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NITRIC OXIDE SIGNALING MODULATES CHOLINERGIC SYNAPTIC INPUT TO PROJECTION NEURONS IN *DROSOPHILA* ANTENNAL LOBES

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Abstract—Biochemical investigations have demonstrated that nitric oxide synthase (NOS) is distributed widely in the olfactory system. However, little is known about the action of NO at the synaptic level on identified neurons in local circuits that process chemosensory signals. Here, using whole-cell recordings, the effect of NO on cholinergic synaptic input to olfactory projection neurons (PNs) is determined in the *Drosophila* antennal lobes (ALs), which has become an excellent model for studying early olfactory-processing mechanisms. We found that the NO donor SNP/SNAP or the NO precursor L-arginine significantly decreased the frequency of cholinergic spontaneous excitatory postsynaptic currents (sEPSCs) in PNs. Conversely, soluble guanylyl cyclase (sGC) inhibitor oxadiazolo-quinoxaline-one (ODQ) significantly increased the frequency of cholinergic sEPSCs in PNs. The subsequent application of 8-bromo-cGMP significantly attenuated the effects of ODQ, indicating the possible involvement of NO–cGMP signaling. To determine the role of NO in quantal release of acetylcholine (Ach) to PNs, cholinergic miniature excitatory postsynaptic currents (mEPSCs) were recorded. SNP significantly decreased the frequency of mEPSCs, but exhibited no effect on the amplitude or the decay time constant of mEPSCs in PNs. The effect of SNP on the frequency of mEPSCs could be eliminated by ODQ as well. Thus, these results suggest that elevated NO concentration decreased cholinergic synaptic input to PNs in a sGC-dependent manner. In this way, NO signaling is suited to fulfill a regulatory role to effectively fine-tune network activity in *Drosophila* ALs.

Key words: nitric oxide, *Drosophila*, projection neuron, cholinergic synapse.

INTRODUCTION

Nitric oxide (NO), served as an endogenous messenger molecule in the central nervous system (CNS), is an important modulator of multiple neuronal processes. NO is generated in an activity-dependent process by Ca^{2+}/calmodulin-stimulated nitric oxide synthases (NOS) (Bredt and Snyder, 1992), which converts L-arginine into NO and citrulline using nicotinamide adenine dinucleotide phosphate (NADPH) as a co-factor (Mayer, 1994). The major target for NO is the soluble form of guanylate cyclase (sGC) (Schuman and Madison, 1994; Esplugues, 2002). Upon NO binding to the heme moiety on sGC, the rate of cyclic guanosine monophosphate (cGMP) production increases dramatically, which initiates the cGMP-signaling pathway and produces subsequent physiological changes (Bryan et al., 2009).

A good model system in which to examine the roles of NO is the olfactory system, particularly the first synaptic neuropil: the olfactory bulb in mammals and the antennal lobe in insects (Breer and Shepherd, 1993). The consistent and widespread localization of NOS and sGC in the olfactory system suggests that NO signaling is likely to be important for olfactory function (Collmann et al., 2004; Murakami et al., 2004; Fujie et al., 2005). Using histochemical methods, most studies on NO in invertebrates have focused on the location of NO-generating neurons or target cells of NO, detailed physiological mechanisms underlying the function of NO in the olfactory system remain largely unknown. For the relatively simpler nervous systems, identified neuronal precursors and nerve cells, the accessibility to genetic dissection, and high degree of conservation in fundamental biological pathways between insects and human (Bicker, 1998; Rubin et al., 2000), the antennal lobes (ALs) of *Drosophila* were used as an intact neural network model to investigate neural circuit function (Ng et al., 2002). In all insects investigated yet, the primary site of olfactory information processing, the ALs exhibit high levels of NOS (Müller and Buchner, 1993; Müller, 1994).

