RESEARCH PAPER

The Mechanistic Action of Carbon Dioxide on a Neural Circuit and NMJ Communication

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ABSTRACT

Previous studies examining behavioral responses to CO_2 revealed that high [CO_2] acts as a natural repellent in a concentration dependent manner for crayfish. Physiologically, CO_2 can rapidly block the autonomic responses in heart rate, as well as, inhibit an escape tail flip reflex in crayfish. Here, we demonstrate that the behavioral observations can be mechanistically explained by CO_2 blocking glutamate receptors at the neuromuscular junction and through inhibition of recruiting motor neurons within the CNS. The effects are not mimicked with a lower pH in the bathing solution. Since spontaneous and sensory-evoked activities in the sensory root and motor neurons are reduced by CO_2, this is an anesthetic effect. We propose this is due to blockage of electrical synapses, as well as, some of the central glutamatergic-drive. We used agonists and antagonists (glutamate, nicotine, domoic acid, cadmium, heptanol) to various synaptic inputs, which are possibly present in the ventral nerve cord (VNC). Results from these chemicals supported the idea that there is electrical as well as chemical drive within the circuit that can modulate intrinsic as well as sensory evoked activity in the motor neurons. We have documented that CO_2 has actions in the periphery as well as in the CNS, to account for the behavioral responses previously shown. Furthermore, we document that gap junctions as well as glutamatergic synapses are potential targets. This study also aids in the dissection of a neural circuitry within the VNC that drives spontaneous and sensory evoked activity of the superficial flexor motor neurons. *J. Exp. Zool. 319A: 340–354, 2013.* © 2013 Wiley Periodicals, Inc.

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Carbon dioxide (CO_2) plays a pivotal role through the complex interactions between many organisms and their environment. Environmental CO_2 is known to be an attractant for many invertebrates at low levels (orientation response), an important cue for host location, and acts as a repellent at higher concentrations. CO_2 as a by-product of cellular respiration is rapidly dealt with in cells and within the whole organism to maintain pH homeostasis. To this day, neurobiologists are still intrigued in the illusive mechanisms of CO_2 in H^+/CO_2 sensing neurons in the periphery and central nervous system in various animals (Putnam et al., 2004; Luo et al., 2009). General actions of CO_2 on cells are likely ubiquitous but it remains unclear how the cellular responses result in diverse outcomes ranging from inhibition to excitation.

Some organisms detect environmental CO_2 by external sensory receptors (Guerenstein and Hildebrand, 2008), while others monitor environmental levels and internal concentrations through intra-receptors (Baker and Honerjiger, '78). Although internal CO_2 levels are constantly monitored and regulated, exact mechanism of detecting the partial pressure of CO_2 (pCO_2) is not fully understood. It is known that there are many cell types capable of sensing CO_2.

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Peripheral carotid and aortic bodies as well as central structures within the brainstem are primary sites for detection in mammals (Ainslie and Duffin, 2009). CO_2 can diffuse across the lipid bilayer and is driven by concentration gradients due to the rapid enzymatic reaction of carbonic anhydrase ($CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$) (Stone and Koopowitz, '74; Breton, 2001). Carbonic anhydrase in mammals is crucial in cellular pH balance.

The mode of action in which CO_2 operates as an anesthetic is not fully established. It is commonly assumed that there is a direct effect of CO_2 and/or indirect effects, through rapid changes in intracellular pH, which are the major contributors to the cellular responses. Empirical evidence to a general mechanism of action of anesthetics involves physical changes in permeability of the cell membrane (Mullins, '75). Permeability changes can come about by alteration in the permeability of leak channels, exchangers or even activity of the various ionic pumps (Desai-Shah and Cooper, 2010) and vesicle recycling/packaging within nerve terminals (Wu and Cooper, 2012a, b).

There are numerous mechanisms in which the internal and external environment can result in alterations of ion channels of primary sensory neurons and in communication between populations of neurons (Bennett et al., '91; Beyer, '93; Peracchia et al., '94). Since it is known that CO_2 has an effect on electrical communication by uncoupling gap junctions (Arellano et al., '90), this could have an effect on the whole animal behavior by altering key steps in communication within the ventral nerve cord (VNC). Some of the earliest findings in this regard were performed on the crayfish lateral giant (LG) interneurons. The escape tail flip circuit involves the LG and electrical communication via gap junctions. The sensory to motor action involved in the tail flip was one of the first neural circuits to be described in detail with individually identifiable neuronal components and physiological properties to be related to a whole animal response (Johnson, '24; Kennedy and Takeda, '65a, b; Kennedy et al., '69; Antonsen and Edwards, 2003).

