insertion 2 with a gray arrow. The insertions do not change the reading frame of the translated product. The alternative C-terminal sequences are shown as colored boxes: A1 (green), A2 (red), B (gray) and C (purple). Four putative transmembrane segments (TMI–IV) are shown by bold black lines. The agonist-binding domains S1 and S2 are indicated by the hatched boxes. The accession number is displayed under the gene number. (B) The sequences of insertions, deletions and the alternatively spliced C-termini.

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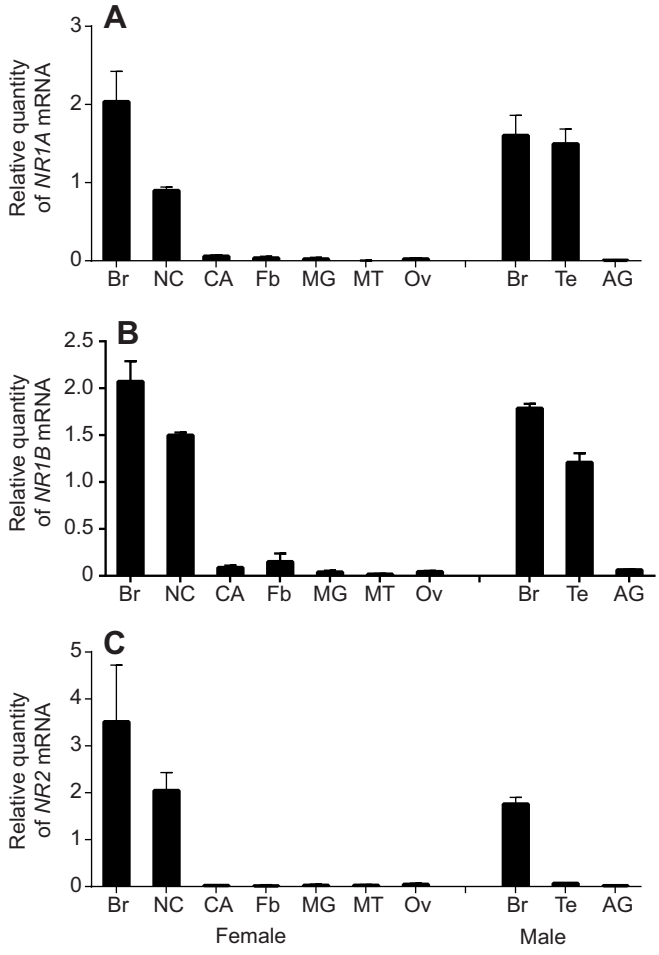

Fig. 4. Graphic representation of the relative tissue distribution of NR1 and NR2 transcripts. (A) *DpNR1A* transcript levels, (B) *DpNR1B* transcript levels and (C) *DpNR2* transcript levels in tissues of day 4 adult male and mated female *D. punctata*. Relative mRNA quantity was normalized against levels of *Tubulin* and *EF1α* mRNA (Marchal et al., 2013b). The data represents an average of three pools (10 animals per pool), run in triplicate. Br, brain; NC, nerve cord; CA, corpora allata; Fb, fat body; Ov, ovary; MG, midgut; MT, Malpighian tubules; AG, accessory gland and Te, testes. Values represent mean±s.e.m.

