The Journal of Experimental Biology 215, 2668-2676

doi:10.1242/jeb.066845

RESEARCH ARTICLE

Changes in the nitric oxide system in the shore crab *Hemigrapsus sanguineus* (Crustacea, decapoda) CNS induced by a nociceptive stimulus

Inessa V. Dyuizen[1,2,*], Elena P. Kotsyuba[1] and Nina E. Lamash[1,2]

[1]A. V. Zhirmunsky Institute of Marine Biology, Far East Branch of Russian Academy of Sciences, 17 Palchevsky Street, Vladivostok 690041, Russia and [2]Far Eastern Federal University, Sukhanova Street, Vladivostok 690950, Russia

*Author for correspondence (duval@mail.ru)

SUMMARY

Using NADPH-diaphorase (NADPH-d) histochemistry, inducible nitric oxide synthase (iNOS)-immunohistochemistry and immunoblotting, we characterized the nitric oxide (NO)-producing neurons in the brain and thoracic ganglion of a shore crab subjected to a nociceptive chemical stimulus. Formalin injection into the cheliped evoked specific nociceptive behavior and neurochemical responses in the brain and thoracic ganglion of experimental animals. Within 5–10 min of injury, the NADPH-d activity increased mainly in the neuropils of the olfactory lobes and the lateral antenna I neuropil on the side of injury. Later, the noxious-induced expression of NADPH-d and iNOS was detected in neurons of the brain, as well as in segmental motoneurons and interneurons of the thoracic ganglion. Western blotting analysis showed that an iNOS antiserum recognized a band at 120 kDa, in agreement with the expected molecular mass of the protein. The increase in nitrergic activity induced by nociceptive stimulation suggests that the NO signaling system may modulate nociceptive behavior in crabs.

Key words: Decapoda, *Hemigrapsus sanguineus*, nitric oxide synthase, NADPH-diaphorase, pain, brain, thoracic ganglion.

Received 14 October 2011; Accepted 5 April 2012

INTRODUCTION

The free radical gas nitric oxide (NO) has been recently identified as a neuronal messenger that performs diverse signaling functions in the nervous system. It is synthesized by the enzyme nitric oxide synthase (NOS, EC 1.14.13.39), which converts L-arginine to L-citrulline, releasing NO in the process. Immunohistochemical studies have shown that NOS is present in some neuronal cell populations in the central nervous system (CNS) of both vertebrate and invertebrate animals (Boehning and Snyder, 2003; Dawson and Snyder, 1994; Moroz, 2001). In invertebrates including crustaceans, insects and mollusks, NO-ergic neurons and nerve fibers are concentrated in primary sensory centers (Elphick et al., 1995; Johansson and Mellon, 1998; Schachtner et al., 2005; Schuppe et al., 2001) and chemo-tactile centers (Ott and Burrows, 1999; Ott and Elphick, 2002; Ott et al., 2007). Based on the cellular localization of NOS and corresponding pharmacological effects of NOS inhibitors, several studies suggest an important role for NO in neural networks that process visual, olfactory and proprioceptive information in invertebrates (Johansson et al., 1996; Robertson et al., 1996; Schuppe et al., 2001).

A significant body of information exists on the role of NO in the transmission of nociceptive signals and modulation of nociceptive reflexes in vertebrates (Millan, 1999; Yoon et al., 1998). Recent studies have demonstrated that acute and chronic noxious stimulation promotes the expression of NOS in nerve centers involved in transmission of nociceptive signals in mammals (Bayir et al., 2005; Esplugues, 2002). NADPH-diaphorase (NADPH-d) activity indicates the presence of putative NOS-containing cells (Hope and Vincent, 1989; Ishide et al., 2003; Okuda et al., 2001). For this reason, the injury-induced expression of NOS and NADPH-d in some nerve centers is considered to be a marker of their nociceptive function (Vetter et al., 2001).

The data on the presence of functional and neurochemical systems that are involved in pain experience in invertebrates are disputable and contradictory (Elwood et al., 2009; Panksepp and Huber, 2004; Tracey et al., 2003). Neurons functionally specialized for nociception have been documented in some invertebrates including the leech *Hirudo medicinalis* (Gu, 1991; Nicholls and Baylor, 1968; Pastor et al., 1996), the nematode *Caenorhabditis elegans* (Kaplan and Horvitz, 1993; Tobin et al., 2002) and the mollusks *Aplysia californica* (Lewin and Walters, 1999; Walters 1996) and *Cepaea nemoralis* (Kavaliers, 1989). Changes in neuronal activity induced by noxious stimuli were registered in the nervous centers of *C. elegans* (Wittenburg and Baumeister, 1999), *Drosophila melanogaster* (Tobin and Bargmann, 2004) and larval *Manduca sexta* (Walters et al., 2001). However, little is known about the action of nitric oxide in local circuits that process nociceptive signals in invertebrates. Changes in NO production induced by exogenous noxious stimulation were recorded in neurons of *Aplysia* (Sung et al., 2004), *H. medicinalis* (Yahyavi-Firouz-Abadi et al., 2007) and *Megalobulimus abbreviates* (Rigon et al., 2009). There is some physiological evidence that NO is involved in behavior elicited by the tissue injury; NO-mediated signals seem to reduce nociception in the land snail *Cepaea nemoralis* (Kavaliers et al., 1998) and are important for long-term hyperexcitability after noxious stimulation in *Aplysia* (Lewin and Walters, 1999).

In view of the potential biological significance of NO in nociception, we investigated the distribution of NO-egric neurons after peripheral nociceptive stimulation (formalin injection into the cheliped) in the CNS of the shore crab *Hemigrapsus sanguineus* (Crustacea, Decapoda).

MATERIALS AND METHODS

Experimental procedures

Subcutaneous injection of formalin into the hind paw has been generally accepted as a reliable method for the study of inflammatory pain in mammalian species (Okuda et al., 2001). In the present work, this method was adapted for studying nociceptive processes in crustaceans. Adult male shore crabs, *Hemigrapsus sanguineus* (De Haan 1853) (Decapoda, Grapsidae), with a carapace width of 43–45 mm and weighing approximately 30–40 g, were used in the present study. The animals were caught in the Peter the Great Bay (Japan Sea) and were kept in aquaria prior to use. The animals were divided into three groups: intact animals, experimental group and control group. Experimentally naive animals with all appendages intact were used as a control. Animals of the experimental group were injected into the propodite of the right cheliped at the propodite–dactylopropodite joint with 1% formalin in artificial seawater at a dosage of 0.0005 ml g^{-1} body mass (15–20 µl per animal). Control animals were injected with the same volume of artificial seawater. Before dissection, animals were anesthetised with an isotonic (0.36 mol l^{-1}) $MgCl_2$ solution injected into the body. Samples were taken at 10-min intervals for 1 h after injection (five to eight animals at each time point).

Determination of NADPH-d/NOS

The brain and thoracic ganglia were rapidly dissected and fixed in 4% paraformaldehyde in 0.1 mol l^{-1} phosphate buffer (PB) pH 7.2 for 2 h at 4°C. After fixation, the tissues were washed in several changes of PB, infiltrated with 30% sucrose, frozen, and sectioned on a cryostat at a thickness of 40 µm. Serial sections were mounted on poly-lysine-coated slides, air-dried and then processed for the visualization of NADPH-d activity or inducible nitric oxide synthase (iNOS) immunoreactivity. For NADPH-d histochemistry (Hope and Vincent, 1989), sections were incubated at 37°C for 1 h in 0.5 mmol l^{-1} β-NADPH (Sigma-Aldrich Inc., St Louis, MO, USA; Cat. No. N1630), 0.5 mmol l^{-1} Nitro Blue Tetrazolium (Sigma-Aldrich Inc.; Cat. No. N6876) and 0.3% Triton X-100 in 0.15 mol l^{-1} Tris-HCl buffer (pH 8.0). Control sections were incubated in the same media but without β-NADPH. The sections were then washed in 0.05 mol l^{-1} Tris-HCl pH 7.6, dehydrated in alcohol and mounted in Canadian balsam.