The AL is the first center of olfactory processing in *Drosophila*. The *Drosophila* AL is composed of ~43

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Abbreviations: Ach, acetylcholine; ALs, antennal lobes; BSA, bovine serum albumin; cGMP, cyclic guanosine monophosphate; CNS, central nervous system; CS, Canton-S; DMSO, dimethyl sulfoxide; EGTA, ethylene glycol tetraacetic acid; EPSPs, excitatory postsynaptic potentials; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPSPs, inhibitory postsynaptic potentials; LNs, local interneurons; MBs, mushroom bodies; mEPSCs, miniature excitatory postsynaptic currents; nAChR, nicotinic acetylcholine receptor; NADPH, nicotinamide adenine dinucleotide phosphate; NADPHd, NADPH diaphorase; NO, nitric oxide; NOS, nitric oxide synthases; ODQ, oxadiazolo-quinoxaline-one; OSNs, olfactory sensory neurons; PKGs, cGMP-dependent protein kinases; PNs, projection neurons; sEPSCs, spontaneous excitatory postsynaptic currents; sGC, soluble guanylyl cyclase; SNAP, S-Nitroso-N-acetyl-penisillamine; SNP, sodium nitroprusside.

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identified glomeruli (Hansson and Anton, 2000). All of the olfactory sensory neurons (OSNs) that express the same odorant receptor project bilaterally onto a pair of glomeruli, one in each AL. There they synapse onto projection neurons (PNs). The glomeruli also contain the neurites of PNs that output to downstream areas of processing and intrinsic local interneurons (LNs) (Ng et al., 2002; Wilson and Laurent, 2005; Olsen et al., 2007; Silbering et al., 2008). PNs, whose cell bodies are located at the periphery of the AL, project their dendrites to single or multiple glomeruli and relay activity to higher brain regions, the mushroom bodies (MBs) and the lateral horns (Stocker et al., 1990). Within the glomeruli, release of NO from one neuron can influence signal processing by acting on synaptic transmission within the glomerular circuits (Müller, 1997).

To determine the roles of NO in the regulation of cholinergic synaptic input to PNs, whole-cell recordings were used. The possible downstream signaling mechanisms involved in NO action on cholinergic synaptic input to PNs were also investigated. We found that NO significantly decreased cholinergic activity dependent- and independent-synaptic input to PNs in a sGC-dependent manner. Our findings provided new insights into the underlying cellular and signaling mechanisms of NO in the olfactory system at the whole-brain level.

EXPERIMENTAL PROCEDURES

Fly strains and isolated whole-brain preparation

Drosophila melanogaster stocks were reared on standard cornmeal agar medium supplemented with dry yeast at 24 °C and 60% relative humidity. All experiments were performed on Canton-S (CS) female flies 2 days before eclosion.

The brains were prepared as previously described (Gu and O'Dowd, 2006). Papain solution (20 U/ml activated by 1 mM L-cysteine) was used to soften the connective tissue sheath surrounding the brain. The brain was isolated by removal of the head capsule, muscles, trachea, the proboscis, antennae, labial palps and compound eyes. The perineural sheath was also gently picked away from the ALs.

Electrophysiological recordings of PNs in *Drosophila* whole-brain

Using an Axioskop FS-1 with IR-DIC optics and a 40× water-immersion object, all recordings were obtained from PN somata. One PN was recorded per fly, except for paired recordings (Fig. 1). The recording saline contained (in mM): 101 NaCl, 1 $CaCl_2$, 4 $MgCl_2$, 3 KCl, 5 glucose, 1.25 NaH_2PO_4, and 20.7 $NaHCO_3$, which were bubbled with 95% oxygen and 5% carbon dioxide. Current-clamp and voltage-clamp recordings were performed by using pipettes (8–10 MΩ) filled with an internal solution containing (in mM): 102 K-gluconate, 0.085 $CaCl_2$, 1.7 $MgCl_2$, 17 NaCl, 0.94 EGTA, 8.5 HEPES, and pH 7.2, 235 mOsm. In current-clamp mode, no current was injected to PNs; in voltage-clamp mode, PNs were held at −70 mV. Miniature excitatory postsynaptic currents (mEPSCs) were recorded using the same recording saline containing TTX (1 μM) and picrotoxin (10 μM). Cells were accepted for recording if R_{input} > 500 MΩ and R_{access} < 40 MΩ. All electrophysiological recordings were acquired at 10 kHz and low-pass filtered at 2 kHz with EPC10 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany). Excitatory postsynaptic potentials (EPSPs), spontaneous excitatory postsynaptic currents (sEPSCs), and mEPSCs were detected using MiniAnalysis (Synaptosoft, Decatur, GA, USA).