Recently, we have shown that acute high [CO_2] alters behavioral responses; specifically, involving the tail flip response in freely moving crayfish. By using a mechanosensory stimulation of a strong tap on the telson, crayfish exposed to water that was bubbled with a 100% CO_2 showed no responsiveness within a 30-min time period. Furthermore, the heart and ventilation rates also showed complete cessation with high acute exposure within the same time period that the unresponsiveness to mechanosensory stimulation occurred. We suggested the CO_2 rises within the animal and has a direct action on the neuromuscular junction as well as on CNS, which accounts for the behavioral and physiological changes (Bierbower and Cooper, 2010).

The crayfish nervous system presents several features which feasibly enable the study of a multitude of questions. Regions of interest in the CNS and well-defined musculature are readily exposed using in situ preparations. An area of particular interest involves a superficial flexor muscle circuit comprised of a "sensory nerve root-ganglia-motor nerve root." The motor root (third root) that innervates the superficial flexor muscle contains five excitor motor neurons and only one inhibitor motor neuron (Velez and Wyman, '78). Importantly, this nerve root contains only motor axons innervating the superficial flexor. These neurons can be activated by stimulating reflex circuits that stimulate the motor neurons to the superficial flexors. Furthermore, these motor nerves are also spontaneously active so an increase or a decrease in activity is readily noted with activation of a sensory drive (Strawn et al., 2000).

The sensory nerve (second root) is comprised of the numerous primary afferent axons which are very small (1–10 μm). The primary mechanosensory neurons have direct connections, by electrical synapses with the LG (Krasne, '69; Zucker, '72). In addition, mechanosensory neurons are known to excite interneurons via chemical synapses.

Glutamate is a major excitatory transmitter in the vertebrate central nervous system (Curtis and Johnston, '74). Glutamate is also the transmitter at excitatory neuromuscular junctions in crustaceans, such as the superficial abdominal slow flexor muscle (Kawagoe et al., '81) and the opener muscle of the crayfish claw or walking legs (Van Harreveld, '36; Kennedy and Takeda, '65a, b). The innervation profile in the limb muscles is also well known. The opener muscle is controlled by two efferent axons, one inhibitor and one excitor (Van Harreveld and Wiersma, '36) and has been used for years as a model preparation for synaptic activity (Cooper and Cooper, 2009). The excitatory motor neuron whose axon branches to the separate muscle fibers is identifiable from preparation to preparation. These motor neurons also produce graded post-synaptic potentials that are non-spiking. To address mechanisms as to why crayfish become unresponsive to a touch or threatening stimuli with high CO_2 exposure, the leg opener muscle was examined in this study for changes in synaptic responses at the NMJ. Since CO_2 has been shown to reduce sensitivity to glutamate at the skeletal NMJs in *D. melanogaster* and given that the glutamatergic receptors at the NMJ in crayfish are also of a quisqualate subtype (Dudel et al., '92; Lee et al., 2009), we expected similar effects for CO_2 at the crayfish NMJs on the opener muscle.

The aims of this study were to examine the mode of action of CO_2 on each component within a crayfish "sensory-ganglia-motor nerve-muscle" circuit within second or third abdominal segments, as well as, synaptic communication at the neuromuscular junction. Experiments were designed to assess the effect of CO_2 on (1) neuromuscular transmission and axon conductance for the opener muscle motor unit in the walking leg; (2) activity of sensory neurons which drive motor neurons; and (3) the activity of superficial flexor motor neurons driven by the central nervous system.

METHODS

Animals

Mid-sized crayfish, *Procambarus clarkii*, measuring 6–10 cm in body length were obtained from Atchafalaya Biological Supply

Co. (Raceland, LA, USA). The animals were housed individually in an aquatic facility and fed dried fish food. All animals were in the laboratory at least 2 weeks before any experimentation. A total of 50 crayfish were used in the study. Both sexes of crayfish were used in this study but differences between the sexes were not analyzed. All dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (in mM: 205 NaCl; 5.3 KCl; 13.5 $CaCl_2 \cdot 2H_2O$; 2.45 $MgCl_2 \cdot 6H_2O$; 5 HEPES adjusted to pH 7.4; Sparks and Cooper, 2004). All chemicals were obtained from Sigma chemical company (St Louis, MO, USA) and all experiments were performed at room temperature (21–22°C).

Opener Muscle—Neuromuscular Junction Physiology

Details of the dissection and electrophysiological recordings are described in Cooper and Cooper (2009). The short-term facilitation (STF) was induced by providing a train of 10 pulses at 40 Hz, at 5-sec intervals, to the excitatory nerve. Intracellular excitatory post-synaptic potentials (EPSPs) recordings were performed by standard procedures (Dudel et al., '83; Cooper et al., '95; Crider and Cooper, 2000; Sparks and Cooper, 2004; Fig. 1). Electrical signals were recorded on-line to a PowerLab/4s interface (ADInstruments, Bella Vista, Australia) and calibrated with the Powerlab Scope software 3.5.4 version.