For iNOS localization, the cryostat sections were incubated in 1% H_2O_2 solution to suppress endogenous peroxidase activity and in 1% normal goat serum to suppress non-specific binding. They were then incubated with rabbit polyclonal antibodies against iNOS (Cayman Chemical Co., Ann Arbor, MI, USA; Cat. No. 160862; 1:100) for 18 h at 4°C. After washing in three changes of 0.1 mol l^{-1} phosphate-buffered saline (PBS) (pH 7.2), the sections were incubated with biotinylated secondary antibodies against rabbit immunoglobulin (Ig) (Vector Laboratories Inc., Burlingame, CA, USA; Cat. No. BA-1000; 1:100) for 2 h. Following another wash, sections were incubated with avidin-peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories Inc.; Cat. No. PK-6100) in darkness at room temperature for 1 h, and washed in three changes of PBS. The reaction products were developed by incubating the sections with the VIP Substrate Kit (Vector Laboratories Inc.; Cat. No. SK-4600) under microscopic control. The sections were then washed in three changes of PBS, dehydrated through an ethanol series of increasing concentrations, and mounted in Canadian balsam.

To reveal cytoarchitectonic structures, some parallel sections were Nissl stained. In describing the structures of the brain, we used the standard anatomical nomenclature proposed by Sandeman et al. (Sandeman et al., 1992). Part of the protocerebrum was not examined in this study, including the eyestalks with lamina ganglionaris, medulla externa, medulla interna and medulla terminalis.

Western blotting

For western blotting, the non-fixed brain and thoracic ganglion, respectively, were thawed and homogenized with a Potter–Elvehjem Teflon®–glass homogenizer in 0.3 ml of ice-cold homogenization buffer (20 mmol l^{-1} Tris-HCl buffer, pH 7.5, containing 0.25 mol l^{-1} sucrose, 10 mmol l^{-1} EGTA, 2 mmol l^{-1} EDTA, and protease inhibitors: 2 mmol l^{-1} PMSF, 50 mg ml^{-1} leupeptin, 25 mg ml^{-1} aprotinin, 10 mg ml^{-1} pepstatin A, and 2 mmol l^{-1} dithiothreitol). The homogenate was centrifuged for 20 min at 15,000 ***g*** in a Beckman Ti50 rotor. All samples (40 µg protein/lane) were separated on 10% SDS-PAGE (Laemmli, 1970). After electrophoresis, the proteins were transferred on a nitrocellulose membrane and left overnight in 0.01 mol l^{-1} TBS (0.01 mol l^{-1} Tris-HCl, pH 8.0, 0.15 mol l^{-1} NaCl) containing 4% BSA. The membranes were rinsed in distilled water and incubated with primary antibody in TBS containing 1% BSA and 0.2% Tween-20 for 3 h at room temperature. The membranes were then washed by shaking in TBS containing 0.2% Tween-20 and incubated with secondary antibodies (goat anti-rabbit alkaline phosphatase conjugated IgG; Vector Laboratories Inc.; Cat. No. AP-1000) in the same buffer for 1 h. After triple washing for 10 min each, the membranes were placed in TNM buffer (0.1 mol l^{-1} Tris-HCl, pH 9.0; 0.1 mol l^{-1} NaCl; 0.005 mol l^{-1} $MgCl_2$) containing 5-bromo-4-chloro-3-indolyl phosphate (100 µg ml^{-1}) and nitro blue tetrazolium (200 µg ml^{-1}). After development of the staining, the membranes were washed in distilled water and dried. The developed blots were scanned with a Bio-Rad GS 670 imaging densitometer to enable quantification of the immunoblots. Molecular mass determinations were based on prestained molecular mass markers (Sigma-Aldrich Inc., St Louis, MO, USA).

Behavior

Immediately after injection, animals were returned into the aquarium and free-moving animals were filmed for 60 min after injury using an S-VHS video camcorder (Hitachi Ltd, Marunouchi, Chiyoda-ku, Tokyo, Japan; Cat. No. VM-57200E). The recordings were analyzed using stop-frame video playback and a computer video acquisition system (miroVideo DC20).

Pain-related behavior was evaluated in three ways. First, the analysis of the direct rubbing was performed according to the method described by Barr et al. (Barr et al., 2007). Briefly, the number of contacts of the injured claw with the untreated cheliped or walking legs was recorded over a 1-min observation period. Second, we analyzed the delay of autotomy of injured cheliped (in seconds) and determined the percentage of animals showing this behavior. Third, we analyzed the comparative usage of affected and unaffected cheliped in locomotion. For this purpose, we counted how many times the tip of the injured or uninjured cheliped, respectively, contacted the floor and was retracted again during walking. This parameter seems to have close analogy with data obtained from mammals using the ‘incapacitance test’; in mammal species, pain activity was often assessed by differences in weight distribution of the injured hindlimb compared with the intact hindlimb using a weight-bearing-test (Min et al., 2001; Nakazato-Imasato and Kurebayashi, 2009). Recently, the weight-bearing deficit of injured limbs has been proposed as an objective index of pain intensity, observed in various types of rodent pain models (Min et al., 2001; Nakazato-Imasato and Kurebayashi, 2009). In our experimental protocol, the locomotion activity of the injured claw was calculated

as a percentage of total placements and repulsions of both chelipeds from the floor of the aquarium during walking.

Data analysis

The NADPH-d activity was estimated using an automatic analysis system, Allegro-MC (Afanasyev et al., 2002). For this purpose, a Nikon-Eclipse E-600 microscope (Nikon Co., Tokyo, Japan) was employed together with VideoTest 5.0 software (VideoTest Software, St Petersburg, Russia). Digitized images of sections were obtained from the selected areas using a high-resolution CCD camera (Pro-Series High Performance Digital Microscopy Camera AxioCam ICc3 Rev.3, Gottingen, Germany). Five sections were taken from the brain and thoracic ganglion of every animal. The optical density of every NO-positive element was measured in five randomly selected vision fields and evaluated in conventional optical density units (ODU). Additionally, the size of NADPH-d-positive neurons was measured using the same software.

The significance of differences was assessed by Student's *t*-test and Mann–Whitney nonparametric *U*-test (for analysis of the data of behavior experiments) or two-way ANOVA Bonferroni test (in the case of histochemical analyses), using Prism 4 software (GraphPad). $P<0.05$ was considered significant. Data are presented as means ± standard error of the mean (s.e.m.).

RESULTS

Behavior

Immediately after the injection of formalin or physiological solution into the cheliped, animals moved quickly into the corner of the aquarium and stopped moving at 2–3 s. In this 'freezing' period, animals were motionless when their untreated claw was in the lifting position [according to the classification of Mariappan et al. (Mariappan et al., 2000)] and the injected claw was in the falling (downward deflection) position. Later on, formalin-treated crabs differed considerably in behavior from the control crabs. Throughout the next period (1–3 min), the experimental animals were fidgety and performed a lot of movements: flexion, extension, shaking or rubbing the affected claw. In the group of formalin-treated animals, considerably more active rubbing behavior (mean frequency 20±4.3 min^{-1}) was observed during the first minute of the observation period compared with saline-treated crabs (mean frequency 0.9±0.2 min^{-1}) (Mann–Whitney *U*-test, $P<0.0001$). Intense rubbing of the claw resulted in autotomy in 20% of animals of the formalin-treated group. In these animals, the time to autotomy was 32.7±7.9 s from the moment of injection. Saline-injected crabs did not autotomize the stimulated cheliped, and there was no difference in movement activity between saline-treated individuals and controls.

During the next 10 min after injection, we analyzed the comparative activity of treated and untreated chelipeds in locomotion. The walking pattern of formalin-treated crabs differed markedly from that of saline-treated animals. During locomotion (in any direction), animals in the formalin-treated group tried to use the intact cheliped, guarding the damaged cheliped. The reduction in usage of the affected cheliped was maximal 3 min post-injury (20.4±3.1% of all claw contacts with the ground), diminished over the next 5 min (39.6±3.8%) and was no longer apparent 10 min post-injection. There were no differences in walking activity of both chelipeds (50%:50%) in saline-treated crabs throughout the entire observation period.