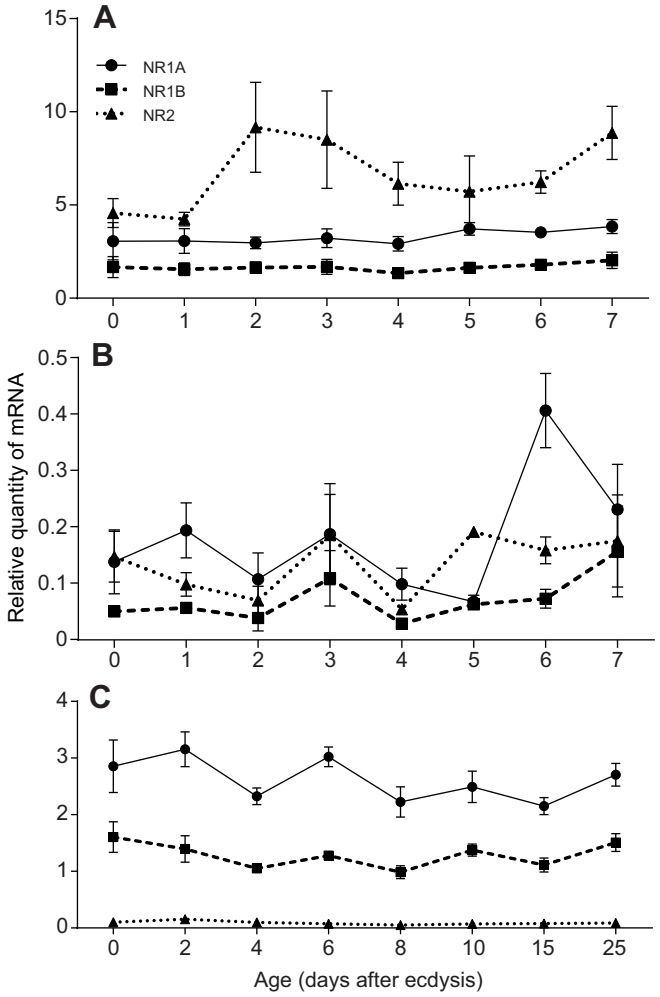

Fig. 5. Relative transcript levels of *DpNR1A*, *DpNR1B* and *DpNR2*. *Diploptera punctata* mRNA levels in (A) brains and (B) CA of mated females from day 0 to day 7 after ecdysis and (C) testes of different ages of males normalized against levels of *Tubulin* and *EF1a* mRNA (Marchal et al., 2013b). The data represent the average of three biologically independent pools (10 animals per pool), run in triplicate. Values represent mean±s.e.m.

Effect of *DpNR2* dsRNA on JH biosynthesis and oocyte growth

To investigate the role of NMDAR in reproduction, *DpNR2* was silenced using RNAi. The injection of 2 µg of *DpNR2* dsRNA on days 0, 1, 2 and 3 resulted in a 48.7% knockdown of *DpNR2* mRNA levels in brains of day 4 females (Fig. 6A). Knockdown of *DpNR2* also resulted in a decrease in the transcript levels of *DpNR1A* and *DpNR1B*. However, JH biosynthetic activity in the CA and basal oocyte length in dsRNA-treated animals did not show any significant difference from control animals (Fig. 6B,C). A similar result was also observed in animals treated with *DpNR1B* dsRNA (supplementary material Fig. S1).

In vitro effect of the NMDAR antagonist MK-801 on JH biosynthesis

A previous study showed a dose-dependent decrease in JH biosynthesis *in vitro* in *G. bimaculatus* CA as a function of MK-801 concentration (Geister et al., 2008). Thus, we determined the *in vitro* effect of MK-801 on JH biosynthesis. Surprisingly, the rates of JH biosynthesis remained at the same level irrespective of the concentration of MK-801 (Fig. 7). MK-801 did not appear to have any significant effect on JH biosynthesis *in vitro* by *D. punctata* CA.

In vivo effect of NMDAR antagonist MK-801 on JH biosynthesis and ovarian development

The effect of NMDAR on JH biosynthesis and ovarian development was also determined by injection of the NMDAR non-competitive antagonist MK-801 into adult females. To determine the optimal dose, newly molted adult females were injected with 0 (control), 3, 12 and 30 µg MK-801 on days 0, 1 and 3. JH biosynthesis and basal oocyte length were measured on day 4. As shown in supplementary material Fig. S2, there was no significant effect on JH biosynthesis in animals treated with 3 µg or 12 µg MK-801.

To further study the effect of MK-801 on reproduction, mated female cockroaches were injected with 30 µg MK-801 on days 0, 1 and 3 following eclosion. Rates of JH biosynthesis and basal oocyte lengths in control and treated animals were determined from day 4 to day 8. As shown in Fig. 8A, application of MK-801 resulted in a 34% and 26% decrease of JH biosynthesis in day 4, and 5 animals, respectively. On day 6, however, MK-801 treated

