Octopamine stabilizes conduction reliability of an unmyelinated axon during hypoxic stress

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Octopamine stabilizes conduction reliability of an unmyelinated axon during hypoxic stress. J Neurophysiol 116: 949–959, 2016. First published June 8, 2016; doi:10.1152/jn.00354.2016.—Mechanisms that could mitigate the effects of hypoxia on neuronal signaling are incompletely understood. We show that axonal performance of a locust visual interneuron varied depending on oxygen availability. To induce hypoxia, tracheae supplying the thoracic nervous system were surgically lesioned and action potentials in the axon of the descending contralateral movement detector (DCMD) neuron passing through this region were monitored extracellularly. The conduction velocity and fidelity of action potentials decreased throughout a 45-min experiment in hypoxic preparations, whereas conduction reliability remained constant when the tracheae were left intact. The reduction in conduction velocity was exacerbated for action potentials firing at high instantaneous frequencies. Bath application of octopamine mitigated the loss of conduction velocity and fidelity. Action potential conduction was more vulnerable in portions of the axon passing through the mesothoracic ganglion than in the connectives between ganglia, indicating that hypoxic modulation of the extracellular environment of the neuropil has an important role to play. In intact locusts, octopamine and its antagonist, epinastine, had effects on the entry to, and recovery from, anoxic coma consistent with octopamine increasing overall neural performance during hypoxia. These effects could have functional relevance for the animal during periods of environmental or activity-induced hypoxia.

locust; DCMD; action potential; conduction velocity; sodium azide

NEW & NOTEWORTHY

Neuronal signaling is energetically expensive, but the cost can be reduced by modulating action potential parameters to trade off performance against energy expenditure. We demonstrate that octopamine reverses some of the effects of hypoxia on axonal conduction of an important visual interneuron (DCMD) in locusts, thus maintaining its high conduction velocity at high firing frequencies. Pharmacological modulation of anoxic comas in intact animals suggests that octopamine has a general effect to enhance neural performance during hypoxia.

INSECTS ROUTINELY EXPERIENCE bouts of hypoxia. These can occur as they move through oxygen-poor regions of the environment or during vigorous activity (Schnitz and Harrison 2004). The hypoxic periods may be acute or seasonal or occur during developmental stages (Hoback and Stanley 2001). The physiological response of an animal to these different hypoxic stressors varies between animals and different tissues (Hochachka 1986; Lutz and Nilsson 1997) and depends on the type, severity, and duration of the hypoxic event. A common theme, however, is that hypoxia creates an energetic stress that results in a change in cellular and whole animal metabolism (Van Voorhies 2009).

The nervous system has a high energetic requirement (Crotty et al. 2006) and is thus particularly sensitive to hypoxic stressors (Buck and Pamenter 2006). It is important, therefore, to understand the physiological consequences of energetic stressors on nervous systems as well as to explore the potential mechanisms that support neural function in the face of these environmental features. An important mechanism to reduce energy utilization of neurons during hypoxia is reduction of firing rate (spike arrest; Lutz 1992). One way this can be accomplished is through a decreased membrane permeability (ion channel arrest; Perezpinzon et al. 1992). Another mechanism for cellular survival in hypoxia-tolerant animals is a sharp reduction of Na⁺-K⁺-ATPase pump activity (Hochachka et al. 1996), allowing the cell to match ATP supply and demand.

In response to a prolonged anoxic stress, most animals enter a coma state that results from a silencing of neural activity. During this event, ion gradients across neuronal membranes are reduced concurrent with a decrease in metabolic activity (Lutz and Nilsson 1997; Rodgers et al. 2007; Wegener and Moratzky 1995). Whereas transient losses of membrane gradients are generally benign (Müller and Somjen 2000), prolonged exposure leads to cell damage and death (Broughton et al. 2009). Anoxia-tolerant animals show a reduction in cellular energy demand (Hochachka et al. 1996), which may help to prevent cellular damage during prolonged anoxic insults.