Between moving episodes, crabs of both groups were motionless, pressing both chelipeds against the carapace. However, the majority of formalin-treated animals demonstrated an asymmetrical position of the chelipeds; they seemed to press their injured cheliped closer to the carapace compared with the intact cheliped until the end of experiment.

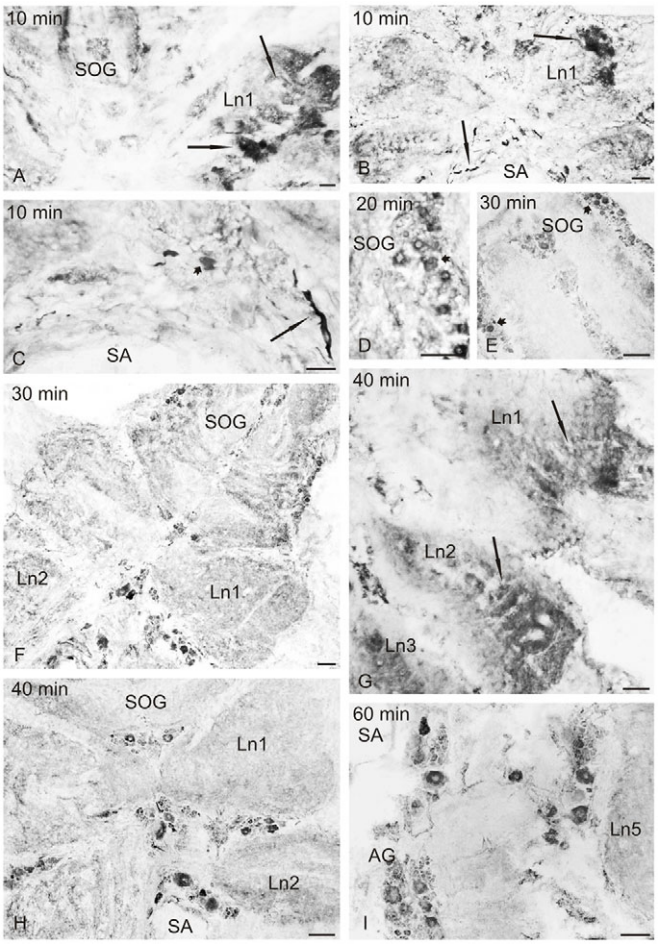

Fig. 1. Dynamics of NADPH-d/iNOS activity in the thoracic ganglion of the shore crab (*Hemigrapsus sanguineus*) after formalin injection. Sections were NADPH-d labeled (A–D and G) or labeled with anti-NOS rabbit antibody (E,F,H,I; 1:200 dilution). (A,B) Ten minutes after nociceptive injury. Fibers in the neuropils of the claw neuromere on the ipsilateral side (arrows) are intensely stained. (C) NADPH-d-positive fibers (arrows) and neurons (arrowheads) in the leg neuromeres 1–2 (Ln 1–2). (D) NADPH-d in neurons (arrowheads) of the subesophageal ganglion (SOG) 20 min after injury. (E) iNOS in neurons (arrowheads) of the SOG 30 min after nociceptive injury. (F) iNOS-immunoreactive neurons in the SOG and Ln 1–2. (G) Increased NADPH-d activity (arrows) in the neuropils of leg neuromeres 1–5. (H,I) Increased iNOS activity in the segmental interneurons and motoneurons of (H) the leg ganglion (LG) and (I) the abdominal ganglion (AG). SA, sternal artery. Scale bars, 100 μm.

NADPH-d/NOS activity

In intact animals, NADPH-d activity was observed in the brain and fused thoracic ganglion (TG), which consists of the subesophageal (SOG), leg (LG) and abdominal ganglia (AG). In the brain, the product of the histochemical reaction was localized mainly in the olfactory deutocerebrum, which corresponds to the data reported previously (Kotsyuba et al., 2010). The thoracic ganglion revealed low NADPH-d reactivity in neuropils and absence of activity in neurons. Distribution of NADPH-d in the brain of control crabs did not differ from that in intact animals and was unchanged throughout

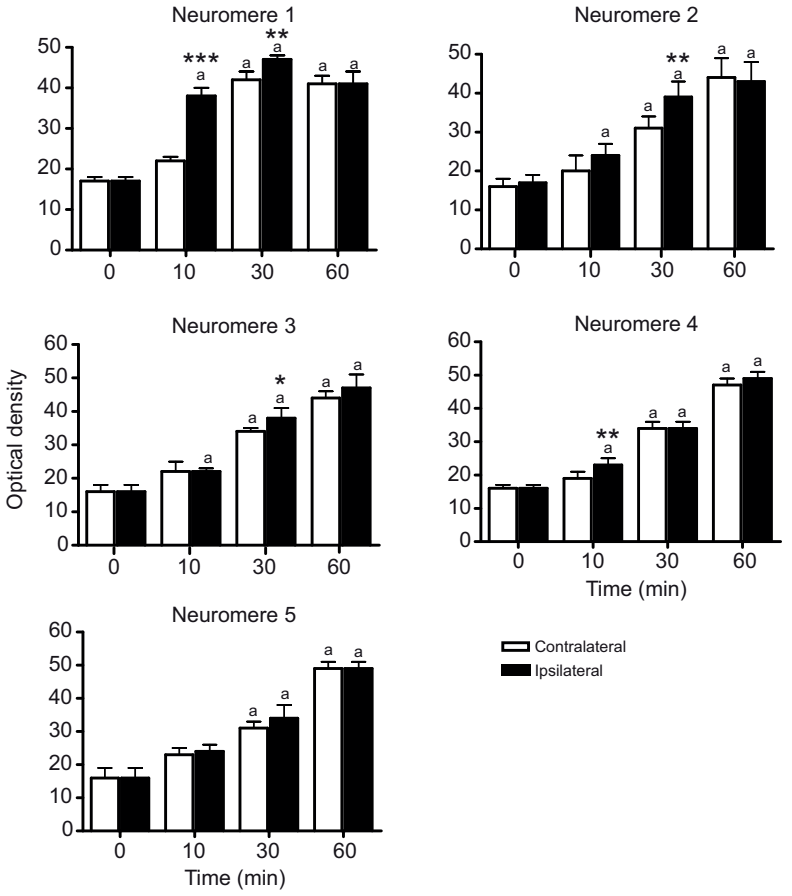

Fig. 2. The temporal dynamics of NADPH-d activity (optical density) in the leg neuromeres 1–5 of *H. sanguineus* induced by nociceptive stimulation. Open bars, the side contralateral to injury; filled bars, the side ipsilateral to injury. In the graphs, lowercase a represents $P<0.05$ when compared with intact (and control) animals whose activity was unchanged during the observation period; asterisks indicate the difference obtained when contralateral and ipsilateral sides of the same neuromere were compared (*$P<0.05$, **$P<0.01$, ***$P<0.001$) (two-way ANOVA, Bonferroni test). Results represent the mean ± s.e.m. from five different sections of five animals.

the experiment. In the nerve centers of intact and control animals, iNOS immunoreactivity was negative throughout the experiment. In experimental animals after formalin injection, a consistent increase in the number and staining intensity of NADPH-d-containing nerve structures and a change in their distribution were registered. In the same period, the expression of iNOS in the neurons of the thoracic ganglion and brain was also detected.

Thoracic ganglion

Ten minutes after formalin injection, on the side of injury, the intensity of stained sensory fibers in the neuropils of the claw neuromere increased more than twofold compared with controls (Fig. 1A, Fig. 2). On the contralateral side, the histochemical staining of neuropils did not significantly differ from the control (Fig. 2). In the LG, NADPH-d activity was visible in large nerve fibers surrounding the sternal artery and in some neurons, with a diameter of 65–75 μm, located anteromedially to the sternal artery (Fig. 1B,C).