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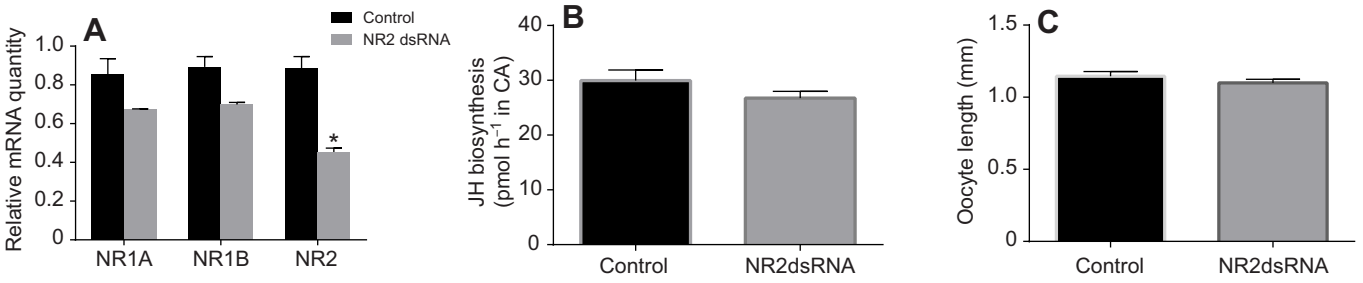

Fig. 6. The effect of *DpNR2* dsRNA treatment on JH biosynthesis and basal oocyte growth, and the interactions among these genes in mated female *D. punctata*. (A) Relative quantity of *DpNR1A*, *DpNR1B* and *DpNR2* mRNA levels in brain between control and dsRNA-treated animals. mRNA levels were normalized against levels of *Tubulin* and *EF1a* mRNA (Marchal et al., 2013b). The data represent the average of three biologically independent pools (five animals each pool) run in triplicate. (B) JH biosynthesis by CA from control and dsRNA-treated animals. (C) Basal oocyte length in control and dsRNA-treated animals. Values represent means±s.e.m. *P<0.05 compared with control.

animals showed higher rates of JH biosynthesis than control animals. On days 7 and 8, there was no significant difference in JH biosynthesis between control and MK-801-treated animals. Furthermore, MK-801 did not have any effect on basal oocyte length (Fig. 8B). All animals oviposited on day 8. To study the effect of MK-801 on vitellogenin (Vg) synthesis, we determined the transcript level of *DpVg* in the fat body of day 4 animals. No significant change was found in the transcription of *DpVg* in MK-801-treated animals (Fig. 8C).

DISCUSSION

We have identified two distinct *NR1* genes and 11 *NR2* variants in *D. punctata*. The major functional domains appear to be well conserved in both *DpNR1* and *DpNR2* amino acid sequences (Figs 1 and 3). The protein contains three hydrophobic transmembrane regions (TM1, 3–4), a hydrophobic pore-forming segment (TM2), and two ligand binding domains (S1 and S2) (Figs 1 and 3) (Dingledine et al., 1999; Kuryatov et al., 1994; Stern-Bach et al., 1994). The asparagine residue, which was predicted to control Ca^{2+} permeability and voltage-dependent Mg^{2+} blockade, is present in the TM2 domain of DpNR1 (N630 in DpNR1A and N608 in DpNR1B) (Burnashev et al., 1992). The overall amino acid sequence identity between DpNR1, DpNR2 and NMDAR subunits in other species is shown in supplementary material Table S1. DpNR1A has higher sequence identity to NR1 in other species than DpNR1B. Both DpNR1 and DpNR2 subunits show highest homology to NMDAR of *A. mellifera*.

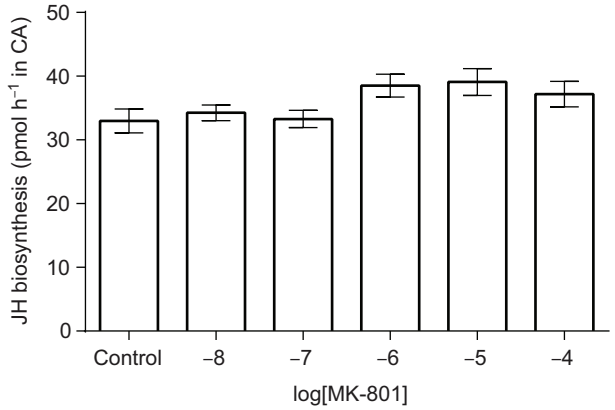

Fig. 7. *In vitro* effect of MK-801 on JH biosynthesis in CA. CA were dissected from day 4 mated females. The JH biosynthesis in normal medium without MK-801 was determined as control. Each data point represents mean± s.e.m. (N=12).