Susceptibility of neural tissue to hypoxic/anoxic stress can be altered through cellular signaling pathways including nitric oxide/cGMP/PKG (Armstrong et al. 2009; Dawson-Scull et al. 2010) and the metabolic regulator AMP-activated protein kinase (AMPK) (Money et al. 2014; Rodgers-Garlick et al. 2011). Furthermore, modulating neurotransmitter systems can affect levels of stress tolerance in animals. Suppression of NMDA receptor function and release of GABA is protective in the brain of anoxia-tolerant turtles (Bickler et al. 2000; Pamerter et al. 2011). The insect hormone octopamine (OA) imparts a protective effect on circuits during environmental stress through its actions on PKA (Armstrong et al. 2006). Indeed, OA responds broadly to stressors (Davenport and Evans 1984) and is known to act directly on both motor (Ramirez and Pearson 1991) and sensory (Leitch et al. 2003) targets.
Using a locust visual interneuron, the descending contralateral movement detector neuron (DCMD), that is involved in predator evasion (Gray et al. 2001; Santer et al. 2006), we have shown previously that recovery from coma slows conduction of action potentials (APs) along the axon (Money et al. 2014). Here we examine the role of OA in supporting conduction during hypoxia. Fast and reliable conduction from the brain to the thoracic motor systems is a vital property of the DCMD, and this neuron has a large-diameter axon capable of supporting high-frequency APs (>400 Hz) at relatively high conduction speeds (>3 m/s at 21°C). The fact that the bursting structure of the DCMD firing pattern contains important information about the properties of looming visual stimuli (McMillan and Gray 2015) suggests that fidelity of axonal conduction would be critical to maintain under different conditions in order to preserve vital reflexive behaviors. These axonal properties make this escape circuit an excellent model for studying the responses of axons to hypoxia, as recordings from this neuron can be made during responses to realistic visual stimuli. We used the DCMD axon to test the effects of hypoxia and OA on conduction fidelity during high-frequency activity. OA is released by insects during stress and times of vigorous activity (Davenport and Evans 1984), making it a compelling target to modulate responses during energetic stress. Our data support the hypothesis that OA can mitigate the effects of hypoxia on axonal conduction.

**METHODS**

**Animals.** Adult male locusts, 3–6 wk after their final molt, were taken from a crowded colony reared in the Queen’s University Department of Biology. Cages were maintained on a 12:12-h circadian period with lights on at 7 AM. The room temperature of the colony was maintained at 25°C. Animals were fed wheatgrass, carrots, and a mixture of bran, yeast, and milk powder. Feeding was done daily in the early afternoon. Animals were collected from the colony between 8 and 10 AM and held in a ventilated plastic container until dissection, 1–8 h after removal from the colony.

**Preparation.** Animals were dissected dorsally to expose the thoracic nervous system, as described in detail elsewhere (Robertson and Pearson 1982). Unlike previous descriptions of this preparation, the thoracic nervous system was lifted onto a plate only for intracellular recording in order to limit the disturbance to the tracheal system during extracellular recording. During the dissection, the thoracic region of the preparation was filled with standard locust saline (in mM: 147 NaCl, 10 KCl, 4 CaCl₂, 3 NaOH, and 10 HEPES buffer; pH = 7.2), and this acted as the tissue bath. The solution was changed regularly with a Pasteur pipette to ensure sufficient bath volume and to maintain the health of the preparation.

**Treatments.** Hypoxia was induced in the thoracic nervous system by cutting the major tracheal connections to the meso- and metathoracic ganglia with scissors. Care was taken not to disrupt other structures. For experiments with the mitochondrial inhibitor sodium azide (Na₃N₃), we used 1 mM Na₃N₃, as this dose has been shown previously to induce an anoxia-like depolarization in locust thoracic ganglia (Rogers-Garlück et al. 2011). We bath applied azide for 5 min, recording DCMD activity before and after exposure. Control animals had fresh saline applied. OA treatments (Sigma-Aldrich) were applied by replacing the normal saline used throughout the dissection with drug + saline via a Pasteur pipette. We used 10⁻³ M OA because this dose has acute effects on the ventilatory rhythm in locusts, and longer-duration treatments protect the rhythm from hyperthermic failure (Armstrong et al. 2006). The solution was changed two or three times to ensure thorough exposure of the nervous system to the drug.

**Visual stimulation protocol.** The visual system was stimulated by projecting the image of an animated moving target onto a translucent screen placed to the left of the animal at 90° to the body axis and at a distance of 7 cm. The target was a black disk on a white background and was animated by using Adobe Flash software to create an apparent forward motion of 1 m/s over a 3-s approach to a maximum target diameter of 38 mm. This stimulus protocol has been shown previously to reliably excite the looming detection system of the locust (Money et al. 2006).

**Extracellular electrophysiology.** After dissection to expose the thoracic nervous system, suction electrodes were used to record neural activity extracellularly from the thoracic nerve cord. The electrodes were made from borosilicate capillary tubes (WPI) that were pulled to a sharp point with a platinum-fluoride electrode puller (model P-97, Sutter Instrument). Electrodes were produced by scoring the tip with a glass cutting tool and then breaking the tip at a size approximately two-thirds that of the thoracic connective nerves. Only electrodes with clean breaks were used, and fire polishing of the tip was not required to achieve good suction seals on the nerve.

