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 alternative spliced variants in *D. 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 JH biosynthesis in the corpora allata (CA) through the influx of extracellular Ca²⁺ in *Diploptera punctata* (Chiang A. et al, 2002). However, our data show that the transcript levels of *DpNR1A*, *DpNR1B* and *DpNR2* were low in the CA. 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 by CA or basal oocyte growth. Injection of animals with a high dose of MK-801 (30 µg/animal/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*.

**Keyword**

- NMDA receptor; JH biosynthesis; MK-801; reproduction; *D. punctata*

**Abbreviation**

- NMDAR, N-methyl-D-aspartate receptor; NR1, NMDA receptor subunit 1; NR2, NMDA receptor subunit 2; JHAMT, juvenile hormone acid methyltransferase; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone

**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 (GluR) 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 (NMDAR) 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).

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).

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

Results

2.1 Identification of DpNR1 subunits

Two DpNR1 were identified with open reading frames of 2871bp (DpNR1A) and 2703bp (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 the NCBI GenBank and received 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. On the other hand, 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.

2.2 Identification of DpNR2 subunits

DpNR2 undergoes alternative splicing, generating eleven different transcripts. Full-length cDNAs for all eleven variants have been isolated and their sequences were deposited in the NCBI GenBank with accession numbers: KJ747200 to KJ747210. In the eleven 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 eleven 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 (Table S1). DpNR2A-1 shares the highest similarity with NR2 subunits from A. mellifera.

2.3 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 \textit{DpNR1A}, \textit{DpNR1B} and \textit{DpNR2} mRNA in day 4 adult male and female cockroaches using q-RT-PCR (Fig. 4). Specific q-RT-PCR primers for each \textit{DpNR1} variant were designed to determine whether transcripts for the different \textit{DpNR1} paralogs are present in the same tissue. Q-RT-PCR primers for \textit{DpNR2} are located in the conserved region of \textit{DpNR2} common to all putative splice variants. In mated female cockroaches, \textit{DpNR1A}, \textit{DpNR1B} and \textit{DpNR2} were highly expressed in brain, followed by nerve cord. Previous study has shown that NMDA stimulated JH biosynthesis by the CA of \textit{Diploptera} (Chiang et al., 2002). However, the transcript level of \textit{DpNR1A}, \textit{DpNR1B} and \textit{DpNR2} in the CA was relatively low compared to other tissues. In male cockroaches, the highest transcript levels of \textit{DpNR1A}, \textit{DpNR1B} and \textit{DpNR2} were measured in the brain (Fig. 4). Interestingly, the transcript level of \textit{DpNR2} in testes was very low whereas both genes encoding NR1 were high.

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

2.4 Effect of \textit{DpNR2} dsRNA on JH biosynthesis and oocyte growth

To investigate the role of NMDAR in reproduction, \textit{DpNR2} was silenced using RNAi. The injection of 2 µg of \textit{DpNR2} dsRNA on days 0, 1, 2 and 3 resulted in a 48.7% knockdown of \textit{DpNR2} mRNA levels in brains of day 4 females (Fig. 8a). Knockdown of \textit{DpNR2} also resulted in a decrease in the transcript levels of \textit{DpNR1A} and \textit{DpNR1B}. However, the JH biosynthetic activity of CA and basal oocyte lengths in dsRNA-treated animals did not show any significant
difference from control animals (Fig. 8b, 8c). A similar result was also observed in DpNR1B dsRNA- treated animals (Fig. S1).

2.5 In vitro effect of 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 concentration of MK-801 (Fig. 9). MK-801 did not appear to have any significant effect on JH biosynthesis in vitro by D. punctata CA.

2.6 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 of injection, 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 lengths were determined on day 4. As shown in 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. 10A, 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 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 10B). 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. 10C).

Discussion
We have identified two distinct NR1 genes and eleven NR2 variants in D. punctata. The major functional domains appear to be well conserved in both DpNR1 and DpNR2 amino acid sequences (Fig. 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) (Fig. 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\textsuperscript{2+} permeability and voltage-dependent Mg\textsuperscript{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 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.