Two conditions were tested and analyzed for comparison. The conditions were: (1) normal saline at pH 7.2 switched to a saline saturated with 100% CO_2 and (2) normal saline at pH 7.2 switched to a saline of pH 5.0. Recording was continuous before, during and after exposure to treatment conditions. Saline adjusted to a pH of 5.0 was performed by the addition of HCl.

To test if CO_2 was directly altering sensitivity of the postsynaptic muscle to the neurotransmitter glutamate, the NMJs were exposed to exogenously applied glutamate (1 mM) while recording EPSPs and membrane potential. The experimental paradigm for examining the effect of CO_2 was to expose the preparation to normal saline, followed by exposure to CO_2-saturated saline, followed by CO_2-saturated saline with glutamate and then finally back to a normal saline. The normal saline washout at the end of the experimental paradigm was to ensure the preparation was not damaged during experimentation. The experimental procedure to examine the effect of saline at a pH 5.0 consisted of exposure to normal saline (pH 7.4), followed by exposure to saline at a pH 5.0, and then by a saline at pH 5.0 with glutamate. The preparation was finally exposed to normal saline (pH 7.4) to test for recovery. The exogenous exposure to glutamate was used to determine postsynaptic sensitivity.

Analysis of the EPSP responses consisted of using the 10th EPSP of the STF train as determined by previously described procedures (Crider and Cooper, 2000). Statistics employed was the Wilcoxon non-parametric test to identify trends of increases or decreases in the percent changes to the various manipulations.

The amplitudes of the EPSPs were measured using the Powerlab program Scope. An average of every 100 sec was used for graphical representation.

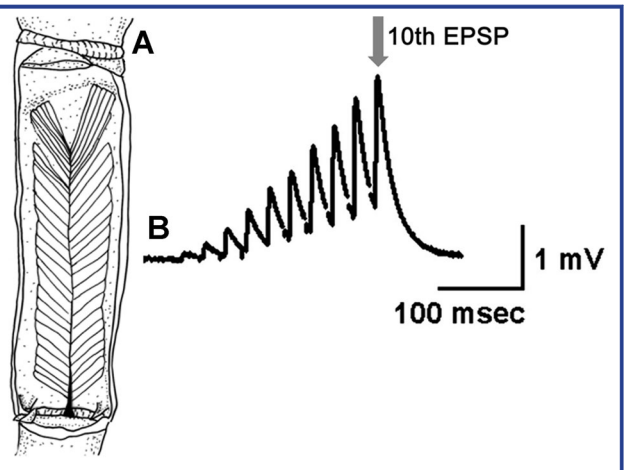

Figure 1. Crayfish walking leg opener muscle preparation: A: Schematic of the opener muscle (propus segment) in the crayfish walking leg (distal is directed downward). B: The EPSP responses recorded intracellularly from the distal muscle fibers and the response shows a marked facilitation that occurs throughout the stimulation train at 40 Hz. Peaks indicate each pulse stimulation in the 10 pulse train with the 10th EPSP indicated by the arrow. The 10th EPSP amplitude was used for analysis.

Recording Action Potentials in the Motor Axon

To measure if the electrical signal of the action potential is affected with CO_2 exposure, the motor nerve (in the merus segment) was stimulated while the pre-terminal of the excitatory axon innervating the leg opener muscle was recorded using a sharp intracellular electrode filled with 3 M KCl. The preparation was exposed in a physiological saline and the bath was exchanged with CO_2 containing saline while stimulating at a frequency of 1 Hz. The amplitudes of the action potentials produced were measured from digital records obtained at a 20 kHz acquisition rate.

Abdominal Neural Circuit Physiology

All animals were sacrificed in less than 5 sec by rapid decapitation followed by removal of the abdomen. As detailed in Strawn et al. (2000) the VNC was cut between T5 and A1, the abdomen was separated from the thorax and pinned ventral side up in a Sylgard coated dish. The nerve roots of the VNC, segmental ganglia and superficial flexor muscles were exposed and bathed in physiological saline solution (schematic, Fig. 2).

Extracellular recordings of neural activity in the second sensory nerve root and third motor nerve root of the third abdominal segment were obtained by *en passant* suction electrodes. For intracellular recordings of EPSPs, the medial muscle fibers of the

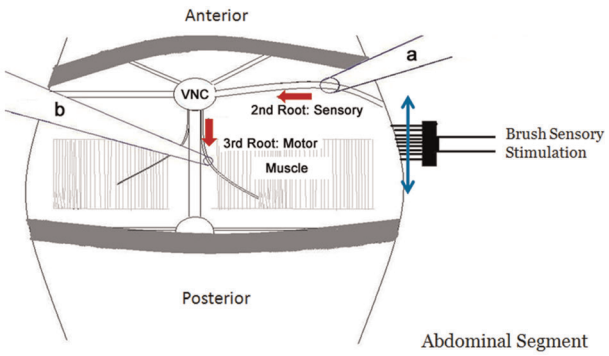

Figure 2. Schematic of ventral view in a dissected abdominal segment of the crayfish abdomen. Stimulation of cuticle occurred along the lateral side of the segment being recorded. Suction electrodes are labeled "a" and "b" above.

superficial flexor were used. All data were recorded by a computer via PowerLab/4s A/D converter (ADInstruments).