Two electrodes were positioned onto the thoracic nerve cord during each experiment. One was placed anterior to the mesothoracic ganglion, approximately halfway between it and the prothoracic ganglion. The second electrode was positioned between the mesothoracic and metathoracic ganglia. In some experiments, a third electrode was positioned immediately anterior to the mesothoracic ganglion.

Recordings from the nerve cord were referenced to a silver-wire ground placed into the animal bath with an A-M Systems model 1700 Differential AC amplifier (low frequency cutoffs were set to 0.1 Hz and high frequency cutoffs were set to 10 kHz) and digitized to computer with a Molecular Devices digitizer (model 1440A) and pCLAMP software. During visual stimulation, DCMD APs were clearly identifiable from background activity, with an amplitude at least twice as large as other activity in the connective.

**Intracellular electrophysiology.** Intracellular electrodes were pulled from borosilicate glass pipettes to a resistance of 20–40 MΩ when backfilled with 3 M KCl. Recordings were made with an A-M Systems Neuroprobe Amplifier (model 1600). The amplifier’s DC offset was zeroed relative to the bath before penetration. Penetration was made just posterior to the mesothoracic ganglion. In experiments where AP parameters were quantified, the resting membrane potential was fixed to −60 mV during the duration of the recording, matching the physiological potential at room temperature (Money et al. 2005) and allowing accurate comparison of AP parameters.

**Whole animal anoxia.** Locusts were exposed to a repeated anoxic stress through two 30-min water immersions. Individual locusts were placed in one of six compartments in a plastic container and then submerged entirely in an aquarium filled with clean, dechlorinated tap water at room temperature (~20°C) for the duration of the trial. The time to succumb to anoxic coma was measured for each locust as the time in which each animal stopped making coordinated movements. At the end of a water immersion trial, the animals were removed from the aquarium, wiped dry, and then weighed on a precision scale. Each locust was then placed in an open air container, and the time required to recover from anoxic coma was observed for each individual. Animals were deemed recovered when they could right themselves with all legs planted on the surface.

Between exposures, animals were allowed to recover for 80 min and were then abdominally injected with 10 μl of 10⁻² M OA, the OA antagonist epinastine (EP; 10⁻² M), or a sham saline injection (control). After the recovery period, the immersion procedure was repeated and a second set of time to succumb and recover measurements were made. To produce a within-animal comparison, measurements of time to succumb to anoxic coma and time to recovery were analyzed as a relative measure: (2nd exposure time − 1st exposure time)/1st exposure time.

**Analysis and statistics.** The primary measure for most experiments was the conduction velocity of APs between the sets of electrodes.
This measure was taken relative to the first AP recorded at the first time point (t = 10 min) for each preparation. Given that the electrodes were not moved through the experiment, relative conduction velocity for any individual AP was calculated from the reciprocal of the time required to pass from one electrode to the next (e.g., anterior to posterior) relative to the time required for the first AP.

The relative conduction velocities of APs within a looming response were sorted according to their instantaneous frequency, calculated as the reciprocal of interspike interval before the AP of interest, and displayed as such, or the response was binned into frequency groups of <100 Hz, 100–200 Hz, and >200 Hz. Bins were chosen to reflect the low, medium, and high firing rates observed in this neuron. The relative conduction velocities within each bin were then used to produce a mean value that was compared across different time points (10 min, 25 min, 45 min) and different treatments (normoxic, hypoxic, OA). Similarly, the relative conduction velocity was also compared across the different electrodes (axon: anterior to middle electrode vs. ganglion: middle to posterior electrode). During azide experiments, relative conduction velocity ratios were compared between the 5-min azide exposure and a time point immediately prior to azide exposure. Differences were statistically assessed with two-way repeated-measures ANOVA (RM-ANOVA) followed by post hoc analysis (Tukey test, \( P < 0.05 \)).

AP fidelity was also assessed by counting the occurrences of conduction failures as a given looming response passed posteriorly down the thoracic connective. The number of failures at each time point for each treatment was quantified as percent failure compared with total spike number. Mean minimum relative conduction velocity was quantified as the lowest relative conduction velocity for a single AP during a looming response expressed as the mean value across all animals for each time point and treatment. Statistical comparisons were made by one-way RM-ANOVA with Holm-Sidak multiple comparisons (\( P < 0.05 \)).

Intracellular measurements of AP amplitude, half-width, and frequency were analyzed and quantified with custom Python scripts using the Stimfit library (Guzman et al. 2014). Relative measures of amplitude and half-width were calculated from the first AP of the recorded response at each time point.

