Localization of NADPH-Diaphorase and Nitric Oxide Synthase Activity in the Eyestalk of the Crayfish, *Procambarus clarkii*

Hong-Shin Zou, Yi-Zhen Chang, Shyh-Chi Chen, Shiou-Mei Yau, Ya-Lun Shen and Chi-Ying Lee*

Department of Biology, National Changhua University of Education, Changhua, Taiwan 500, R.O.C.

Hong-Shin Zou, Yi-Zhen Chang, Shyh-Chi Chen, Shiou-Mei Yau, Ya-Lun Shen, and Chi-Ying Lee (2002) Localization of NADPH-diaphorase and nitric oxide synthase activity in the eyestalk of the crayfish, *Procambarus clarkii*. *Zoological Studies* **41**(3): 244-250. The distribution of nitric oxide synthase (NOS) in the eyestalk tissues of the crayfish, *Procambarus clarkii*, was studied using NADPH-diaphorase (NADPH-d) histochemistry and an NOS biochemical assay. In the retina, NADPH-d activity was detected in the rhabdom of the ommatidium and axons of retinular cells that terminate in the lamina ganglionaris. In the eyestalk ganglia, NADPH-d was localized to the fibers in the 1st chiasma that connect the lamina ganglionaris and medulla externa, and 2 prominent fiber tracts that appear to originate from the medulla terminalis, project distally, and terminate in the synaptic layers of the medulla interna. In addition, NADPH-d-positive somata were found in the periphery of the medulla externa. Specific staining of NADPH-d activity was eliminated by DPIP, an inhibitor of NOS-associated NADPH-d. The distribution pattern of NADPH-d activity described above is much broader than that of NOS-immunoreactivity reported in a previous study (Lee et al. 2000). Biochemical analysis revealed that the regions (the medulla interna and medulla terminalis) where only NADPH-d activity (but not NOS-immunoreactivity) was detected also contained significant levels of NOS activity, suggesting that the observed NADPH-d activity is associated with NOS. The combined results indicate that, in contrast to a highly restricted pattern suggested by immunohistochemical data, NOS is widely distributed in the crayfish eyestalk and implicates NO in the processing of visual information. http://www.sinica.edu.tw/zool/zoolstud/41.3/244.pdf

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Nitric oxide (NO) is formed by NO synthase (NOS) from L-arginine with stoichiometric formation of citrulline. A family of isoenzymes is currently recognized in mammalian tissues: the brain (bNOS) and endothelial (eNOS) isoforms are constitutively expressed and Ca^{2+}/calmodulin-regulated, whereas the cytokine/endotoxin-induced isoform (iNOS) is Ca^{2+}-independent (Griffith and Stuehr 1995). Recent comparative studies have shown that NOS is present in the central and peripheral nervous systems of various invertebrate species and suggested a wide variety of functional roles for NO (Elofsson et al. 1993, Martinez 1995, Müller 1997).

In crustaceans, localizations of NOS in the cerebral ganglion of the crayfish (*Pacifastacus leniusculus* and *Cambarellus montezumae*) and in

the cerebral ganglion and the ventral nerve cord of the lobster (*Homarus americanus*) have been investigated by nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry and NOS immunohistochemistry, respectively (Johansson and Carlberg 1994, Talavera et al. 1995, Scholz et al. 1998). In addition, Ca^{2+}/calmodulin-dependent NOS activity was detected in the crayfish brain (Johansson and Carlberg 1994). It was suggested that NO may participate in crayfish olfactory processes and the development of lobster neural networks (Johansson and Mellon 1998, Scholz et al. 1998). Recently, we showed that the eyestalk ganglia of the crayfish, *Procambarus clarkii*, contain Ca^{2+}/calmodulin-dependent NOS activity and localized NOS immunoreactivity to the fibers present in an optic

*To whom correspondence and reprint requests should be addressed. Tel: 886-4-7247756. Fax: 886-4-7211156. E-mail: bicylee@cc.ncue.edu.tw

chiasma (the 1st chiasma) and a neurohemal organ (the sinus gland) (Lee et al. 2000). Because the antibody used in that study was produced against mammalian enzymes, it is possible that there are other NOS enzymes that are not immunoreactive to that antibody and hence were undetected. In mammalian tissues, it has been shown that neuronal NOS activity and NADPH-d activity reside in the same molecule (Dawson et al. 1991, Hope et al. 1991). Thus, NADPH-d activity is considered a reliable histochemical marker for NOS (Norris et al. 1995) and has been widely used for NOS localization in studies of invertebrates where an antibody against native enzymes is unavailable (Cooke et al. 1994, Johansson and Carlberg 1994, Martinez et al. 1994, Talavera et al. 1995, Leake and Moroz 1996, Moroz and Gillette 1996).