Immunohistochemistry

The morphology and identification of PNs were confirmed by post hoc staining of biocytin as described (Wilson et al., 2004). In most experiments, 0.4% biocytin was added to the internal pipette solution for at least 30 min. After electrophysiological recordings, brains were fixed overnight in 4% paraformaldehyde in PBS, rinsed in PBS, permeabilized in PBST (0.1 M PBS containing 0.2% Triton) for 20 min, and blocked in 5% bovine serum albumin (BSA) in PBST (PBST-BSA) for 30 min. The nc82 antibody from the Developmental Studies Hybridoma Bank (Wagh et al., 2006) was then applied for 24 h at a dilution of 1:10 in PBST-BSA at 4 °C. After four washes in PBST, the brains were incubated overnight at 4 °C with goat anti-mouse: Alexa Fluor 488 (A11001, Invitrogen, Carlsbad, CA, USA) and streptavidin-CY3 (Molecular Devices, America) diluted at 1:200 in PBST-BSA. The brains were then washed for 30 min in PBST, mounted in Vectashield on a slide flanked by two #1 coverslips to prevent flattening.

Confocal fluorescence microscopy was performed on Zeiss LSM 710 (Oberkochen, Germany) using a 40X C-Apochromat water-immersion objective lens to acquire 3-μm slices through the ALs (Fig. 1B). Wider-field images (Fig. 1A) were acquired using a 10× objective in 20-μm slices. 3D postrecording image processing was done using Imaris 6.4.2 software (Bitplane, Zurich, Switzerland).

Drugs

S-Nitroso-N-acetyl-penisillamine (SNAP), sodium nitroprusside (SNP), and 8-bromoguanosine 3′,5′-cyclic monophosphate (8-bromo-cGMP) were dissolved in distilled water; oxadiazolo-quinoxaline-one (ODQ) was dissolved in 0.05% dimethyl sulfoxide (DMSO). SNP/SNAP solutions were fresh and protected from light. Drugs obtained from Sigma (www.sigmaaldrich.com) were prepared as stock solutions and diluted to the required concentrations.

Data analysis

Electrophysiological data analysis was carried out with Clampfit 10.2 (Molecular Devices, America) and Fitmaster 2.6 (HEKA Elektronik, Lambrecht/Pfalz, Germany). Data represent means ± SEM. Unless otherwise indicated, statistical significance was assessed with paired *t*-test $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

RESULTS

Projection neurons (PNs) in the *Drosophila* antennal lobe produce cholinergic excitatory rhythmic activities

To examine the roles of NO in the regulation of cholinergic excitatory synaptic input to PNs, whole cell patch-clamp recordings in *Drosophila* antennal lobe were used. All of the PNs were identified morphologically from dye fills (Fig. 1A, B). In the ALs, PNs typically received a continuous barrage of EPSPs, and fired spontaneously (mean rate 3.01 ± 0.9 Hz). In contrast to action potentials in local neurons (LNs), which were always ⩾40 mV in amplitude, spikes recorded in PN somata were ⩽15 mV (Fig. 1C). Consistent with previous results (Wilson et al., 2004), spontaneous EPSPs in PNs were blocked by a