RESULTS

Performance in the axon varies depending on oxygenation. The conduction velocity of APs in the axon of DCMD was examined under both normoxic and hypoxic conditions. In normoxic preparations, animals were dissected to expose the thoracic nervous system. Care was taken not to disrupt the tracheal system that supplies the region. In hypoxic preparations, respiratory connections to the prothoracic and mesothoracic ganglia were deliberately cut with dissecting scissors. In both preparations, responses in DCMD were generated by displaying a looming visual stimulus to the eye of the animal and recorded extracellularly from the thoracic connective at two locations (Fig. 1A).

In normoxic preparations, responses were stable throughout the experiment. The triphasic shape of the extracellularly recorded AP showed very little change at either the anterior electrode or the posterior electrode over 45 min (Fig. 1B). Conduction velocity in the DCMD axon was calculated by measuring the time from the trough of the AP waveform at the anterior electrode to the trough of the AP at the posterior electrode. Normoxic preparations had APs with similar conduction velocities at time points of 10 min, 25 min, and 45 min. There was a mild but reliable effect of AP instantaneous frequency, with higher-frequency APs showing a slightly reduced conduction velocity (Fig. 1B). In contrast, conduction velocity of APs in hypoxic preparations slowed considerably throughout the course of the experiment. This decrease in conduction velocity was particularly evident at higher AP instantaneous frequencies, with some APs showing a decrease of >50% (Fig. 1, B and C).

Chemical anoxia slows conduction velocity and reduces fidelity of APs in the axon. To further investigate how the DCMD’s performance changed because of hypoxia, we used the mitochondrial toxin NaN₃. Measurements of AP properties were made in the before-coma period, as prolonged exposure can arrest neural function (see below) and produce an abrupt

Fig. 1. An individual response to hypoxia showing reduced conduction velocity in the DCMD axon. A: axonal action potentials recorded extracellularly in the right connective between the prothoracic (Pro) and mesothoracic (Meso) ganglia and between the Meso and metathoracic (Meta) ganglia generated in response to a 1 m/s looming object presented to the left eye. B: overlaid traces of action potentials recorded from the anterior and posterior electrodes at different times and with different instantaneous frequencies. Conduction delay between the electrodes was measured at 10 min, 25 min, and 45 min under normoxic or hypoxic conditions. C: relative conduction velocity derived from the delay measurements was stable in normoxic preparations over time and across different instantaneous firing frequencies. Hypoxic preparations, however, showed a steadily decreasing conduction velocity over time, particularly at instantaneous firing frequencies above 200 Hz.
change in extracellular potassium in locust metathoracic ganglia similar to anoxia (Rodgers-Garlick et al. 2011).

Animals were treated with either 1 mM NaN₃ or saline control. The DCMD’s response to a looming visual stimulus was recorded before (0 min) and after 5 min of azide exposure (Fig. 2, A and B). Exposure to azide resulted in a significant reduction in conduction velocity (Fig. 2C). Reduction in conduction velocity was observed across APs firing at different frequency ranges of <100 Hz (Holm-Sidak pairwise multiple comparison, $t = 4.90, P < 0.001$), 100–200 Hz (Holm-Sidak pairwise multiple comparison, $t = 5.37, P < 0.001$), and >200 Hz (Holm-Sidak pairwise multiple comparison, $t = 5.32, P < 0.001$). We also found an overall decrease in the number of APs elicited at the highest frequencies (>200 Hz) compared with control animals (control 19.9 ± 2.8 APs vs. azide 12.9 ± 1.5 APs; Holm-Sidak multiple comparison, $t = 2.17, P < 0.05$).

We next explored how azide affects the DCMD’s AP amplitude, as amplitude can be a predictor of sodium load and energy consumption (Hallermann et al. 2012; Sengupta et al. 2010). We used intracellular electrodes and recorded the DCMD’s activity in response to hand-waving visual stimuli before and after 5-min exposure to 1 mM azide (Fig. 3A). We observed a clear reduction in AP amplitude during azide treatment (Fig. 3B), quantified as a percent change in amplitude relative to the pretreatment (Mann-Whitney rank sum test, $t = 63.0, P < 0.001$). Concomitantly, there was a greater percent reduction in the maximum rising slope of the first AP during azide treatment ($-38.2$ ± 4.5%) compared with control ($-12.6$ ± 3.0%) (data not shown; $t$-test, $P < 0.001$). To examine the dependence of firing rate on amplitude attenuation distinct from the overall amplitude loss observed in azide, we analyzed the amplitude of APs relative to the first AP recorded within each azide or control treatment. The change in amplitude reduction in the presence of azide was particularly pronounced in APs with high instantaneous frequencies (Fig. 3C; Holm-Sidak pairwise multiple comparison, $t = 2.59, P < 0.05$), whereas amplitude attenuation during high-frequency firing was limited in control experiments.