In order to study the functional roles of nitric oxide, its cellular sites of production must be clarified. In this study, localization of NOS in the eyestalk of the crayfish, *P. clarkii*, was investigated by NADPH-d histochemistry and NOS biochemical assay. A distinct staining pattern of NADPH-d was revealed that is much broader than that of the NOS-immunoreactivity described previously (Lee et al. 2000). Specific staining of NADPH-d activity was completely eliminated by an inhibitor of NOS-associated NADPH-d. Furthermore, biochemical analysis showed that NADPH-d-positive but NOS-immunonegative regions of eyestalk tissues also contain significant levels of NOS activity. These results suggest that NOS is widely distributed in the crayfish eyestalk and that NO plays important roles in visual functions.

MATERIALS AND METHODS

Animals

Animals (*Procambarus clarkii* Girard, 1852) were collected in rivers of northern Taiwan by a local fisherman, kept in freshwater tanks (water temperature, 24-26°C; photoperiod, 12L:12D), and fed with commercial shrimp food (Shanghai, Pingtung, Taiwan). Adult animals of both sexes weighing ≥ 12 g were used in the present study.

NADPH-diaphorase histochemistry

Immediately after the eyestalks were ablated from ice-anesthetized animals, eyestalk tissues (Fig. 1) were dissected out in ice-cold HEPES-buffered Van Harreveld saline (200 mM NaCl, 5.4 mM KCl, 2.6 mM $MgCl_2$, 13.5 mM $CaCl_2$, pH 7.4), fixed in 0.1 M phosphate-buffered saline (PBS, pH 7.5) containing 4% paraformaldehyde (4°C, 2 h), cryoprotected in 15% sucrose in PBS (4°C, overnight), embedded in Tissue-Tek (Miles, Elkhart, IN, USA), and sectioned longitudinally (20 μm, -20°C). Tissue sections were analyzed for NADPH-d activity according to Hope et al. (as described in Beesley 1995). Briefly, tissue sections were incubated at 37°C in the dark with reaction solution (50 mM Tris-HCl, pH 8.0, 1 mM β-NADPH, 0.5 mM nitroblue tetrazolium, 0.2% Triton X-100) for 1 h, rinsed with Tris buffer, air-dried, and mounted in Canada balsam. In some experiments, sections were incubated as described above with the addition of 2,6-dichlorophenol-indophenol (DPIP), an inhibitor of NOS-associated NADPH-d (Spessert et al. 1994, Moroz and Gillette 1996), to the reaction solution to a final concentration of 1 mM. Specificity of NADPH-d staining was tested in control experiments, in which tissue sections were incubated in the reaction solution as described except that β-NADPH or nitroblue tetrazolium was omitted, or that β-NADPH was substituted by β-NADP, β-NADH, or β-NADPH; no specific staining was observed in these control sections.
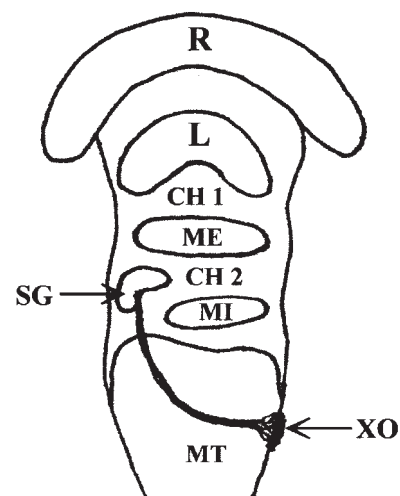

Fig. 1. A schematic diagram showing the crayfish retina (R) and eyestalk ganglia, including the lamina ganglionaris (L), medulla externa (ME), medulla interna (MI), and medulla terminalis (MT). The 1st and 2nd chiasmata (CH 1 and 2) are situated between L and ME and between ME and MI, respectively. On the lateral surface of the ganglia lies the sinus gland (SG), a discrete aggregation of nerve terminals of a cluster of neurosecretory cells, the X-organ (XO). Modified from Lee et al. (2000).