For stimulation of the cuticle, a stiff-bristled paintbrush was mounted on a micromanipulator to control pressure and movement. The brush was positioned along the lateral side of the segment being monitored and moved an approximate distance of 2.54 cm in a consistent and continuous forward and then backward motion.

The analysis of the neural circuit used direct counting of the evoked spikes and was presented as frequency of activity. A 30-msec time period prior to bush stimulation and a 30-msec time period during the stimulation was used for analysis of spike frequency. This was repeated five times and the average percent change in frequency was used. This provided an index to describe the effect of stimulating the circuit. The average activity "prior to stimulation" and "during stimulation" were also used to assess the effect of exogenously applied compounds on the activity without and with stimulation. In every individual trial, direct counts were then used to obtain an average value for each condition. These average values were then used to obtain a percent change in spike frequency for comparison across experimental conditions.

Chemical Agents

All chemicals were obtained from Sigma chemical company.

Nicotine. Acetylcholine (Ach) is a major excitatory neurotransmitter in the crustacean and insect nervous system, in which many of its actions involve nicotinic receptors (Tsunoyama and Gojobori, '98). To test for nicotinic cholinergic receptors influencing the sensory or motor roots, a 10-μM nicotine containing saline was bathed on the preparation. The effect of nicotine was also examined in the presence of CO_2 containing saline to determine if the presence of CO_2 blocked the action of nicotine within the CNS.

Glutamate. To induce spike activity in the sensory root, glutamatergic interneurons and/or the motor nerve root, saline containing 1 mM glutamate was used. The effect of glutamate was also examined in the presence of CO_2 containing saline to determine if the presence of CO_2 blocked the action of glutamate within the CNS. The saline was rapidly exchanged with saline containing 1 mM glutamate. The concentration of glutamate at synaptic clefts is estimated to be in the range of 1–10 mM (Heckmann and Dudel, '98). Thus, using 1 mM is reasonable to examine the effects on the neural circuitry.

Domoic Acid. A kainate receptor agonist, domoic acid, was used to examine if a quisqualate-type glutamate receptor may have a role in the sensory-CNS-motor neuron circuit. Since it was shown at the NMJ in *D. melanogaster* that quisqualate-type glutamate receptors are blocked by CO_2, as well as domoic acid (Badre et al., 2005; Lee et al., 2009), it was of interest to know if domoic acid would have an effect in crayfish and if the effect of CO_2 might be mimicked by domoic acid. Since it was shown that 1 mM is sufficient to inhibit quisqualate-type receptors at the NMJ in *D. melanogaster*, the same concentration was used in this study.

Cadmium. Cadmium is known to block chemical transmission, as seen in the crayfish *Pacifastacus leniusculus* (Heitler et al., '91). In order to understand the role of chemical transmission in the CNS circuit used herein, saline containing 1 mM cadmium was used. A high concentration of cadmium is used to ensure blocking of chemical transmission. The effect of CO_2 after chemical synapses were blocked with Cd^{2+} was also examined, to determine if any additional action of CO_2 occurred. The action of CO_2 would likely block any electrical junctions present after the chemical synapses are blocked since low intracellular pH is known to block gap junctions.

Heptanol. The uncoupling effect of heptanol on gap junctions was first described in crayfish septate axons by Johnston et al. ('80). This effect was soon confirmed in vertebrate and invertebrate systems where electrical communication was known to occur (Bernardini et al., '84; Meda et al., '86). To understand the role of electrical communication in this circuit, a saline with 1 mM 1-heptanol was used. The effect of CO_2 after electrical synapses are blocked was also examined to determine if any additional action by CO_2 occurred.

RESULTS

Opener Muscle—NMJ

Since bubbling 100% CO_2 in crayfish saline causes the external pH (pH_o) to drop to pH of 5.0, the possibility that low pH_o is mediating some of the identified effects needed to be addressed independently of CO_2 exposure. Thus, the EPSP amplitude was measured over time during exposure to normal saline, saline saturated with

CO_2, saline saturated with CO_2 plus glutamate. Also, saline adjusted to a pH of 5.0 was used to examine any effect on the amplitude of the EPSPs. All procedures were followed by normal saline to examine if recovery of the preparation occurred. When the preparation was exposed to CO_2 saturated saline, EPSPs rapidly became smaller and faded out all together. In five out of five individual preparations, there was a consistent attenuation of the EPSP within 1 min resulting in a significant effect with CO_2 exposure (five out of five animals; $P < 0.05$, non-parametric analysis sign test for paired-sampled data). In addition, the resting membrane potential (RMP) did not change with CO_2 exposure. A typical response for one preparation is shown in Figure 3. Furthermore, the membrane potential did not depolarize upon exposure to exogenous glutamatein a CO_2 incubated preparation (Fig. 4). With washout using normal saline, thus the removal of CO_2 saturated saline, the EPSPs returned (Fig. 3D).