Twenty minutes after injury, NADPH-d expression was observed in small (15-20 μm in diameter) and larger (more than 30 μm) neurons in all neuromeres of the SOG (Fig. 1D). Thirty minutes after injection, these cells were also stained with anti-iNOS (Fig. 1E,F). In the LG, iNOS immunoreactivity appeared in the medial and lateral group of neurons located in the midline and laterally from it, between the neuropils, in both the first and second leg neuromeres, predominantly on the side of injury (Fig. 1F). In each group, the single segmental neuron with a diameter of 40–60 μm with high enzyme activity and several small cells (less than 30 μm in diameter) with low and moderate enzyme activity were labeled. However, despite the marked histochemical changes, the immunoblotting results suggest that iNOS expression is weak during this period (Fig. 3).

Forty to sixty minutes after injury, histochemical changes were determined in the LG on the ipsi- and contralateral sides and also in the AG. In this period, NADPH-d activity increased in all neuropils and in neurons of leg neuromeres 1–5 (Fig. 1G, Fig. 2). The high iNOS immunoreactivity appeared in large segmental neurons with a diameter of 60–80 μm, located in the leg neuromeres

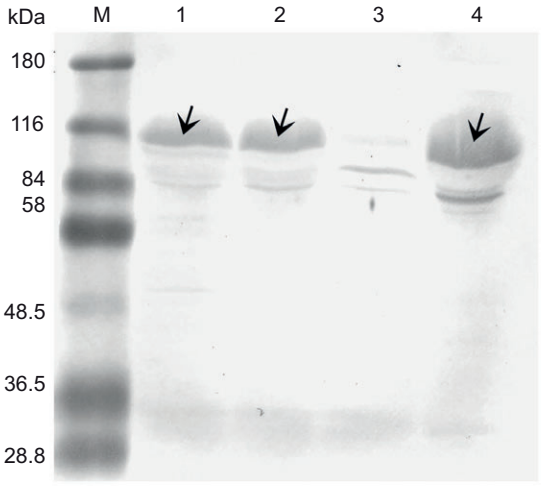

Fig. 3. Western blot showing the expression of iNOS (arrows) in the thoracic ganglion and brain of *H. sanguineus* in different periods after nociceptive stimulation. M, molecular-mass markers (kDa); 1, brain, 30 min; 2, brain, 60 min; 3, thoracic ganglion, 30 min; 4, thoracic ganglion, 60 min after nociceptive injury.

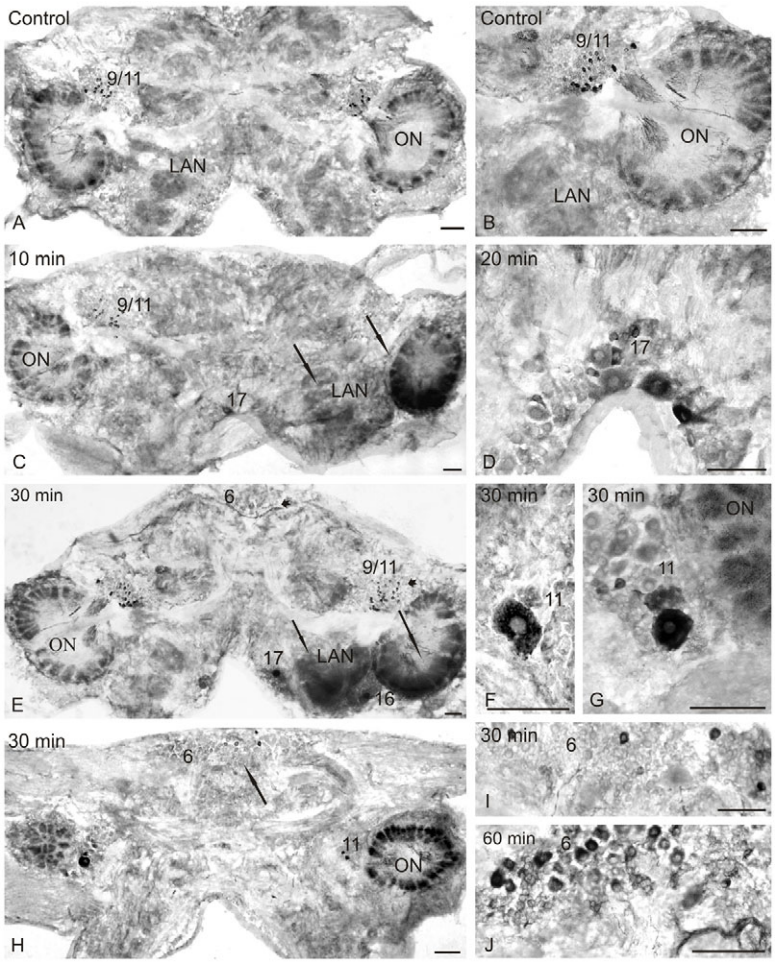

Fig. 4. Dynamics of NADPH-d/iNOS activity in the brain of *H. sanguineus* after nociceptive stimulation. Horizontal sections are NADPH-d labeled (A–E,G–I) or anti-iNOS immunostained (F). (A,B) Distribution of NADPH-d in the olfactory lobe (ON), the lateral antenna 1 neuropils (LAN), antenna II neuropils (AnN) and cluster 9/11 is shown in control animals. (B) Only the right brain hemisphere is shown. (C) Ten minutes after nociceptive injury, NADPH-d staining increased in the ON, LAN (arrows) and cluster 17. (D) Twenty minutes after nociceptive injury, the activity of NADPH-d in neurons of cluster 17 increased on the side of injury. (E–I) Thirty minutes after injury, NADPH-d activity maximally increased in the ON and LAN (arrows), and solitary NADPH-d-positive neurons appeared in clusters 9/11 and 6 (arrowheads). (F) iNOS immunoreactivity in a large neuron of cluster 11 30 min after injury. (G) Localization of NADPH-d in a large neuron of cluster 11. (H,I) NADPH-d-positive neurons that appeared in cluster 6. (J) Sixty minutes after nociceptive injury, the number and activity of NADPH-d-positive neurons gradually increased in cell cluster 6. Scale bars, 100 μm.

1–5 on both sides of the TG (Fig. 1H). More dorsally, in the fused AG, the iNOS-immunoreactive neurons with a diameter of 60–80 μm and smaller cells 15–20 μm (Fig. 1I) were intensely stained. The immunoblotting showed that iNOS expression reached maximum in neurons of the TG 60 min after formalin injection (Fig. 3).

Brain

In the brain of intact and control crabs, NADPH-d activity was observed mainly in the glomerular neuropil of the olfactory lobes (ON), the lateral antenna I neuropils (LAN), and the medial cell group [MC, or cluster 9/11 according to the Sandeman's terminology (Sandeman et al., 1992)] (Fig. 4A,B).

Ten minutes after injury, in the ipsilateral deutocerebrum, NADPH-d activity increased markedly in the periphery of olfactory neuropils and in the lateral antenna I neuropils (Fig. 4C, Fig. 5). On the contralateral side, NADPH-d activity did not change compared with controls (Fig. 4C). In the tritocerebrum, in the same period, 1–2 pear-shaped neurons 50–60 μm in diameter were stained in a group of posterior medial cells (cluster 17). They displayed high NADPH-d activity. In addition, an increase in NADPH-d activity in the ipsilateral antenna II neuropil (AnN) was observed (Fig. 5).

Twenty minutes after formalin injection, the number of NADPH-d-positive neurons in cluster 17 was increased; they are located at the middle plane of the brain between the roots of the esophageal connectives. In particular, small elliptical cells 15–20 μm in diameter were stained (Fig. 4D).