Nitric oxide synthase assay

In a previous study, we demonstrated the presence of nitric oxide synthase activity in homogenates of eyestalk ganglia (Lee et al. 2000). In order to examine the distribution of NOS activity, several well-delineated regions of the eyestalk ganglia were dissected out for the determination of NOS activity. These include (1) the sinus gland, (2) the lamina ganglionaris and medulla externa, and (3) the medulla interna and medulla terminalis (see Fig. 1). The retina was not included for analysis because its endogenous coloring would interfere with the subsequent colorimetric assay. These dissected regions were separately homogenized in extraction buffer (20 mM Tris-HCl, pH 7.2, 0.5 mM EDTA) containing 100 µg/ml phenylmethylsulfonyl fluoride, and centrifuged (10,000 xg, 4°C, 15 min). Supernatants were collected, concentrated using Microcon-30 (Millipore, Bedford, MA, USA), and used for determination of NOS activity as described previously (Lee et al. 2000). Briefly, an aliquot of the concentrate was added to the extraction buffer containing 2 mM β-NADPH, 0.45 mM $CaCl_2$, 25 U/ml calmodulin, 0.2 mM L-arginine, and 1 µM tetrahydro-L-biopterin to initiate the NOS reaction. The reaction was incubated (20°C, 6 h), terminated by heating (100°C, 10 min), and centrifuged (10,000 xg, 4°C, 15 min). The supernatant was collected and analyzed using a nitric oxide assay kit (no. 482702, CalBiochem, San Diego, CA, USA). The assay kit is based on a modified Griess method that quantifies the combined levels of nitrite and nitrate (both stable NO metabolites) as an indicator of NOS activity (Leone et al. 1995). The assay sensitivity is 0.5 nmol nitrite/well. The NOS activity was normalized to tissue proteins determined according to Bradford (1976).

RESULTS

The crayfish eyestalk contains the retina and 4 ganglia that are, from distal to proximal, the lamina ganglionaris, medulla externa, medulla interna, and medulla terminalis (Fig. 1). The first 3 ganglia, connected to each other by fibers forming the 1st and 2nd chiasmata, are collectively called the optic ganglia devoted to processing visual information received by photoreceptor cells (retinular cells) of the retina (Blaustein et al. 1988, Sandeman et al. 1992), whereas the medulla terminalis, based on its connections with other parts of the brain, probably functions as a multimodal higher-order center involved in processing olfactory, visual, and mechanical information (Blaustein et al. 1988, Derby and Blaustein et al. 1988). The medulla terminalis also contains neurosecretory cells of the X-organ whose axonal tract projects distally and ends in a neurohemal organ, the sinus gland (Andrew 1983).

Histochemical analysis revealed that NADPH-d activity is widely distributed in the crayfish eyestalk. Although staining intensity varied from preparation to preparation, a distinct staining pattern was consistently observed as described below. First, the rhabdom of the ommatidium was stained for NADPH-d (Fig. 2). It is difficult to tell whether or not the retinular cell bodies were NADPH-d-positive because these cells are heavily pigmented. Nonetheless, the less pigmented proximal portion of retinular cell axons was clearly found to be NADPH-d-positive. Fascicles of the retinular cell axons terminate in columnar optic cartridges of the lamina ganglionaris (Fig. 2). The monopolar cells situated at the distal border of the lamina ganglionaris were not stained for NADPH-d (Fig. 2). NADPH-d-positive fibers were also pre-
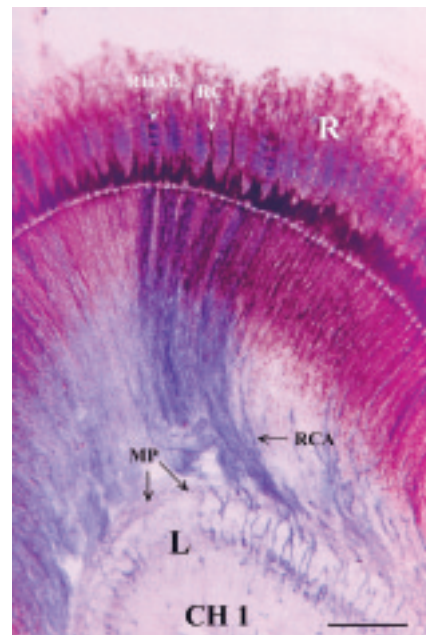