The two NR1 subunits are 65.6% identical at the amino acid level and the differences are distributed throughout the entire gene, which suggests that the two *NR1* paralogs result from gene duplication (Fig. 1). The other *NR1* paralogs were isolated from the Zebrafish (Cox et al., 2005); they encode the same length of protein with only a few amino acid differences. No *NR1* paralogs other than in Zebrafish have been identified in vertebrate or invertebrate species. Phylogenetic analysis of DpNR1 with NR1s from other insect species shows that the two DpNR1s are grouped, which suggests that these paralogs result from a gene duplication event in *D. punctata* that did not occur in other insects. However, given that our dataset was composed primarily of higher insects, and the small number of insect species in which NR1 has been identified, it is difficult to make definitive conclusions about the origin of the paralogs. *DpNR2* undergoes alternative splicing, generating 11 different transcripts that may encode nine protein isoforms (Fig. 3). Unlike *NR2* in *Drosophila* in which alternative splicing occurs mainly at the 5′ untranslated region, *DpNR2* undergoes alternative splicing principally at the 3′-end (Xia et al., 2005).

Tissue distribution shows that both *DpNR1* and *DpNR2* mRNA accumulated in brain and nerve cord, which is consistent with the role of NMDAR in learning and memory (Xia et al., 2005). The NR1 subunit is essential for the basic channel activity of NMDAR, whereas the NR2 subunit is regarded as the rate-limiting molecule in controlling the optimal channel properties of NMDAR (Monyer et al., 1992; Sprengel et al., 1998; Tang et al., 1999). In *D. punctata*, we observed that both *DpNR1* paralogs are stably expressed in brain, whereas the relative *DpNR2* mRNA level underwent a slight change, probably to regulate the activity of NMDAR (Fig. 5A). In rats, D-aspartic acid (D-Asp) can induce testosterone synthesis through the activation of NMDAR in testis (Santillo et al., 2014). A high transcript level of *DpNR1* was observed in adult *D. punctata* testes, indicating that NMDAR may also play a role in regulation of reproduction in our model insect (Fig. 5B). However, the transcript level of *DpNR2* in testes was negligible compared with that of *DpNR1*, which suggests that NR1 may form a homomeric functional channel, as observed in an earlier study in which expression of *Drosophila* NR1 alone produced a weak but significant NMDA response (Ultsch et al., 1993; Xia et al., 2005). An alternative explanation for the low expression of *DpNR2* in the testes could be the existence of one or more additional *DpNR2* subunits in *D. punctata*. Four distinct NR2 subunits (A–D) were identified in mammalian species. All four *NR2* subunits are expressed in brain (Cull-Candy et al., 2001). In testis, however, expression of the *NR2* subunits varies (Hu et al., 2004; Santillo et al., 2014). In the rat testis, only *NR2A* and *NR2D* are strongly

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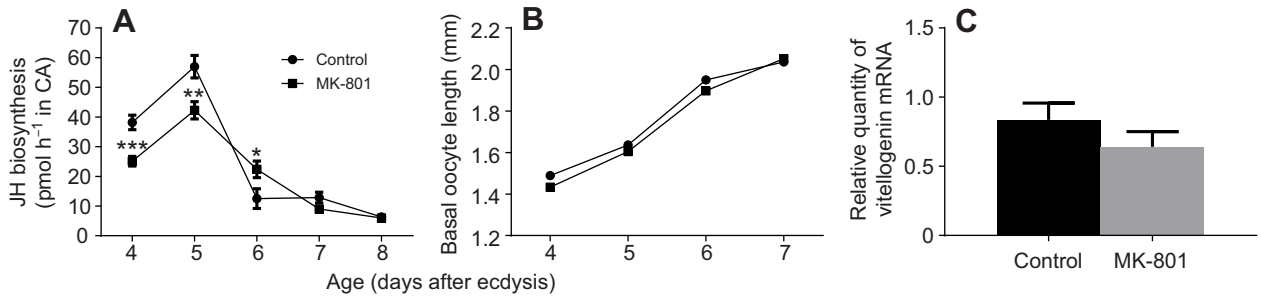