Thirty minutes after nociceptive injury, the maximum of NADPH-d activity was observed in the olfactory neuropils, lateral antenna I neuropils and antenna II neuropils (Fig. 5) and in a solitary neuron in a group of posterior medial cells (cluster 16) on the side of injury (Fig. 4E). In the dorsal part of cluster 11, 1–2 large olfactory interneurons (60 μm in diameter) were intensely stained. These neurons demonstrated a high level of iNOS immunoreactivity at this point (Fig. 4F,G). In the protocerebrum, some NADPH-d-positive neurons (20–30 μm in diameter) in a group of anterior medial cells (cluster 6) were stained (Fig. 4H,I). They had rounded or elliptic perikarya and displayed no iNOS immunoreactivity. Their number and histochemical activity gradually increased during 60 min after injury (Fig. 4J).

Western blotting

Using immunoblotting, no traces of iNOS were detected in the total brain extract of intact and control animals throughout the experiment. In experimental animals, anti-iNOS labeling was also negative during the first half of the experiment. Thirty minutes after formalin injection, immunoblotting showed the presence of a 116–120 kDa polypeptide antigen reactive to rabbit antibodies against iNOS (Fig. 3). Sixty minutes after formalin injection, its concentration in the crab CNS increased noticeably.

DISCUSSION

As determined in this study, the tissue-damaging stimulus evokes specific nociceptive behavioral responses and consistent

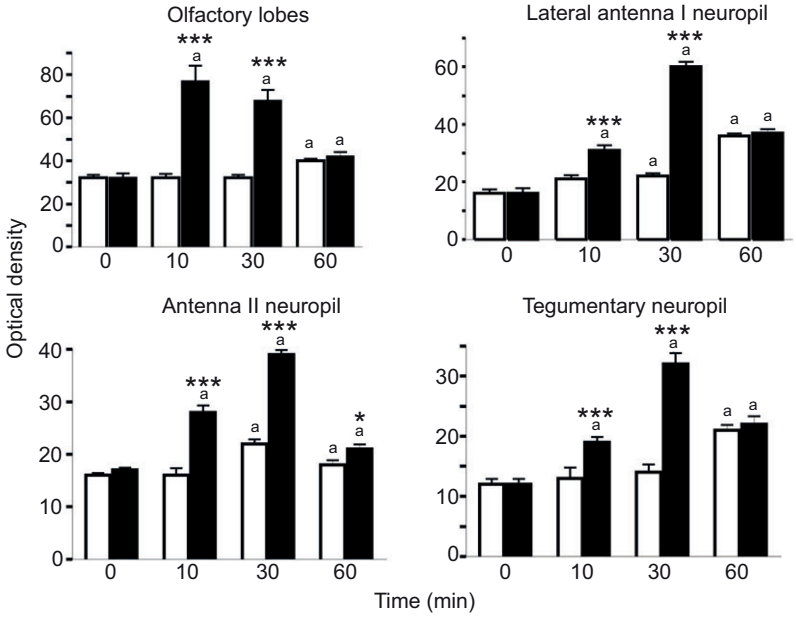

Fig. 5. The temporal dynamics of NADPH-d activity (optical density, mean ± s.d.) in brain regions of *H. sanguineus* after nociceptive stimulation. Open bars, the side contralateral to injury; filled bars, the side ipsilateral to injury. In the graphs, lowercase a represents $P<0.05$ when compared with intact (and control) animals whose activity was unchanged during the observation period; asterisks indicate the difference obtained when contralateral and ipsilateral sides of the same neuromere were compared (*$P<0.05$, **$P<0.01$, ***$P<0.001$) (two-way ANOVA, Bonferroni test). Results represent the mean ± s.e.m. from five different sections of five animals.

neurochemical responses of the shore crab *Hemigrapsus sanguineus*. Injection of formalin into the cheliped triggers a complex behavioral pattern composed of the immediate withdrawal reflexes (rapid escape) and integrated pain-related responses. Immediate reaction to the injection occurred in the nearest 3 s, when animals quickly moved into the corner of their aquarium and stopped moving. This protective motor reaction has been described as an escape response to nociceptive stimulation in many decapod crustaceans as well as insects (Barr et al., 2007; Kavaliers, 1989; Tobin and Bargmann, 2004). It enables the animal to move away from the source of aversive stimulus (Elwood and Appel, 2009). Because this behavior was observed in both formalin-treated and saline-treated crabs, it may be considered as an escape response to the injection *per se*.

Subsequent to the initial protective motor reaction, behavior changed significantly. Formalin-treated animals responded to injury by intense rubbing of the injured claw during the first 3 min after injection. In saline-treated animals, this reaction was inconsequential. Similar behavior has been seen previously in prawn, *Palaemon elegans*, as a response to chemical stimulation of antenna (Barr et al., 2007). The authors suggest that the animal is attempting to ameliorate the painful effect of the stimulus by grooming or rubbing the affected area. This specifically directed activity may be interpreted as a variant of pain-related behavior and might indicate an awareness of the specific site of stimulus application and seems to be more than a reflex reaction (Elwood et al., 2009; Elwood, 2011).

Another well-known type of protective motor response seen in arthropods is autotomy of the injured body segment (Elwood et al., 2009). In the present study, formalin, which typically induces pain in vertebrates (Okuda et al., 2001), can cause autotomy of injured chelipeds when injected into the claw of a crab, whereas saline injection does not. It has been shown in several studies in invertebrates, including crustaceans, that the autotomy of appendages may be elicited by heat stimulation (Fiorito, 1986), application of a minor electric shock and acetic acid injection (Elwood et al., 2009). The autotomy in crustaceans seems to be dose dependent (I.V.D., E.P.K. and N.E.L., unpublished observation) and environment dependent (Kotsyuba et al., 2010), and results obtained previously are consistent with pain mediation of the autotomy response (see Elwood, 2011).

Besides, our study reveals the asymmetrical usage of the chelipeds in crab locomotion during the first 10 min after injury. Separate evaluation of 'steps' performed by damaged and undamaged chelipeds during walking was attempted here by analogy with the incapacitance test, which has been widely employed for quantitative assessment of pain in mammals (Min et al., 2001; Nakazato-Imasato and Kurebayashi, 2009). In our study, there was a great reduction in the walking activity of the damaged cheliped whereas use of the intact cheliped in locomotion was increased. With reference to asymmetrical hindlimb weight bearing under neuropathic pain conditions in mammals, the present results obtained in crabs may be indicative of pain experience rather than relating to a simple nociceptive reflex.

The aforementioned alterations in animal behavior were accompanied by visible changes in NO-ergic activity in neurons and neuropils of the brain and thoracic ganglia and promoted expression of iNOS as revealed by immunocytochemical analysis and western blotting. The localization, intensity, and dynamics of NADPH-d activity and iNOS immunoreactivity imply dynamic neurochemical alterations in the CNS of experimental animals. Visible changes were more pronounced on the side of injury, suggesting their specific, injury-induced nature. We did not observe an increase in NADPH-d activity or any iNOS-like activity throughout the experiment in the brains of control (saline-treated) animals. Therefore, it can be assumed that an increase in NOS activity in the CNS of formalin-treated animals appears to be among the responses to chemical stimulation of cheliped peripheral receptors, and NO may participate in the neuronal circuits involved in pain modulation.

Histochemical changes were initially recorded in thoracic ganglion and, with a slight delay, in the brain. The earliest changes in NADPH-d activity were observed in nerve fibers of the thoracic ganglion sensory neuropils, and the most prominent changes were seen in structures related to modulation of cheliped reflexes. The first minutes after injury are a time when the most prominent alterations in crab behavior were observed – intense rubbing and autotomy of the injured cheliped. Therefore, it may be assumed that the increase of NO-ergic activity in ipsilateral sensory neuropils is caused by stimulation of peripheral nociceptors. It has been shown in crustaceans that the NO-ergic nerve fibers of sensory neuropils originate from the sensory neurons of peripheral tissues (Ott et al.,

2007). Their physiology is similar to mammalian low-threshold mechanoreceptors (Byrne et al., 1974) or typical nociceptors (Illich and Walters, 1997) and their noxious-induced responses has been characterized in some invertebrate species (Shen et al., 2002), including crustaceans (Appel and Elwood, 2009; Barr et al., 2007; Elwood and Appel, 2009; Puri and Faulkes, 2010).