The effect of the acidic environment was also tested by using saline adjusted to pH 5.0. In each preparation, EPSP amplitudes remained unchanged with exposure of the acidic saline (Fig. 5; five out of five animals; $P < 0.05$, Wilcoxon non-parametric analysis). Interestingly, the RMP of the muscle fiber was shown to become more negative (hyperpolarization) with exposure. Application of exogenous glutamate caused the RMP to quickly depolarize to approximately 0 mV within 1 min and quickly returned to resting membrane potential, indicating a quick depolarization and then receptor desensitization (Fig. 5C). EPSPs amplitude attenuates during exposure and then quickly attenuates to the baseline, indicating depolarization of the muscle with application of glutamate (Fig. 6; five out of five animals; $P < 0.05$, Wilcoxon non-parametric analysis).

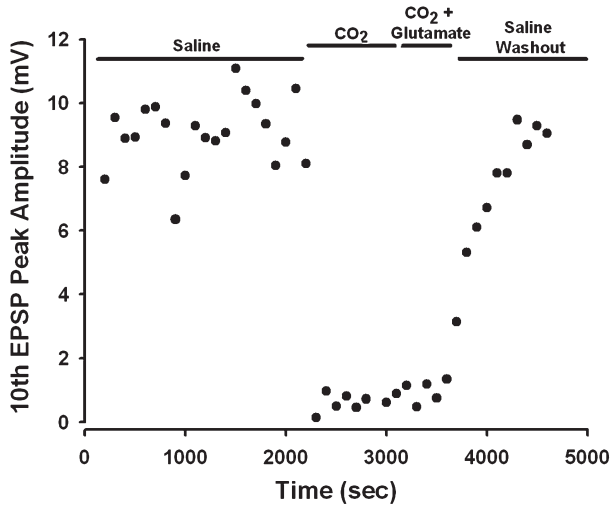

Figure 4. The effect of CO_2 on EPSP amplitude in opener muscle. Exposure to CO_2 resulted in a complete attenuation of the EPSP in five out of five preparations within 1 min without any change in resting membrane potential ($P < 0.05$, non-parametric analysis). Scatter dots indicate 100 sec averages of EPSP amplitudes. The black lines at the top indicate treatment conditions. Shown are the results for as typical preparation. Note that each preparation exhibits different amplitude for the facilitated 10th EPSP response.

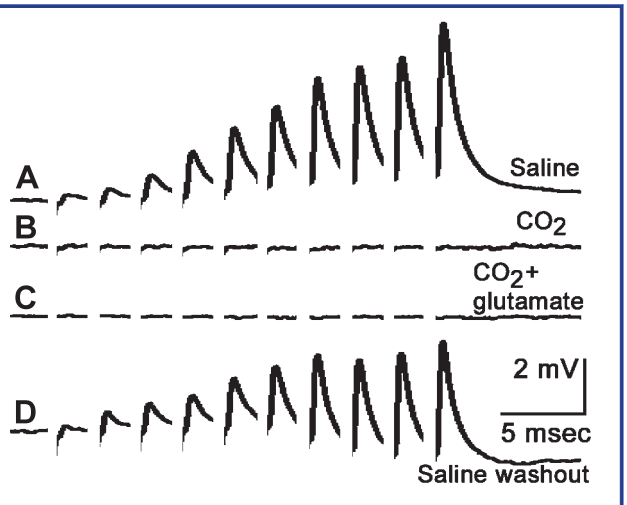

Figure 3. Intracellular recordings of junction potentials. A: EPSPs in normal physiological saline before CO_2 exposure, (B) EPSPs during CO_2 exposure, (C) EPSPs during CO_2 + glutamate exposure, (D) EPSPs after returning to physiological saline without CO_2 or glutamate. Junction potentials show significant reductions with CO_2 exposure (five out of five preparations, $P < 0.05$, Wilcoxon non-parametric analysis).

Glutamate application showed no effect on the CO_2 saturated NMJ; whereas, NMJs without CO_2 exposure did respond to glutamate the same as they did for exposure to pH 5.0 saline. The reduction in the RMP was used as an assay for sensitivity to exogenous glutamate. Due to the insensitivity of the muscle to exogenously applied glutamate, the site of action for the effect of CO_2 is likely postsynaptic. Furthermore, the reduced EPSP amplitude and attenuation could not be explained by the low pH but rather can be explained by a direct effect of CO_2 exposure itself. A summary of EPSP and resting membrane responses are summarized in Table 1. These results clearly indicate that CO_2 blocks the responsiveness of the muscle to exogenously applied glutamate. This is also likely to explain the reduced EPSP amplitude with endogenously evoked glutamate release, as well as, unresponsiveness to exogenously applied glutamate.