Fig. 8. *In vivo* effect of MK-801 on JH biosynthesis, basal oocyte growth and relative vitellogenin mRNA levels. Females were injected with MK-801 on days 0, 1 and 3 following ecdysis (30 µg/animal). Control was injected with ddH_2O. JH biosynthesis (A) and oocyte length (B) were determined on day 4 to day 8. All animals oviposited on day 8. The mRNA level of *DpVg* was determined in the fat body of day 4 animals. (C) mRNA levels were normalized to levels of *Tubulin* and *EF1a* mRNA (Marchal et al., 2013b). The data represent the average of three biologically independent pools (five animals each pool), run in triplicate. Values represent means±s.e.m. (A) $N \geq 15$; (B) $N \geq 10$. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

expressed, whereas *NR2B* and *NR2C* are undetectable (Santillo et al., 2014). In mouse testis, by contrast, only the *NR2B* subunit is highly expressed (Hu et al., 2004). Thus, it is possible that an additional *NR2* gene is expressed in the testis of *D. punctata*.

The function of NMDAR in reproduction is well studied in mammals (Mahesh and Brann, 2005). However, clear evidence linking NMDAR to reproduction in insects remains limited. The possible role of NMDAR in reproduction of insects was described by Chiang et al. (2002). In that study, NMDA stimulates JH biosynthesis by inducing a Ca^{2+}-influx from the extracellular environment into the CA of *D. punctata*; this stimulation could be significantly reduced by MK-801 treatment (Chiang et al., 2002). This experiment was performed in a minimum incubation medium (150 mmol l^{-1} NaCl, 12 mmol l^{-1} KCl, 4 mmol l^{-1} HEPES, 100 nmol l^{-1} dopamine, 5 µmol l^{-1} glycine, 2% Ficoll). However, following incubation of CA in TC199 medium, which better mimics the cockroach hemolymph environment, we found that MK-801 did not show any significant effect on JH biosynthesis (Fig. 7). Chiang et al. (2002) also reported that the stimulation by NMDA of JH biosynthesis is age dependent, which is suggested to be controlled by the expression of NMDAR. However, our study has clearly demonstrated that the transcript levels of *DpNR1* and *DpNR2* in the CA are very low throughout the first gonadotrophic cycle and did not exhibit any significant changes. Those results suggest that although NMDA is able to stimulate JH biosynthesis *in vitro*, NMDAR may not function as a regulator of JH biosynthesis in *D. punctata*. To answer this question, the role of NMDAR in JH biosynthesis and reproduction was further examined by the administration of MK-801 *in vivo* in *D. punctata*.

MK-801 did not have an *in vivo* effect on JH biosynthesis at doses up to 12 µg per animal (about 60 µg g^{-1} body mass) (supplementary material Fig. S2). In *D. punctata*, the production of vitellogenin in fat body and the uptake of vitellogenin by the basal oocytes are JH-dependent events (Marchal et al., 2013a; Rankin and Stay, 1984; Stay and Tobe, 1978). Although a higher dose of MK-801 resulted in a significant decrease in JH biosynthesis on days 4 and 5, the change in transcript level of *DpVg* was not significant relative to controls (Fig. 8C), and the pattern of JH biosynthesis during the first gonadotrophic cycle is similar to that in control animals (Fig. 8A). Oocyte growth in treated animals is also similar to the controls (Fig. 8B). All females oviposited on day 8. Overall, administration of MK-801 did not have a significant effect on reproduction in female *D. punctata*. This is consistent with the MK-801 results. In *G. bimaculatus*, in which MK-801 treatment *in vitro* resulted in a dose-dependent inhibition of JH biosynthesis by the CA, egg size and egg number were significantly affected by the application of MK-801 (Geister et al., 2008). Thus, the regulatory role of NMDAR on JH biosynthesis appears to be species specific.

It is interesting that treatment of animals with a high dose of MK-801 *in vivo* significantly reduced JH biosynthesis on days 4 and 5, even though MK-801 did not show any significant effect on JH biosynthesis *in vitro*. Partial knockdown of *DpNR2* did not show any effect on JH biosynthesis or on basal oocyte growth (Fig. 6). How does MK-801 change the rate of JH biosynthesis whereas NMDAR does not appear to have an *in vivo* effect on JH biosynthesis or reproduction? To date, there is no clear evidence showing that the effect of MK-801 on JH biosynthesis is mediated through NMDAR. In rats, injection of 0.2 µg g^{-1} MK-801 resulted in a failure to elevate LH, FSH or progesterone (Luderer et al., 1993). In rats and mice, a dose of 0.1 µg g^{-1} MK-801 was the maximum dose that could be used without causing sensorimotor impairments and/or signs of intoxication (Van der Staay et al., 2011). Injection of 30 µg of MK-801 paralyzed the cockroaches for 4–5 h. Therefore, it is possible that the significant decrease in JH biosynthesis on days 4 and 5 in *D. punctata* was the result of physiological stress induced by MK-801 treatment, rather than the action of NMDAR.