In the sensory neuropils, fibers from cheliped receptors make synaptic contacts with dendrites of interneurons and motoneurons (Elson, 1996; Mulloney et al., 2003). In our study, these appeared to be the neurons that display maximum NADPH-d activity shortly after injury, and iNOS immunoreactivity thereafter. Using NOS donors and inhibitors, Araki and colleagues showed that NO enhances the activity of the motoneurons modulating the local tailfan reflexes in the crayfish (Araki et al., 2004). This suggests that NO-signaling, which is enhanced in the thoracic ganglion of shore crabs in response to nociceptive chemical stimulation, could be involved not only in sensory processing but also in motor program modulation. Gradual upregulation of NOS activity in neuromere 1, and with a slower time course in neuromeres 2–5, of the thoracic ganglion may be linked to a sequential modulation of motor circuits, thus causing the specific behavioral pattern observed in response to nociceptive stimulation. The precise mechanisms, however, of how NO modulates pain-related activity have yet to be determined.

Specifically directed behavioral responses determined here (rubbing and guarding the affected cheliped during locomotion) might indicate the involvement of high nerve centers in discrimination of the injury locus and in organization of this complex behavior. Activation of the NO-ergic system in the brain, as shown in the present study, may suggest that NO is involved in the pain-related modulation of integrative brain centers. The most prominent changes in NO-ergic activity were observed in the fibers of olfactory lobes and lateral antenna I and II neuropils. These structures seem to be the main neuropils participating in multimodal sensory integration (Fedotov, 2009). Besides, an expression of NADPH-d and iNOS activity was also found in the proto-, deuto- and tritocerebrum nerve cells; under normal conditions, these neurons lack endogenous NOS.

The injury-related increase of NADPH-d and iNOS activity in the crab CNS is consistent with data obtained on insects and mollusks (Rigon et al., 2009). Thermal nociceptive stimulation enhances NOS activity in peripheral sensory neurons, nerve cells of the thoracic ganglion, and mushroom bodies of *Drosophila* (Xu et al., 2006). Walters performed a detailed study of nociceptive processes in sensory circuits of invertebrates and showed that injury-induced changes of their nociceptive neurons have some common features with central and peripheral pain mechanisms in vertebrates (Walters, 1996). Numerous studies have demonstrated that the time course of NO-ergic activity in pain-related systems of vertebrates reflects the intensity and duration of the nociceptive signal (Aley et al., 1998; Ishide et al., 2003; Luo and Cizkova, 2000; Millan, 1999). The expression of NADPH-d and/or NOS in neurons of primary nociceptive centers in mammals is mediated by the activation of glutamatergic (Gadotti et al., 2006; Soen et al., 2007; Ishide et al., 2003), substance P-ergic (Coderre and Yashpal, 1994; Sun et al., 2003) and cholinergic (Zhuo et al., 1993) neurotransmission in afferent fibers. It is possible that NO production in invertebrates is enhanced by similar mechanisms. Glutamate is known to be a neurotransmitter of afferent pathways in invertebrates (Groome and Vaughan, 1996), and the expression of NMDA-glutamate receptors functionally similar to mammalian receptors has been determined in crayfish visual interneurons (Pfeiffer-Linn and Glantz, 1991) and in mechanosensory neurons in the snail *Helix lucorum* (Bravarenko et al., 2003). According to Ushizawa and colleagues, some mechanosensory afferents in crustaceans are cholinergic (Ushizawa et al., 1996). Substance P and its specific receptors are widespread in the crustacean brain too. In crustaceans, the SP-ergic afferents are similar to nociceptive afferents in vertebrates in terms of their immunochemical profile and morphology (Schmidt, 1997). Thus, the NO production enhancement in our experiment may be partially related to activation of glutamatergic, SP-ergic or cholinergic neurotransmission in afferent fibers of the injured cheliped.

The immunocytochemical and western blotting analyses showed that an increase of NADPH-d activity in the thoracic ganglion segmental neurons and in the large medial group interneurons of the shore crab brain following nociceptive injury is mainly caused by iNOS-like expression. Only limited data exist on the induction of an iNOS-like enzyme in the invertebrate tissues. To date, Ca^{2+}/calmodulin-independent constitutive isoforms of non-neuronal NOS have been found in hemocytes of horseshoe crabs (Radomski et al., 1991), in hemocytes of some molluscs (Novas et al., 2004; Wright et al., 2006) and in Malpighian tubules of insects (Choi et al., 1995). Invertebrate iNOS was found to have a 130 kDa apparent molecular mass, and its amino acid sequence lacked a part of the N-terminal domain when compared with mammalian type I and II NOS (Kalsner, 2000). The expression of iNOS (NOS II) in mammalian neurons under acute or chronic pain conditions has been shown in several studies using immunoblotting and detection of mRNA iNOS (Riedel and Neeck, 2001). In mammals, induction of iNOS is elicited by bacterial toxins (Heneka and Feinstein, 2001) and some intercellular signaling factors, including long-term nociceptive stimulation (Millan, 1999). In the present study, iNOS-like activity was detected in neurons of the thoracic ganglion and brain, which under control conditions lack endogenous NOS. We did not perform the double staining for co-localization of iNOS and NADPH-d in this work but we analyzed the serial sections using both procedures. It makes it possible to assume that iNOS and NADPH-d were expressed in the same neurons. A number of studies demonstrate that, along with neuronal NOS, iNOS may contribute to the neuroplastic changes accompanying the acute and chronic pain conditions in mammals (see Millan, 1999).

The present results show that peripheral nociceptive injury elicits a specific pattern of behavioral reactions and activates the NO-ergic system in the crab CNS mostly by expression of an iNOS-like NOS. Increased NO levels may affect sensory signaling, central neural processing and the generation of efferent motor signals. Further investigations will be required to determine the neurochemical mechanisms of pain-modulation systems in invertebrates and to elucidate whether injury-induced expression of iNOS-like activity in their CNS has both adverse and protective effects.

LIST OF ABBREVIATIONS

AG	abdominal ganglion
AnN	antenna II neuropil
BSA	bovine serum albumin
CNS	central nervous system
iNOS	inducible nitric oxide synthase
LAN	lateral antenna I neuropil
LG	leg ganglion
Ln	leg neuromer
MC	medial cell group
NADPH-d	NADPH-diaphorase
NO	nitric oxide
NOS	nitric oxide synthase
ON	olfactory lobes
SOG	subesophageal ganglion
TG	thoracic ganglion

FUNDING

This research was supported by the Far East Branch of the Russian Academy of Sciences [grant numbers 12-III-B-06-104, 12-III-A-06-090; 12-I-P7-02] and by Russian Government [grant number 11.G34.31.0010].

REFERENCES

Afanasyev, A. A., Kotsyuba, A. E. and Chertok, V. M. (2002). The system Allegro-MC for automatic analysis of images of micro- and macrostructures. *Tihookeansky Meditsinsky Zhurnal* **3**, 65-68.

Aley, K. O., McCarter, G. and Levine, J. D. (1998). Nitric oxide signaling in pain and nociceptor sensitization in the rat. *J. Neurosci.* **18**, 7008-7014.

Appel, M. and Elwood, R. W. (2009). Motivational trade-offs and potential pain experience in hermit crabs. *Appl. Anim. Behav. Sci.* **119**, 120-124.

Araki, M., Schuppe, H., Fujimoto, S., Nagayama, T. and Newland, P. L. (2004). Nitric oxide modulates local reflexes of the tailfan of the crayfish. *J. Neurobiol.* **60**, 176-186.

Barr, S., Laming, P., Dick, J. T. A. and Elwood, R. W. (2007). Nociception or pain in a decapod crustacean? *Anim. Behav.* **75**, 745-751.

Bay, H., Kagan, V. E., Borisenko, G. G., Tyurina, Y. Y., Janesko, K. L., Vagni, V. A., Billiar, T. R., Williams, D. L. and Kochanek, P. M. (2005). Enhanced oxidative stress in iNOS-deficient mice after traumatic brain injury: support for a neuroprotective role of iNOS. *J. Cereb. Blood Flow Metab.* **25**, 673-684.