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 together, which suggests that these are paralogs resulting 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 eleven 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. 5). 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 \textit{D. punctata} testes, indicating that NMDAR may also play a role in regulation of reproduction in our model insect (Fig. 6). However, the transcript level of \(DpNR2\) in testes was negligible compared to that of \(DpNR1\), which suggests that NR1 may form a homomeric functional channel, as was observed in an earlier study in which expression of \textit{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 \textit{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, the expression of \(NR2\) subunits varies (Hu et al., 2004; Santillo et al., 2014). In the rat testis, only \(NR2A\) and \(NR2D\) are strongly expressed, whereas \(NR2B\) and \(NR2C\) are undetectable (Santillo et al., 2014). In mouse testis, on the other hand, only \(NR2B\) subunit is highly expressed (Hu et al., 2004). Thus, it is possible that an additional \(NR2\) gene is expressed in the testis of \textit{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 \textit{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 mM NaCl, 12 mM KCl, 4 mM Hepes, 100 nM dopamine, 5 \(\mu\)M glycine, 2% Ficoll). However, following incubation of CA in TC199 medium, which better mimics the cockroach hemolymph environment, MK-801 did not show any significant effect on JH biosynthesis (Fig. 9). On the other hand, the study of Chiang et al. (2002) showed 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 change. Those results suggest to that although NMDA is able to stimulate JH biosynthesis, NMDAR may not function as a regulator of JH biosynthesis in \textit{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.

Administration of MK-801 in vivo did not have an effect on JH biosynthesis at doses up to 12 µg/animal/injection (about 60 µg/g body mass) (Fig. S2). In D. punctata, the production of Vg in fat body and the uptake of Vg 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. 10C), and the pattern of JH biosynthesis during the first gonadotrophic cycle is similar to that in control animals (Fig. 10A). Oocyte growth in treated animals is also similar to the controls (Fig. 10B). 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 vitro in G. bimaculatus, in which MK-801 treatment 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. 8). How does MK-801 change the rate of JH biosynthesis while 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 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 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/animal 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 eleven 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, in the female cockroach, although a previous study suggested that NMDAR mediates JH biosynthesis in *D. punctata in vitro*, our data 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 reexamination of the function of NMDA receptors in the reproduction of insects now appears to be appropriate and timely.

**Materials and Methods**

**4.1 Insects**

*D. punctata* were reared in cages and fed with lab chow and water *at 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.

**4.2 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 mM NaCl, 12 mM KCl, 10 mM CaCl$_2$.2H$_2$O, 3 mM MgCl$_2$.6H$_2$O, 10 mM HEPES, 40 mM Glucose, pH 7.2), flash-frozen in liquid nitrogen to prevent RNA degradation and stored at -80 °C until further processing.

**4.3 RNA extraction and cDNA synthesis**

Selected tissues were collected from adult females and male for gene sequence, 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, 3 biologically independent pools of brain and fat body were collected, each pool containing tissue from 5 animals.

4.4 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 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 Table S2.

4.5 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.

4.6 Quantitative Real Time-PCR (q-RT-PCR)

Primers used for q-RT-PCR are shown in 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 (R²) can be found in 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).

4.7 RNA interference (RNAi)

Primers for DpNR2 and control pJET dsRNA constructs with T7 promoters are shown in 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 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 mM Ca²⁺, 2% Ficoll, methionine-free) for use in the radiochemical assay (RCA) (see below). Basal oocyte length was measured during dissection.

4.8 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 CaCl₂ to a final concentration of 1.3mM, and fortified with 2% Ficoll) containing different concentrations of MK-801, and the JH production was examined using the radiochemical assay (RCA). The concentrations of MK-801 ranged from
10^{-4} \text{ M to } 10^{-8} \text{ M. JH biosynthesis in medium without MK-801 was used as control. RCA was
performed as described by Feyereisen and Tobe (1981) and modified by Tobe and Clarke (1985).

4.9 MK-801 in vivo assay

MK-801 was dissolved in ddH2O to a concentration of 6 µg/µl 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 mM Ca^{2+}, 2% Ficoll, methionine-free) for use in the
RCA (see below). Basal oocyte length was also measured.