Fig. 2. Distribution of NADPH-diaphorase activity in the retina. NADPH-d activity was present in the rhabdom (RHAB) and the retinular cell axons (RCA) that project proximally and terminate in the lamina ganglionaris (L). Note that the monopolar cells (MP) located at the distal border of L were not stained for NADPH-d activity. Bar = 200 µm. Abbreviations are as indicated in figure 1.

sent in the 1st chiasma situated between the lamina ganglionaris and medulla externa. It appears that these fibers were confined to the 1st chiasma without deeply penetrating the lamina ganglionaris or medulla externa (Fig. 3A). More proximally, NADPH-d activity was detected in a prominent fiber tract located at the lateral side of the medulla terminalis (Fig. 3B). Examination of serial sections revealed that this tract branched profusely as it projected distally toward the medulla interna with fine branches terminating in the most proximal synaptic layer of the medulla interna, which was also NADPH-d-positive (Fig. 3B, C). Another NADPH-d-positive fiber tract is located at the medial side of the medulla terminalis (Fig. 3B). It then runs along the distal border of the medulla interna. Fine branches of this fiber tract were found to terminate in the most distal synaptic layer of the medulla interna, which was also NADPH-d-positive (Fig. 3B, D). Cellular origins of neither fiber tract, however, were observed. NADPH-d-positive cell somata were found scattered around the medulla externa (Fig. 3E). The staining pattern described above was completely abolished in DPIP-treated sections (data not shown).