Recording Action Potentials in the Motor Axon

In order to examine the influence of CO_2 on the size and shape of the action potential in the presynaptic (pre-terminal) of the excitatory axon, intracellular recordings were carried out on the

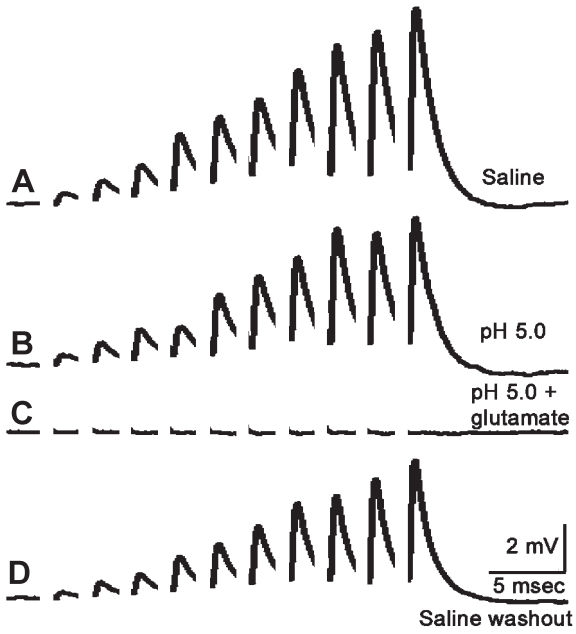

Figure 5. Intracellular recordings of junction potentials. A: EPSPs in normal physiological saline before pH 5.0 saline exposure, (B) EPSPs during low pH exposure, (C) EPSPs during low pH + glutamate exposure, (D) EPSPs in physiological saline after returning to normal saline. Recordings of junction potentials did not show a significant change in EPSP attenuation with pH 5.0 exposure in five out of five preparations ($P < 0.05$, Wilcoxon non-parametric analysis).

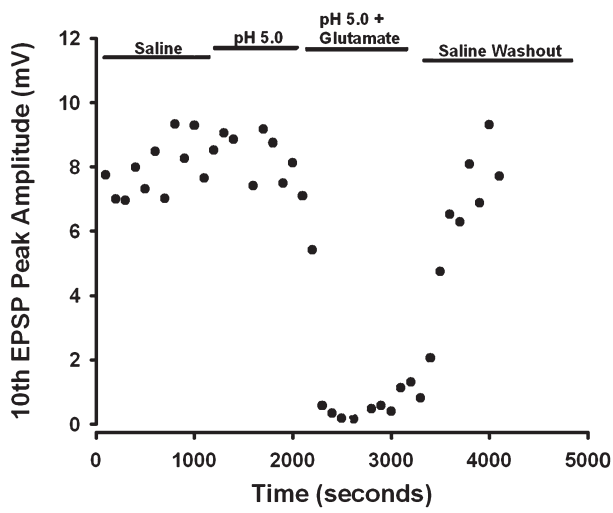

Figure 6. Timing in the effect to low pH 5.0 and CO_2 exposure on the 10th EPSP amplitude in the opener muscle. Exposure to pH 5.0 did not result in an attenuation of the EPSP in five out of five preparations as seen with CO_2 exposure. The resting membrane potential depolarized in acidic saline when exposed to glutamate ($P < 0.05$, non-parametric analysis). Scatter dots indicate 100 sec averages of EPSP amplitudes. The black lines at the top indicate treatment conditions.

crayfish tonic motor neuron which innervates the opener muscle in the first walking leg (Fig. 7A). The electrode impaled axon, showed that the action potential was typical (Sparks et al., 2003) and when normal saline was replaced with CO_2 saturated saline, no change in action potential amplitude or shape was noted (three out of three preparations, Fig. 7B).

The Effect of Carbon Dioxide and Low pH 5.0 on the Spike Activity in a Superficial Flexor Motor Circuit

To record from a "sensory root-ganglia-motor root" neural circuit, the sensory nerve root from the cuticle to the VNC was left intact to provide a means of stimulating the circuit. En passant recordings of the intrinsic activity as well as evoked (through cuticular stimulation with the brush) activity from the sensory and motor roots was captured simultaneously.

Stimulation of cuticular sensory neurons increased the firing frequency in both the sensory root and motor root, which is indicated by increased firing activity when compared to basal activity (traces within the boxes are during stimulation, Fig. 8). Augmented activity induced by cuticular stimulation was observed in all preparations initially to ensure that the "sensory root-ganglia-motor root" circuit was activated prior to further experimentation. The effect on firing frequency is shown in the representative single traces in the presence of saline, CO_2 or low pH 5.0. In each of the five preparations, application of CO_2-satuared saline to the abdominal preparation resulted in a continuous firing in the sensory root but showed a rapid decrease, to the point of cessation of spike activity, in the motor root (Fig. 8A part g). The rate of change in the sequence of spikes in the motor nerve root was determined by counting the number of events for 30 msec before and during periods of cuticular stimulation. For low pH 5.0, the firing frequency persists in both the sensory and motor roots (Fig. 8B).