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Author contributions

Conceived and designed the experiments: JH, SST. Performed the experiments: JH, EFH, EM.
Analyzed the data: JH, EFH, EM. Contributed reagents/materials/analysis tools: SST. Wrote the
paper: JH, EFH, EM, SST. Principal investigator, responsible for the management of the projects
that provided financial support: SST. All authors read and approved the final manuscript.

Figure captions

Figure 1. Amino acid sequence alignment of the two Diploptera NR1 subunit (DpNR1A,
DpNR1B), and homologous receptors from 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.

Figure 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.

Figure 3. (A) **Schematic representation of the structures of the 11 variants of the NR2.** The 5’ end untranslated region is indicated with black or dashed lines, whereas the open reading frames (ORF) 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 shown by the black arrow and insertion 2 by a gray arrow. The insertions do not change the reading frame of the translated product. The alternative carboxyl-terminal sequences are indicated by colored boxes: A1 (Green), A2 (Red), B (Gray), and C (Purple). Four putative transmembrane segments (TM I – 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 carboxyl-termini.**

Figure 4. **Graphic representation of the relative tissue distribution of (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 3 pools (10 animals per pool), run in triplicate. Abbreviations used: 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 ± SEM.

Figure 5. **Relative transcript levels of *DpNR1A*, *DpNR1B* and *DpNR2* in brains of mated female *D. punctata* from day 0-day 7 after ecdysis.** mRNA levels were normalized against levels of *Tubulin* and *EF1α* mRNA (Marchal et al., 2013b). The data represent the average of 3 biologically independent pools (10 animals per pool), run in triplicate. Values represent mean ± SEM.
Figure 6. **Relative transcript levels of** *DpNR1A*, *DpNR1B* and *DpNR2* **in CA of mated female *D. punctata from day 0-day 7 after ecdysis.** mRNA levels were normalized against levels of *Armadillo* and *EF1a* mRNA (Marchal et al., 2013b). The data represent the average of 3 biologically independent pools (10 animals per pool), run in triplicate. Values represent mean ± SEM.

Figure 7. **Relative transcript levels of** *DpNR1A*, *DpNR1B* and *DpNR2* **in testes of different ages of male *D. punctata.** mRNA levels were normalized against levels of *Tubulin* and *EF1a* mRNA (Marchal et al., 2013b). The data represent the average of 3 biologically independent pools (10 animals per pool), run in triplicate. Values represent mean ± SEM.

Figure 8. **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 *DippuNR1A*, *DippuNR1B* and *DippuNR2* 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 3 biologically independent pools (5 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 mean ± SEM. Levels of significance to the control are indicated with the asterisk symbol: *P < 0.05

Figure 9. **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 ± SEM (n = 12).

Figure 10. **In vivo effect of MK-801 on JH biosynthesis** (A), **basal oocyte growth** (B) **and relative Vg mRNA levels** (C). Females were injected with MK-801 on days 0, 1 and 3 following ecdysis (30µg/animal). Control was injected with ddH2O. 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 *Vg* was determined in the fat body of day 4 animals. mRNA levels were normalized to levels of *Tubulin* and *EF1a* mRNA (Marchal et al., 2013b). The data represent the average of 3 biologically independent pools (5 animals each pool), run in triplicate. Values represent mean ± SEM. (A) N≥15; (B) N≥10. Levels of significance to the control are indicated with an asterisk: *P < 0.05, **P < 0.01, ***P < 0.001


A

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B

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| C-terminal A2 | CTTGCAGGACAGAAATAGCGGAGATGGAAACTGTTCTGTGA |
| C-terminal B | TTGTTGAATGCAGATGACGTCCCTCAAAAACCGCGTGATTAGACAGAAATCCACGTGACATCTACAGAATTCTCCGGCGTTTTCACTAGTGGCGCCAGTATTACAGGCTTTGA |
| C-terminal C | TATTTATCTGGACACGAACGTAG        |
Relative mRNA quantity

- NR1A
- NR1B
- NR2
JH biosynthesis (pmol/h/CA) vs. log[MK-801]