Boehning, D. and Snyder, S. H. (2003). Novel neural modulators. *Annu. Rev. Neurosci.* **26**, 105-131.

Bravarenko, N. I., Korshunova, T. A., Malyshev, A. Y. and Balaban, P. M. (2003). Synaptic contact between mechanosensory neuron and withdrawal interneuron in terrestrial snail is mediated by L-glutamate-like transmitter. *Neurosci. Lett.* **341**, 237-240.

Byrne, J., Castellucci, V. and Kandel, E. R. (1974). Receptive fields and response properties of mechanoreceptor neurons innervating siphon skin and mantle shelf in *Aplysia*. *J. Neurophysiol.* **37**, 1041-1064.

Choi, S. K., Choi, H. K., Kadono-Okuda, K., Taniai, K., Kato, Y., Yamamoto, M., Chowdhury, S., Xu, J., Miyanoshita, A., Debnath, N. C. et al. (1995). Occurrence of novel types of nitric oxide synthase in the silkworm, *Bombyx mori*. *Biochem. Biophys. Res. Commun.* **207**, 452-459.

Coderre, T. J. and Yashpal, K. (1994). Intracellular messengers contributing to persistent nociception and hyperalgesia induced by L-glutamate and substance P in the rat formalin pain model. *Eur. J. Neurosci.* **6**, 1328-1334.

Dawson, T. M. and Snyder, S. H. (1994). Gases as biological messengers: nitric oxide and carbon monoxide in the brain. *J. Neurosci.* **14**, 5147-5159.

Elphick, M., Rayne, R., Riveros-Moreno, V. V., Moncada, S. and Shea, M. (1995). Nitric oxide synthesis in locust olfactory interneurones. *J. Exp. Biol.* **198**, 821-829.

Elson, R. C. (1996). Neuroanatomy of a crayfish thoracic ganglion: sensory and motor roots of the walking-leg nerves and possible homologies with insects. *J. Comp. Neurol.* **365**, 1-17.

Elwood, R. W. (2011). Pain and suffering in invertebrates? *ILAR J.* **52**, 175-184.

Elwood, R. W. and Appel, M. (2009). Pain experience in hermit crabs? *Anim. Behav.* **77**, 1243-1246.

Elwood, R. W., Barr, S. and Patterson, L. (2009). Pain and stress in crustaceans? *Appl. Anim. Behav. Sci.* **118**, 128-136.

Esplugues, J. V. (2002). NO as a signalling molecule in the nervous system. *Br. J. Pharmacol.* **135**, 1079-1095.

Fedotov, V. P. (2009). Systems of chemoperception in decapod crayfish. *Zh. Evol. Biokhim. Fiziol.* **45**, 3-24.

Fiorito, G. (1986). Is there 'pain' in invertebrates? *Behav. Processes* **12**, 383-388.

Gadotti, V. M., Tibola, D., Paszcuk, A. F., Rodrigues, A. L., Calixto, J. B. and Santos, A. R. S. (2006). Contribution of spinal glutamatergic receptors to the antinociception caused by agmatine in mice. *Brain Res.* **1093**, 116-122.

Groome, J. R. and Vaughan, D. K. (1996). Glutamate as a transmitter in the sensory pathway from prostomial lip to serotonergic Retzius neurons in the medicinal leech *Hirudo*. *Invert. Neurosci.* **2**, 121-128.

Gu, X. N. (1991). Effect of conduction block at axon bifurcations on synaptic transmission to different postsynaptic neurones in the leech. *J. Physiol.* **441**, 755-778.

Heneka, M. T. and Feinstein, D. L. (2001). Expression and function of inducible nitric oxide synthase in neurons. *J. Neuroimmunol.* **114**, 8-18.

Hope, B. T. and Vincent, S. R. (1989). Histochemical characterization of neuronal NADPH-diaphorase. *J. Histochem. Cytochem.* **37**, 653-661.

Illich, P. A. and Walters, E. T. (1997). Mechanosensory neurons innervating *Aplysia* siphon encode noxious stimuli and display nociceptive sensitization. *J. Neurosci.* **17**, 459-469.

Ishide, T., Maher, T. J. and Ally, A. (2003). Role of nitric oxide in the ventrolateral medulla on cardiovascular responses and glutamate neurotransmission during mechanical and thermal stimuli. *Pharmacol. Res.* **47**, 59-68.

Johansson, K. U. I. and Mellon, D., Jr (1998). Nitric oxide as a putative messenger molecule in the crayfish olfactory midbrain. *Brain Res.* **807**, 237-242.

Johansson, K. U. I., Wallen, R. and Hallberg, E. (1996). Electron microscopic localization and experimental modification of NADPH-diaphorase activity in crustacean sensory axons. *Invert. Neurosci.* **2**, 167-173.

Kalsner, S. (2000). *Nitric Oxide and Free Radicals in Peripheral Neurotransmission*. New York: Springer-Verlag.

Kaplan, J. M. and Horvitz, H. R. (1993). A dual mechanosensory and chemosensory neuron in *Caenorhabditis elegans*. *Proc. Natl. Acad. Sci. USA* **90**, 2227-2231.

Kavaliers, M. (1989). Evolutionary aspects of the neuromodulation of nociceptive behaviors. *Am. Zool.* **29**, 1345-1353.

Kavaliers, M., Choleris, E., Prato, F. S. and Ossenkopp, K. (1998). Evidence for the involvement of nitric oxide and nitric oxide synthase in the modulation of opioid-induced antinociception and the inhibitory effects of exposure to 60-Hz magnetic fields in the land snail. *Brain Res.* **809**, 50-57.

Kotsyuba, E. P., Dyuizen, I. V. and Lamash, N. E. (2010). Stress-induced changes in the nitric oxide system of shore crabs living under different ecological conditions. *Russ. J. Mar. Biol.* **36**, 201-208.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lewin, M. R. and Walters, E. T. (1999). Cyclic GMP pathway is critical for inducing long-term sensitization of nociceptive sensory neurons. *Nat. Neurosci.* **2**, 18-23.

Luo, Z. D. and Cizkova, D. (2000). The role of nitric oxide in nociception. *Curr. Rev. Pain* **4**, 459-466.

Mariappan, P., Balasundaram, C. and Schmitz, B. (2000). Decapod crustacean chelipeds: an overview. *J. Biosci.* **25**, 301-313.

Millan, M. J. (1999). The induction of pain: an integrative review. *Prog. Neurobiol.* **57**, 1-164.

Min, S. S., Han, J. S., Kim, Y. I., Na, H. S., Yoon, Y. W., Hong, S. K. and Han, H. C. (2001). A novel method for convenient assessment of arthritic pain in voluntarily walking rats. *Neurosci. Lett.* **308**, 95-98.

Moroz, L. L. (2001). Gaseous transmission across time and species. *Am. Zool.* **41**, 304-320.

Mulloney, B., Tschuluun, N. and Hall, W. M. (2003). Architectonics of crayfish ganglia. *Microsc. Res. Tech.* **60**, 253-265.

Nakazato-Imasato, E. and Kurebayashi, Y. (2009). Pharmacological characteristics of the hind paw weight bearing difference induced by chronic constriction injury of the sciatic nerve in rats. *Life Sci.* **84**, 622-626.

Nicholls, J. G. and Baylor, D. A. (1968). Specific modalities and receptive fields of sensory neurons in CNS of the leech. *J. Neurophysiol.* **31**, 740-756.

Novas, A., Cao, A., Barcia, R. and Ramos-Martinez, J. I. (2004). Nitric oxide release by hemocytes of the mussel *Mytilus galloprovincialis* Lmk was provoked by interleukin-2 but not by lipopolysaccharide. *Int. J. Biochem. Cell Biol.* **36**, 390-394.