Continued exposure to azide caused a failure of conduction through the mesothoracic ganglion (Fig. 3D), which recovered when normal saline was returned to the bath (Fig. 3E). During the process of failure and recovery the rising phase of the AP separated into two distinct components. A similar phenomenon during hyperthermic failure of conduction has been interpreted as a separation into an initial component due to passive conduction from a point of low safety factor followed by an active component with slowed conduction velocity (Money et al. 2009). Before failure resting membrane potential depolarized, consistent with azide causing a failure of the Na⁺–K⁺-ATPase and loss of the pump current. An equivalent hyperpolarization was not evident during recovery because the pump current is necessary to restore function, i.e., the hyperpolarization associated with pump operation occurs before AP recovery. At the point of failure the rapid loss of AP amplitude, before conduction block, in the intracellular electrode was not reflected in the downstream electrode, which suggests that the axon at the downstream region of the connective could support a full-size AP if it managed to propagate through the ganglion.

Octopamine mitigates hypoxia-induced loss of conduction velocity and conduction failure. Conduction velocity of APs from normoxic preparations did not show any change throughout the
45-min experiment (2-way RM-ANOVA, $F = 1.14$, $P = 0.38$). There was an effect of instantaneous frequency of an AP on its conduction velocity (2-way RM-ANOVA, $F = 166.34$, $P < 0.001$), but this was unaltered by the duration of the experiment (2-way RM-ANOVA, $F = 0.77$, $P = 0.56$). Hypoxic preparations, however, showed a significant decrease in conduction velocity throughout the experiment (Fig. 4; 2-way RM-ANOVA, $F = 35.20$, $P < 0.001$) for all instantaneous frequency bins ($<100$ Hz, 100–200 Hz, >200 Hz; Holm-Sidak pairwise multiple comparison, $P < 0.05$).

In a subset of hypoxic preparations, the control saline was changed to saline containing OA ($10^{-4}$ M). Conduction velocity in these preparations was still slowed over the course of the 45-min experiment (2-way RM-ANOVA, $F = 15.70$, $P < 0.001$).

At 10 min and for APs with low instantaneous frequencies ($<100$ Hz), there was no significant difference in the conduction velocity among the three treatment groups (Fig. 4). At higher frequency bins of 100–200 Hz and >200 Hz, however, both hypoxic groups (untreated and OA treated) show a significant decrease in conduction velocity of APs down the DCMD axon (Holm-Sidak pairwise multiple comparisons, $P < 0.05$) compared with normoxic preparations. Likewise, by 25 min the three treatment groups have APs with similar conduc-
tion velocities for low frequency activity (<100 Hz), but the hypoxic and OA-treated groups have significantly decreased conduction velocities (Holm-Sidak pairwise multiple comparisons, \( P < 0.05 \)).

At 45 min, however, OA-treated groups show significantly less reduction in conduction velocity compared with untreated hypoxic preparations at instantaneous frequency groups of 100–200 Hz (Fig. 4; Holm-Sidak pairwise multiple comparison, \( t = 2.22, P < 0.05 \)) and at >200 Hz (Holm-Sidak pairwise multiple comparison, \( t = 2.87, P < 0.05 \)). For APs with instantaneous frequencies < 100 Hz, normoxic preparations have relative conduction velocities that are maintained at 45 min compared with hypoxic preparations (Holm-Sidak pairwise multiple comparison, \( t = 2.79, P < 0.05 \)) but they are indistinguishable from the OA-treated hypoxic group (Holm-Sidak multiple comparison, \( t = 1.56, P = 0.13 \)). Both OA-treated and untreated hypoxic preparations in 100–200 Hz and >200 Hz frequency groups have significantly decreased conduction velocities compared with normoxic preparations (Holm-Sidak pairwise multiple comparison, \( P < 0.05 \)).

Further examination of the responses in hypoxic preparations revealed that some APs failed to propagate between the electrodes, resulting in a spike in the anterior electrode with no matching spike at the posterior electrode. Failure of APs always occurred late in the response, where firing frequency tends to be at a peak. We therefore examined the relationship between changes in the axon conduction velocity during hypoxia and the loss of fidelity in the axon. To make this comparison, we quantified the relative conduction velocity of the slowest spike during a looming response for each animal and averaged these across all animals at 10 min, 25 min, and 45 min time points during each experiment to calculate a mean minimum relative conduction velocity. When plotted against firing frequency, we found that a decreased mean minimum relative conduction velocity was associated with decreased fidelity in response to hypoxia (Fig. 5). From a starting fidelity of 100% at 10 min, there was a significant loss of fidelity in hypoxic control animals (1-way RM-ANOVA on ranks, \( \chi^2 = 8.43, P < 0.05 \)), falling to 97% at 25 min and further to 95% by 45 min. The OA treatment group, however, did not show a significant loss of fidelity through the experiment (1-way RM-ANOVA, \( F = 3.34, P = 0.06 \)). Fidelity was 100% at 10 min and 99% at both 25 and 45 min.