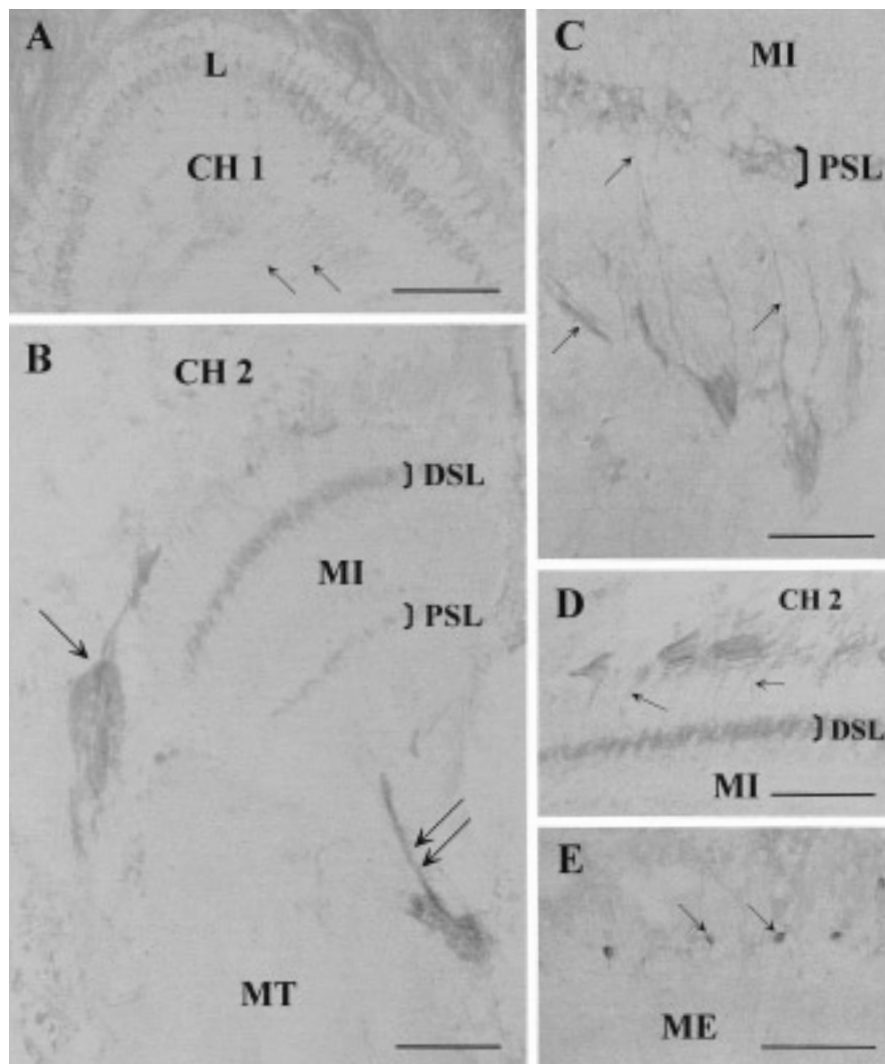The distribution of NADPH-d activity

Fig. 3. Distribution of NADPH-diaphorase activity in the eyestalk ganglia. (A) NADPH-d-positive fibers (arrows) located within CH 1; (B, C, D) NADPH-d activity present in prominent fiber tracts located at the lateral (double arrows in B) and medial (single arrow in B) sides of MT with fine branches (arrows in C, D) terminating, respectively, in the most proximal (PSL) and distal (DSL) synaptic layers of MI; (E) NADPH-d-positive cell somata (arrows) found in the distal border of ME. Bar = 200 μm(A, B), 100 μm (C, D, E). Abbreviations are as indicated in figure 1.

described above is much broader than that of NOS-immunoreactivity reported in a previous study, in which the immunoreactivity was found to be restricted to the sinus gland and the 1st chiasma (Lee et al. 2000). A question arises as to whether the NADPH-d activity described above is truly associated with NOS. An indirect way of answering this question is to determine whether or not the NADPH-d-positive but NOS-immunonegative regions contain NOS activity. Thus, the following well-delineated regions of the eyestalk ganglia were separately dissected out and analyzed for NOS activity: 1) the sinus gland, 2) the lamina ganglionaris and medulla externa (L-ME), and 3) the medulla interna and medulla terminalis (MI-MT). It was found that enzymatic activity in the L-ME and MI-MT was 2.38 ± 0.08 and 2.11 ± 0.03 pmol nitrite/mg protein/min, respectively (n = 3 each). The sinus gland contained an insignificant amount of NOS activity. Analysis of proteins extracted from up to 300 sinus glands showed that the NOS activity was below the detection limit of the assay (n = 3).

DISCUSSION

The present study provides evidence indicating that NOS is widely distributed in the eyestalk tissues of the crayfish, *Procambarus clarkii*. In a previous study, it was demonstrated that Ca^{2+}/calmodulin-dependent NOS activity was present in the eyestalk ganglia; immunological analysis indicated the presence of a 138 kDa NOS-immunoreactive protein and localized NOS-immunoreactivity (IR) to the sinus gland and 1st chiasma (Lee et al. 2000). Histochemical analysis presented in this study (Figs. 2, 3) reveals a distribution pattern of NADPH-d activity much broader than that of NOS-IR. Specifically, none of the NADPH-d-positive cell somata and fibers described in the present study were NOS-immunoreactive except for the fibers in the 1st chiasma that were both NADPH-d-positive and NOS-immunoreactive. The observed NADPH-d activity was completely inhibited by DPIP, an inhibitor of NOS-associated NADPH-d (Spessert et al. 1994, Moroz and Gillette 1996). Furthermore, biochemical analysis reveals that the MI-MT, which contained only NADPH-d activity (but not NOS-IR), also contains significant levels of NOS activity, as do the L-ME. Overall, these results indicate that the observed NADPH-d is truly NOS-associated. The observation that NADPH-d activity did not co-

localize perfectly with NOS-IR indicates the existence of NOS molecules that do not react with the antibody used in our previous study (Lee et al. 2000). In fact, studies of invertebrate tissues often detect NADPH-d activity in regions where there is no apparent NOS-IR (Cooke et al. 1994, Moroz et al. 1994). This is probably due to the fact that antibody against native NOS is not available, and the antibodies employed were raised against mammalian enzymes.

On the other hand, it seems paradoxical that both NADPH-d and NOS activities were undetectable in the sinus gland in which NOS-IR has been detected (Lee et al. 2000). A similar inconsistency has also been shown in other invertebrates (Cooke et al. 1994, Hurst et al. 1999). It has been suggested that the NOS-IR may represent inactive enzymes (Hurst et al. 1999) or that the NOS molecule may contain a NADPH binding site that is inactive after fixation (Cooke et al. 1994). In fact, a recent article pointed out that the quality of NADPH-d staining in the crustacean nervous system is relatively poor and suggested that the poor quality might be due to an unusual sensitivity of crustacean NOS to paraformaldehyde or glutaraldehyde fixation (Scholz 2001). Alternatively, the apparent NOS-IR may be due to the presence of cross-reacting non-NOS molecules. Further investigation is required to determine whether or not the sinus gland, a neurohemal organ where several well-characterized neurohormones are stored and released (Keller 1992), contains NOS.