In conclusion, two NR1 paralogs and 11 NR2 alternative splicing variants have been identified in *D. punctata*. The expression of NMDAR subunits suggests that NMDAR may play a role in the reproduction of male cockroaches. However, although a previous study suggested that NMDAR mediates JH biosynthesis in *D. punctata in vitro*, our data in the female cockroach reveal a different story. Neither *in vitro* treatment of MK-801 nor partial knockdown of *DpNR2* has any effect on JH biosynthesis. The decrease in JH biosynthesis at a high dose in MK-801-treated animals appears to result from physiological stress, rather than a direct action on NMDAR. In addition, no reproductive events were affected following the blocking of NMDAR activity through RNAi or MK-801 treatment. A re-examination of the function of NMDA receptors in the reproduction of insects now appears to be appropriate and timely.

MATERIALS AND METHODS

Insects

Diploptera punctata Eschscholtz 1822 were reared in cages and fed with lab chow and water *ad libitum* at 27–28°C in a dark room. Newly molted male and female adult cockroaches were picked from the colony and raised in separate containers. Mated status in the females was confirmed by the presence of a spermatophore.

Tissue collection

Cockroach tissues were dissected under a dissecting microscope. Basal oocyte length was measured to determine the physiological age of female cockroaches. Selected tissues were dissected and cleaned in

sterile cockroach ringer solution (150 mmol l^{-1} NaCl, 12 mmol l^{-1} KCl, 10 mmol l^{-1} $CaCl_2 \cdot 2H_2O$, 3 mmol l^{-1} $MgCl_2 \cdot 6H_2O$, 10 mmol l^{-1} HEPES, 40 mmol l^{-1} Glucose, pH 7.2), flash-frozen in liquid nitrogen to prevent RNA degradation and stored at −80°C until further processing.

RNA extraction and cDNA synthesis

Selected tissues were collected from adult females and males for gene sequencing, tissue distribution and developmental profiling. RNA extraction and cDNA synthesis were performed as described by Marchal et al. (2013b). For the tissue distribution and developmental profiling, three biologically independent pools of 10 animals each were collected. For RNAi and MK-801 treatment experiments, three biologically independent pools of brain and fat body were collected, each pool containing tissue from five animals.

Sequencing of *DpNR1A*, *DpNR1B* and *DpNR2*

Degenerate primer sequences were designed for *DpNR1A*, *DpNR1B* and *DpNR2* based on conserved amino acid sequences of several insect orthologs. Primers used for degenerate PCR are listed in supplementary material Table S2. Partial sequences were obtained using these primers in a standard T-gradient PCR using Taq DNA polymerase (Sigma-Aldrich) and a *D. punctata* brain cDNA sample. After purification, the resulting DNA fragments were subcloned into a pJET 1.2/blunt cloning vector (CloneJet PCR Cloning Kit, Thermo Scientific) and sequenced following the protocol outlined in the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems). The complete sequence of *DpNR1A*, *DpNR1B* and *DpNR2* was obtained using 5′-RACE (Rapid Amplification of cDNA Ends) and 3′-RACE strategies, following the protocol outlined in the Roche 5′/3′ RACE Kit. Primers used for RACE are listed in supplementary material Table S2.

Phylogenetic analysis

For the phylogenetic analysis of *DpNR1* genes, the NR1 sequences of 15 insect species (identified or predicted) were used. These sequences were aligned using ClustalW as implemented within MEGA 6.06 (Tamura et al., 2013). Poorly aligned positions and gaps were removed, which resulted in 854 amino acid residues. The obtained alignment was used to construct a phylogenetic tree in PhyML 3.0 (Guindon and Gascuel, 2003) based on the maximum-likelihood principle, using the WAG substitution model (Whelan and Goldman, 2001). Four substitution rate categories were used to estimate the gamma parameter shape with 100 bootstrap replicates to assess branch support (Felsenstein, 1985; Yang, 1994). The resulting tree was then rooted using the sea slug, *Aplysia californica* as outgroup.