Comparing the treatments directly, we found no difference at 10 min between hypoxic and OA-treated hypoxic preparations in terms of mean minimum relative conduction velocity (Fig. 5; hypoxic mean 0.61 ± 0.02 vs. OA mean 0.66 ± 0.02; Holm-Sidak pairwise multiple comparison, \( t = 1.32, P = 0.19 \)). There was also no difference in fidelity with both treatments at 100%.

At 25 min, we again found no difference between hypoxic and OA-treated hypoxic preparations in terms of mean minimum relative conduction velocity (Fig. 5; hypoxic mean 0.52 ± 0.03 vs. OA mean 0.55 ± 0.03; Holm-Sidak pairwise multiple comparison, \( t = 0.87, P = 0.39 \)). There was, however, a significant difference in fidelity between the treatments (99% vs. 97%; Holm-Sidak pairwise multiple comparison, \( t = 2.07, P < 0.05 \)).

By 45 min, we observed a significant difference in mean minimum relative conduction velocity between hypoxic and OA-treated hypoxic preparations (Fig. 5; hypoxic mean 0.43 ± 0.02 vs. OA mean 0.55 ± 0.03; Holm-Sidak pairwise multiple comparison, \( t = 3.08, P < 0.05 \)). At this time point there was also a significant difference in fidelity (99% vs 95%; Holm-Sidak pairwise multiple comparison, \( t = 4.30, P < 0.001 \)).

**Increased sensitivity of conduction velocity to hypoxia through the ganglion.** We next wanted to determine whether the loss of conduction velocity during hypoxia was generalized to the axon as a whole, or whether there were particular sites of vulnerability. Local changes in the extracellular environment within the ganglion could have a profound effect on the signaling properties of the axon. The DCMD axon does not have a constant diameter and also branches significantly within the mesothoracic ganglion, both of which could be sources of vulnerability. Varicosities and branch points are well established as regions of low safety factor due to changes in the electrical load caused by increased axonal membrane area (Debanne 2004). Signaling was examined by comparing the change of conduction velocity in the connective with the change as the APs passed through the mesothoracic ganglion.

After 10 min of hypoxia, the relative conduction velocities of APs firing at lower instantaneous frequencies were not significantly different between axon and neuropil sections (Fig. 6; Holm-Sidak pairwise multiple comparisons; <100 Hz, \( t = 0.06, P = 0.96; 100–200 Hz, t = 1.34, P = 0.21 \)). Firing rate was found to be a factor, as spikes at rates > 200 Hz conducted
During hypoxia, the fidelity of signaling in the DCMD decreased, with APs failing to propagate from the anterior to the posterior recording electrode. The loss was most pronounced at 45 min in hypoxic preparations, and this was reduced by \(10^{-4}\) M OA bath application. *Significant differences between treatments, \(P < 0.05\).

Prolonged hypoxia exacerbated the difference between connective and ganglion relative conduction velocity. While there was again no difference at low firing rates after 45 min (Fig. 6; <100 Hz, Holm-Sidak pairwise multiple comparison, \(t = 2.38, P = 0.07\)), both moderate firing rates (100–200 Hz) and high firing rates (>200 Hz) showed a significant slowing of conduction velocity through the ganglion (100–200 Hz, Holm-Sidak pairwise multiple comparison, \(t = 3.44, P < 0.05\); >200 Hz, Holm-Sidak pairwise multiple comparison, \(t = 6.07, P < 0.01\)).

OA affects whole animal responses to anoxia. Whole animal responses to anoxia were examined by immersion in water. The time required to succumb to the anoxic stress as well as the time required to recover once removed from the water were measured. To minimize the effects of variation in individuals, responses to the stress were compared within animals during a repeated trial following recovery (80 min after removal from water) to derive a relative measure comparing second and first immersions. The effects of OA or the OA antagonist EP were evaluated among animals by comparing the relative measures under different treatment conditions. Treatments included an abdominal injection during the recovery period of 10 \(\mu\)l of \(10^{-2}\) M OA, \(10^{-2}\) M EP, or a control saline injection. Given a hemolymph volume in gregarious *Locusta migratoria* around 200 \(\mu\)l (Ayali and Pener 1992), the approximate dosage of either drug was \(0.5 \times 10^{-2}\) M. Compared with EP (Fig. 7A; Holm-Sidak pairwise multiple comparisons, \(t = 2.69, P < 0.05\)) or saline vehicle (Holm-Sidak pairwise multiple comparisons, \(t = 2.40, P < 0.05\)), OA injection significantly decreased the relative time to succumb to the water immersion.