Okuda, K., Sakurada, C., Takahashi, M., Yamada, T. and Sakurada, T. (2001). Characterization of nociceptive responses and spinal releases of nitric oxide metabolites and glutamate evoked by different concentrations of formalin in rats. *Pain* **92**, 107-115.

Ott, S. R. and Burrows, M. (1999). NADPH diaphorase histochemistry in the thoracic ganglia of locusts, crickets, and cockroaches: species differences and the impact of fixation. *J. Comp. Neurol.* **410**, 387-397.

Ott, S. R. and Elphick, M. R. (2002). Nitric oxide synthase histochemistry in insect nervous systems: methanol/formalin fixation reveals the neuroarchitecture of formaldehyde-sensitive NADPH diaphorase in the cockroach Periplaneta Americana. *J. Comp. Neurol.* **448**, 165-185.

Ott, S. R., Aonuma, H., Newland, P. L. and Elphick, M. R. (2007). Nitric oxide synthase in crayfish walking leg ganglia: segmental differences in chemo-tactile centers argue against a generic role in sensory integration. *J. Comp. Neurol.* **501**, 381-399.

Panksepp, J. B. and Huber, R. (2004). Ethological analyses of crayfish behavior: a new invertebrate system for measuring the rewarding properties of psychostimulants. *Behav. Brain Res.* **153**, 171-180.

Pastor, J., Soria, B. and Belmonte, C. (1996). Properties of the nociceptive neurons of the leech segmental ganglion. *J. Neurophysiol.* **75**, 2268-2279.

Pfeiffer-Linn, C. and Glantz, R. M. (1991). An arthropod NMDA receptor. *Synapse* **9**, 35-42.

Puri, S. and Faulkes, Z. (2010). Do decapod crustaceans have nociceptors for extreme pH? *PLoS ONE* **5**, e10244.

Radomski, M. W., Jenkins, D. C., Holmes, L. and Moncada, S. (1991). Human colorectal adenocarcinoma cells: differential nitric oxide synthesis determines their ability to aggregate platelets. *Cancer Res.* **51**, 6073-6078.

Riedel, W. and Neeck, G. (2001). Nociception, pain, and antinociception: current concepts. *Z. Rheumatol.* **60**, 404-415.

Rigon, P., de Castilhos, J., Saur, L., Rodrigues, M. F., Achaval, M. and Xavier, L. L. (2009). NADPH-diaphorase activity in the nociceptive pathways of land snail *Megalobulimus abbreviatus*: the involvement of pedal ganglia. *Invert. Neurosci.* **9**, 155-165.

Robertson, J. D., Bonaventura, J., Kohm, A. P. and Hiscat, M. (1996). Nitric oxide is necessary for visual learning in *Octopus vulgaris*. *Proc. Biol. Sci.* **263**, 1739-1743.

Sandeman, D. C., Sandeman, R. E., Derby, C. and Schmidt, M. (1992). Morphology of the brain of crayfish, crabs, and spiny lobsters: a common nomenclature for homologous structures. *Biol. Bull.* **183**, 304-326.

Schachtner, J., Schmidt, M. and Homberg, U. (2005). Organization and evolutionary trends of primary olfactory brain centers in tetraconata (crustacea + hexapoda). *Arthropod Struct. Dev.* **34**, 257-299.

Schmidt, M. (1997). Distribution of presumptive chemosensory afferents with FMRFamide- or substance P-like immunoreactivity in decapod crustaceans. *Brain Res.* **746**, 71-84.

Schuppe, H., Aonuma, H. and Newland, P. L. (2001). NADPH-diaphorase histochemistry in the terminal abdominal ganglion of the crayfish. *Cell Tissue Res.* **303**, 289-299.

Shen, D. W., Lu, Q. W., Wang, L. and Zhang, R. J. (2002). Ultrastructure of electrical synapses between nociceptive and anterior pagoda neurons in the CNS of the leech (*Whitmania pigra*). *Invert. Neurosci.* **4**, 193-198.

Soen, M., Minami, T., Tatsumi, S., Mabuchi, T., Furuta, K., Maeda, M., Suzuki, M. and Ito, S. (2007). A synthetic kainoid, (2S,3R,4R)-3-carboxymethyl-4-(phenylthio)pyrrolidine-2-carboxylic acid (PSPA-1) serves as a novel anti-allodynic agent for neuropathic pain. *Eur. J. Pharmacol.* **575**, 75-81.

Sun, X. C., Li, W. B., Li, S. Q., Li, Q. J., Chen, X. L. and Ai, J. (2003). Intrathecal injection of Sar9, Met(O2)11-substance P, neurokinin-1 receptor agonist, increases nitric oxide synthase expression and nitric oxide production in the rat spinal cord. *Sheng Li Xue Bao* **55**, 677-683.

Sung, Y. J., Walters, E. T. and Ambron, R. T. (2004). A neuronal isoform of protein kinase G couples mitogen-activated protein kinase nuclear import to axotomy-

induced long-term hyperexcitability in *Aplysia* sensory neurons. *J. Neurosci.* **24**, 7583-7595.

Tobin, D., Madsen, D., Kahn-Kirby, A., Peckol, E., Moulder, G., Barstead, R., Maricq, A. and Bargmann, C. (2002). Combinatorial expression of TRPV channel proteins defines their sensory functions and subcellular localization in *C. elegans* neurons. *Neuron* **35**, 307-318.

Tobin, D. M. and Bargmann, C. I. (2004). Invertebrate nociception: behaviors, neurons and molecules. *J. Neurobiol.* **61**, 161-174.

Tracey, W. D., Jr, Wilson, R. I., Laurent, G. and Benzer, S. (2003). painless, a *Drosophila* gene essential for nociception. *Cell* **113**, 261-273.

Ushizawa, T., Nagayama, T. and Takahata, M. (1996). Cholinergic transmission at mechanosensory afferents in the crayfish terminal abdominal ganglion. *J. Comp. Physiol.* **179**, 1-13.

Vetter, G., Geisslinger, G. and Tegeder, I. (2001). Release of glutamate, nitric oxide and prostaglandin E2 and metabolic activity in the spinal cord of rats following peripheral nociceptive stimulation. *Pain* **92**, 213-218.

Walters, E. T. (1996). Comparative and evolutionary aspects of nociceptor function. In *Neurobiology of Nociceptors* (ed. C. Belmonte and F. Cervero), pp. 92-114. New York: Oxford University Press.

Walters, E. T., Illich, P. A., Weeks, J. C. and Lewin, M. R. (2001). Defensive responses of larval Manduca sexta and their sensitization by noxious stimuli in the laboratory and field. *J. Exp. Biol.* **204**, 457-469.

Wittenburg, N. and Baumeister, R. (1999). Thermal avoidance in *Caenorhabditis elegans*: an approach to the study of nociception. *Proc. Natl. Acad. Sci. USA* **96**, 10477-10482.

Wright, B., Lacchini, A. H., Davies, A. J. and Walker, A. J. (2006). Regulation of nitric oxide production in snail (*Lymnaea stagnalis*) defence cells: a role for PKC and ERK signalling pathways. *Biol. Cell* **98**, 265-278.

Xu, S. Y., Cang, C. L., Liu, X. F., Peng, Y. Q., Ye, Y. Z., Zhao, Z. Q. and Guo, A. K. (2006). Thermal nociception in adult *Drosophila*: behavioral characterization and the role of the painless gene. *Genes Brain Behav.* **5**, 602-613.

Yahyavi-Firouz-Abadi, N., Tahsili-Fahadan, P. and Ostad, S. N. (2007). Effect of mu and kappa opioids on injury-induced microglial accumulation in leech CNS: involvement of the nitric oxide pathway. *Neuroscience* **144**, 1075-1086.

Yoon, Y. W., Sung, B. and Chung, J. M. (1998). Nitric oxide mediates behavioral signs of neuropathic pain in an experimental rat model. *Neuroreport* **9**, 367-372.

Zhuo, M., Meller, S. T. and Gebhart, G. F. (1993). Endogenous nitric oxide is required for tonic cholinergic inhibition of spinal mechanical transmission. *Pain* **54**, 71-78.