Quantitative Real Time-PCR

Primers used for q-RT-PCR are shown in supplementary material Table S3. Primer sets were validated by determining relative standard curves for each gene transcript using a five-fold serial dilution of a calibrator cDNA sample. Efficiency and correlation coefficient (R^2) can be found in supplementary material Table S3. Reactions were performed in triplicate on a CFX384 Touch™ Real-Time PCR Detection System (Bio-Rad) as described previously by Marchal et al. (2013b). Target specificity was confirmed by running a few representative q-RT-PCR products on an agarose gel containing GelRed (Biotium). The optimal housekeeping genes for target gene profiling and RNAi experiments were chosen according to a previous study (Marchal et al., 2013b). The quantity of mRNA for each tested gene relative to reference genes was determined as described by Vandesompele et al. (2002).

RNA interference

Primers for *DpNR2* and control *pJET* dsRNA constructs with T7 promoters are shown in supplementary material Table S4. Double-stranded RNA (dsRNA) constructs were prepared using the MEGAscript RNAi Kit (Ambion). A PCR using forward and reverse primers with attached T7 promoters was performed to amplify the fragment, which was subcloned and sequenced to verify the presence of the T7 promoter. The amplified fragment was used in an RNA transcription reaction which was incubated overnight to obtain a high yield of annealed dsRNA construct. A nuclease digestion was subsequently performed to remove ssRNA and DNA remaining in the product. The dsRNA was further purified according to the manufacturer's instructions (Ambion). Concentration of the dsRNA construct was determined using a Nanodrop instrument (Thermo Fisher Scientific Inc., Canada). Five-fold diluted dsRNA was run on a 1.2% agarose gel to examine the quality and integrity of the construct.

Newly molted adult female cockroaches (day 0) were injected with 2 µg of either *DpNR2* dsRNA or *pJET* dsRNA diluted in 5 µl of cockroach saline. This treatment was repeated on days 1, 2 and 3, and the effect of *DpNR2* dsRNA was determined on day 4. Brains were dissected and stored in liquid nitrogen prior to RNA extraction. CA were dissected and cleaned in TC199 medium (GIBCO; 1.3 mmol l^{-1} Ca^{2+}, 2% Ficoll, methionine-free) for use in the radiochemical assay (RCA) (see below). Basal oocyte length was measured during dissection.

MK-801 *in vitro* assay

To determine the *in vitro* effect of MK-801 on JH biosynthesis, CA were incubated in TC199 medium (M 7653 (Sigma), supplemented with $CaCl_2$ to a final concentration of 1.3 mmol l^{-1} and fortified with 2% Ficoll) containing different concentrations of MK-801; JH production was examined using a radiochemical assay (RCA). The concentrations of MK-801 ranged from 10^{-4} mol l^{-1} to 10^{-8} mol l^{-1}. JH biosynthesis in medium without MK-801 was used as a control. RCA was performed as described by Feyereisen and Tobe (1981) and modified by Tobe and Clarke (1985).

MK-801 *in vivo* assay

MK-801 was dissolved in ddH_2O to a concentration of 6 µg $µl^{-1}$ and injected into animals using a Hamilton syringe. Newly molted adult females were injected with 30 µg of MK-801 on days 0, 1 and 3, and the effect of MK-801 was determined from day 4 to day 8. Fat body was dissected from day 4 animals and stored in liquid nitrogen prior to RNA extraction. CA were dissected and cleaned in TC199 medium (GIBCO; 1.3 mmol l^{-1} Ca^{2+}, 2% Ficoll, methionine-free) for use in the RCA (see below). Basal oocyte length was also measured.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

J.H. and S.S.T. conceived and designed the experiments. J.H., E.F.H. and E.M. performed the experiments. J.H., E.F.H. and E.M. analyzed the data. J.H. and S.S.T. interpreted the findings being published. J.H. wrote the paper. E.F.H., E.M. and S.S.T. revised the paper. S.S.T. was the Principal Investigator, responsible for the management of the projects that provided financial support. All authors read and approved the final manuscript.

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Supplementary material

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