During recovery from the anoxic coma induced by the second water immersion, animals that had been injected with EP between trials showed a significantly slower recovery compared with control animals (Fig. 7B; Holm-Sidak pairwise multiple comparisons, \(t = 2.54, P < 0.05\)) as well as OA-treated animals (1-way ANOVA with Holm-Sidak pairwise multiple comparisons, \(t = 3.06, P < 0.05\)). Together, these results indicate that OA affected whole animal responses to anoxic coma. As anoxic coma and recovery have been shown to result from disruption and recovery, respectively, of ion homeostasis within ganglia (Armstrong et al. 2009; Rodgers et al. 2007), this is suggestive that OA helps to mitigate the effects of anoxia on neural circuits as a whole, in addition to the axon of the DCMD that we investigated.

**DISCUSSION**

The structure of DCMD bursts contains important information related to its role in detecting the looming approach of predators (McMillan and Gray 2015). This structure of intraburst firing rate will be affected during conduction along the axon by a decrease in conduction velocity with increasing firing rate. Hence mechanisms that could maintain conduction velocity at high firing rates and during abiotic stress are likely to have provided a selective advantage. We have found that AP signaling in the axon of DCMD undergoes a loss of fidelity during hypoxia. These changes occurred slowly over time (~45 min), with a gradual loss of performance. Similar but more severe effects on AP signaling were observed in response to mitochondrial impairment with NaN3. The reduction in conduction velocity could be mitigated by bath application of OA. Modulation by OA was also shown to affect whole animal responses to oxygen stress, further suggestive of a role for OA in supporting circuit function during stress.

In these experiments, the effects of hypoxia on conduction velocity and fidelity were local to the thoracic regions affected by the surgically lesioned trachea. Care was taken during dissection to prevent tracheal damage to regions other than intended targets, and we found no unusual changes in visual processing and signal strength in hypoxic preparations, supportive that outside of the surgical intervention the animal was indeed normoxic. Clearly this specific experimental intervention would not occur in nature. During periods of intense activity, however, insects show a remarkably high aerobic metabolic rate, particularly during flight (Suarez et al. 1996).

Such high levels of activity can lead to metabolic and respira-
OCTOPAMINE, HYPOXIA, AND AXONAL CONDUCTION

Fig. 6. Reductions in conduction velocity are more pronounced during conduction through the ganglion compared with conduction within the connective. Three extracellular electrodes were used to measure conduction velocity of DCMD action potentials, both along the thoracic connective (Connective) as well as across the mesothoracic ganglion (Ganglion). During 45 min of hypoxia, conduction velocity in the axon was reduced more through the ganglion than along the connective. The difference is particularly evident at higher instantaneous firing frequencies. *Significance between treatments, P < 0.05.

Fig. 7. Octopamine (OA) affects whole animal time to succumb and time to recover from anoxic coma. Time to succumb to anoxic coma and time to recover are expressed as a within-animal relative measure of (2nd exposure time − 1st exposure time)/1st exposure time. A: OA-treated locusts showed significantly faster times to succumb than epinastine (EP)-treated or control (C) animals. B: EP-treated animals showed significantly slower recovery times (standing upright) compared with OA-treated and control animals. Significance was assessed by a 1-way ANOVA and is indicated by letters: bars with different letters are significantly different from each other. nC = 13; nEP = 14; nOA = 12.
result in APs of smaller amplitude and slower conduction velocity, as we show in these experiments.

The impact of hypoxia on both conduction velocity and AP fidelity can be modulated by OA. OA has a known role in increasing excitability of thoracic motor systems in the locust, where it makes an important contribution to the flight central pattern generator (Ramirez and Pearson 1991). As well, OA has actions in sensory afferents associated with flight circuitry (Leitch et al. 2003), where its presence extends the duration of flight bouts (Brembs et al. 2007). OA also contributes to responsiveness of DCMD-mediated escape behavior through its effect on looming detection circuitry in the brain (Rind et al. 2008). Furthermore, endogenous OA release has been shown to be supportive of thoracic circuit function during environmental stress (Armstrong et al. 2006). That OA is elevated during vigorous activity or environmental stress suggests that it could act to support signaling during these times. Dorsal unpaired median neurons have been identified as putative sites of OA release within the mesothoracic ganglion (Braunig and Burrows 2004). Any OA released either locally or into the hemolymph during the present experiments would be expected to have been washed out by the controlled bathing solution, thereby blocking any endogenous protective effect to the hypoxia. The pharmacological results, however, point to OA release within the ganglion as a potential mechanism to alleviate conduction deficits associated with tissue hypoxia. Our experiments did not address the mechanism or pathway by which OA exerted its effect. However, the fact that OA modulated AP excitability and axonal conduction in the connective strongly implies that the axon possesses OA receptors. In the crustacean stomatogastric nervous system there is excellent evidence for OA directly modulating axons of a projection neuron (Goaillard et al. 2004) and for dopamine improving fidelity of AP propagation via \( I_h \) (Ballo et al. 2012). There is increasing awareness in different systems that AP propagation can be modulated in axons (e.g., in mammalian cortical pyramidal neurons; Yang et al. 2013), thus affecting information processing between spatially separated sites (reviewed in Bucher and Goaillard 2011). It remains to be determined how OA modulates AP propagation in the axon of the DCMD in locusts.