With the stimulation, the associated increases in activity were quantified by taking the response of 30 msec of basal rates ($n = 5$) and the response during cuticle stimulation ($n = 5$) in five preparations. This was used to determine quantifiable measures on spike frequency with exposure to CO_2 and low pH 5.0. These enhancements of the stimulus-associated drive of the superficial flexor neural circuit were observed in five out of five preparations (Wilcoxon non-parametric test, $P < 0.05$). The mean percentage values reported are an average of five subsequent trials in saline and with exposure. The saline bath was exchanged with the one containing saline saturated with CO_2. In the sensory root, the

Table 1. Responses at the NMJ during exposure of CO_2 or pH 5.

	RMP (saline)	Exposure to agent	Add glutamate with agent	Saline wash
CO_2	∼(−75 mV)	No EPSPs ∼(−75 mV)	No depolarization	EPSPs slow to return
pH 5.0	∼(−75 mV)	EPSPs ∼(−85 mV)	Quick depolarization and desensitization	EPSPs

The response is characteristic for both CO_2 and pH 5.0 exposure (five out of five animals, $P < 0.05$, non-parametric analysis). The main point is the CO_2 blocks the responsiveness of the muscle to exogenously applied glutamate.

average change with cuticle stimulation was shown to be significantly decreased (−25%, ANOVA; $^{*}P > 0.001$, Holm-Sidak post hoc analysis) upon exposure to CO_2 (Fig. 9A, see Fig. 8a–b and c–d). For the motor root, the average change with cuticle stimulation was also shown to be significantly decreased (−100%, ANOVA; $^{*}P > 0.001$, Holm-Sidak post hoc analysis) upon exposure (Fig. 9A and see Fig. 8e–f and g–h). These effects on both sensory and motor roots with CO_2 exposure were shown to be significantly different from saline control frequency measures (Fig. 9A).

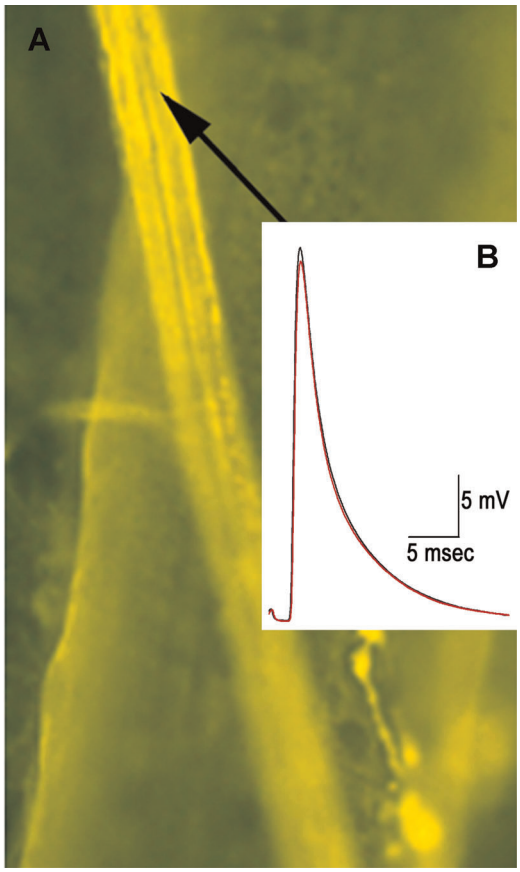

Figure 7. Intracellular recording of an action potential in excitatory crayfish opener motor neuron. A: Presynaptic (pre-terminal) motor axon. B: Schematic of amplitude and shape of action potential with CO_2 exposure. There was no noticeable change in either amplitude or shape of the action potential from normal saline to CO_2 saturated saline. Black trace in normal saline and red trace in saline bubbled with CO_2.

Exposure to low pH 5.0 saline did not show the same effects as seen with CO_2 (Fig. 8). Specifically, cuticle stimulation during pH 5.0 significantly increased activity in the sensory root (75%, ANOVA; $^{*}P > 0.001$, Holm-Sidak post hoc analysis) and most importantly the motor root increased (50%, ANOVA; $^{*}P > 0.001$, Holm-Sidak post hoc analysis) upon exposure. Thus, effects on the motor root seen with CO_2 exposure are unlikely to be the result of low pH.