The broad distribution of NADPH-d activity implies that NO plays various regulatory roles in the crayfish eyestalk. It is most likely that NO is involved in processing visual information, since NOS-containing elements are widely found in the retina and optic ganglia. Similarly, NADPH-d activity and/or NOS-IR have been found in the optic ganglia of various insect species (Bicker and Schmachtenberg 1997, Müller 1997, Gibbs and Truman 1998). It was suggested that NO modulates the sensitivity of photoreceptor cells in adult locust, *Schistocerca gregaria* (Bicker 1998), and regulates retinal patterning in developing *Drosophila* (Gibbs and Truman 1998). Potentially, the crayfish visual system could also be exploited as a model for investigating the roles of NO in visual functions.

The observation that the 2 NADPH-d-positive fiber tracts that appear to originate from the medulla terminalis form synaptic contacts in the neuropile of the medulla interna is interesting.

Blaustein et al. (1988) described anatomical connections between the medulla terminalis and medulla interna (the most proximal optic ganglion) and suggested that specific regions of the medulla terminalis are also involved in processing visual information. Based on its location and projection, the NADPH-d-positive fiber tract located at the lateral side of the medulla terminalis probably contributes to the optic-globular tract identified by Blaustein et al. (1988); we were unable to identify a structure described by Blaustein et al. that is analogous to the NADPH-d-positive fiber tract located at the medial side of the medulla terminalis. Whatever their identity, the presence of NADPH-d activity in these fiber tracts, which run between the medulla terminalis and medulla interna, implies that NO may be one of the neural inputs that the medulla terminalis feeds into the medulla interna with regard to the processing of visual information.

In summary, this report provides histochemical and biochemical evidence suggesting that NOS is widely distributed in the crayfish eyestalk, in contrast to a highly restricted pattern suggested by earlier immunological evidence. Presumably, more than 1 NOS isoform is present in the eyestalk. It is argued that immunological studies utilizing antibodies against mammalian enzymes may underestimate the abundance of NOS in invertebrate tissues. With proper control, NADPH-d can be used as a reliable histochemical marker for NOS. Finally, the distribution pattern suggests that NO participates in processing visual information at multiple steps. Elucidation of the specific roles played by NO awaits further investigation.

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NADPH-黃遞酶與氧化氮合成酶活性在螯蝦 (*Procambarus clarkii*) 眼柄組織之分布

鄒浤鑫　　張伊甄　　陳仕鎮　　姚秀美　　沈雅倫　　李奇英

本研究利用 NADPH-黃遞酶組織化學染色法與氧化氮合成酶生化分析法探討螯蝦 (*Procambarus clarkii*) 眼柄組織中氧化氮合成酶之分布。在視網膜中，視軸與感光細胞的軸突具有 NADPH-黃遞酶活性。在眼柄神經節，NADPH-黃遞酶活性被發現於連結視層與外髓的第一視叉中之神經纖維，以及可能源起於終髓且往遠端延伸，而終止於內髓的突觸層之兩束神經纖維束。此外，具有 NADPH-黃遞酶活性之細胞本體也在外髓的周遭被發現。在 DPIP (氧化氮合成酶-相關之 NADPH-黃遞酶抑制劑) 處理下，NADPH-黃遞酶專一性染色被完全清除。上述 NADPH-黃遞酶活性之分布遠比先前研究 (Lee et al. 2000) 所發現之氧化氮合成酶免疫活性之分布更廣泛。進一步的生化分析顯示只具有 NADPH-黃遞酶活性但不具有氧化氮合成酶免疫活性之區域 (內髓與終髓) 也具有顯著之氧化氮合成酶生化活性，顯示本研究所觀察到的 NADPH-黃遞酶即是氧化氮合成酶。這些結果指出，不同於免疫組織化學分析所顯示的侷促性分布，氧化氮合成酶在眼柄組織中有廣泛之分布，並且意含氧化氮可能參與視覺訊息之處理。

關鍵詞：氧化氮，眼柄神經節，視網膜，甲殼類動物。

國立彰化師範大學生物學系暨生物學研究所