To investigate the general applicability of the results we obtained on conduction in the axon of a single identified neuron, we examined the effects of OA and its antagonist, EP, on the response of whole animals to anoxia. The times to succumb and recover from anoxia induced by water immersion are useful indicators of whole animal resistance to anoxia, which can be increased by heat shock pretreatments targeting ion homeostasis in the CNS (Hou et al. 2014). Similar to the effects of a heat shock pretreatment, we found that an anoxic pretreatment increased resistance in control animals by increasing the time to succumb and decreasing the time to recover. This modulation is mediated via the AMPK pathway and is associated with a reduction in performance of DCMD and decreased energy consumption (Money et al. 2014). Against this background, the acute effect of OA was to reduce the time to succumb, suggesting an increased metabolic rate associated with generally improved neural performance. Whereas OA did not affect time to recover compared with control animals, EP increased the time to recover, suggesting that the struggling and convulsions during immersion prior to coma increased endogenous levels of OA, which improved neural performance during recovery and was blocked by EP. Consistent with this interpretation is the observation that EP had no effect on time to succumb, indicating low levels of OA before immersion. Together the results show that manipulation of OA affected whole animal responses to hypoxia/anoxia in ways that can be interpreted as improving neural performance, as shown specifically for axonal conduction of DCMD, at the expense of increased energy consumption.

An important future direction will be to examine the role of second messengers known to be involved in OA signaling in the locust during environmental stress, particularly the cAMP/PKA pathway (Armstrong et al. 2006). The cAMP agonist forskolin reduces the effect of hypoxia on DCMD (Money et al. 2014), although OA has yet to be linked to this action. Another approach could be the use of RNAi to block OA production (Farooqui et al. 2004), and therefore release during hypoxia. We have not yet demonstrated a functional consequence of the decreased conduction velocity and reduced fidelity. DCMD is known to be important during escape behaviors such as jumping (Rind and Simmons 1992) and steering avoidance during flight (Santer et al. 2006), where activity in DCMD at rates in excess of 200 Hz is associated with triggering the behavior. A testable prediction from our results is that animals suffering this type of hypoxia may respond more slowly or less reliably to approaching threats, because of the decreased performance of DCMD. Locusts recovering from a prior stress also show slower DCMD conduction and an associated decrease in the reliability of their responses to looming targets (Money et al. 2014), suggestive that reduced function in DCMD may indeed impair behavior.

There is an extensive literature on axonal excitability in mammalian white matter (e.g., spinal cord or optic nerve preparations) because of its importance in human pathologies of stroke (Matute et al. 2013) or pain hypersensitivity (Waxman and Zampini 2014). Attention has mostly focused on the amplitude of extracellularly recorded compound action potentials (CAPs), which provide a monitor of conduction reliability under different conditions. In these preparations hypoxia causes a reduction followed by a loss of the evoked CAP amplitude in rat optic nerve, and the severity depends on developmental age associated with the stage of myelination (Fern et al. 1998). It is also increasingly clear that neurotransmitter mechanisms exist to modulate conduction in peripheral pathways (Butt et al. 2014) in ways that are not related to pathology (Kawai et al. 2007). Thus there are parallels between our findings and results from mammals. The latter studies, however, are often restricted by the small size of myelinated axons to monitoring CAPs rather than single-unit responses. The ability to focus study on identified, single axons provides a considerable advantage for dissecting the modulatory mechanisms that support AP conduction at high firing frequencies (Cross and Robertson 2016).

We have shown that transmission of long-distance signals through the ganglia can be compromised during hypoxic episodes and that OA helps to reduce this loss of fidelity. We suggest that this could be an important mechanism in the behaving animal during periods of tissue hypoxia.
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DISCLOSURES

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AUTHOR CONTRIBUTIONS


REFERENCES


Pame stern ME, Hogg DW, Ormond J, Shin DS, Woodin MA, Buck LT. Endogenous GABA_A and GABA_B receptor-mediated electrical suppression is critical to neuronal anoxia tolerance. Proc Natl Acad Sci USA 108: 11274–11279, 2011.