Pharmacological Characterization of a "Sensory Root-Ganglia-Motor Root" Circuit Using Selective Agonists and Antagonists, Reveals the Complexity of the Superficial Flexor Motor Circuit

A range of selective agonists and antagonists was used to attempt a pharmacological classification of the receptors causing activation of superficial flexor motor neurons and abdominal muscles. Ligand-gated ion channels normally mediate rapid chemical synaptic transmission. After establishing the actions of these pharmacological agents, their actions were examined in regards of the responses observed for CO_2 saturated saline to determine actions of the CO_2 conditions on the neural circuit.

Application of nicotine to the preparation showed significant increased spike frequency on both sensory and the motor roots before and during cuticle stimulation (Fig. 9, ANOVA; $^{***}P > 0.001$, Holm-Sidak post hoc analysis). Nicotine in combination with CO_2, caused significant inhibition of the activity of the motor root (ANOVA; $^{***}P > 0.001$, Holm-Sidak post hoc analysis), but did not significantly inhibit evoked activity in the sensory nerve root (Fig. 9). We interpret the results that there is some cholinergic drive through nicotinic receptors in the central nervous system; however, the exact influence is still undetermined.

There was activation of the neural circuit during application of glutamate for both sensory and motor roots, indicated by further spike activity before and during cuticle stimulation (Fig. 9, ANOVA; $^{*}P > 0.001$, Holm-Sidak post hoc analysis, Fig. 9). Glutamate in combination with CO_2, caused significant inhibition of the activity of the motor root (ANOVA; $^{***}P > 0.001$, Holm-Sidak post hoc analysis), as well as activity in the sensory nerve

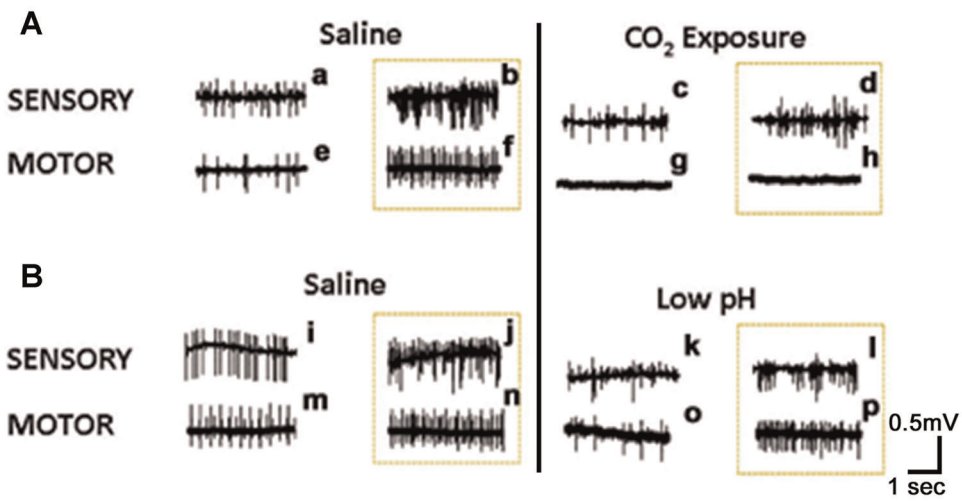

Figure 8. Effects of carbon dioxide on spike firing frequency in a "sensory root-ganglia-motor root" neural circuit. A: Spike frequency of the sensory and motor nerve roots during CO_2 exposure. B: Spike frequency of the sensory and motor nerve roots during low pH 5.0 exposure. The box indicates the responses during cuticular stimulation.

root (ANOVA; $^{*}P > 0.05$, Holm-Sidak post hoc analysis, Fig. 9). There was a consistent effect in five out of five preparations ($P < 0.05$, Wilcoxon non-parametric analysis). Results suggest some glutamatergic drive through glutamate receptors in the central nervous system. It is plausible that many of the interneuronal synaptic connections are glutamatergic and that this would explain the increase with glutamate application. The mechanistic action on the drive of the motor root is still undetermined.

Domoic acid did not show a significant decrease in spike activity from saline controls, for either sensory or the motor root in non-stimulated or stimulated preparations (Fig. 9). However, glutamate application showed a significant increase in spike activity. Thus, it appears domoic acid does not act as an antagonist or an agonist in the CNS. It is possible, that domoic acid might not be able to perfuse readily into the CNS tissue or that the quisqualate type receptors found at the NMJ are not present in the CNS. The NMJ responses are blocked rapidly by domoic acid. This would explain the continued motor nerve activity with application of domoic acid and the lack of inducing EPSPs on the muscle fibers.

Cadmium (Cd^{2+}) is known to block chemical synapses in the crayfish preparation; thus, Cd^{2+} was used to dissect chemical versus electrical communication in this superficial flexor circuit. Cd^{2+} showed no effect on spike activity from saline controls for the sensory root for up to 15 min of recording. However, there is a significant decrease in motor root activity after 10 min in time (ANOVA; $^{**}P > 0.02$, Holm-Sidak post hoc analysis, Figs. 9B and 10B). There was activation of the neural circuit during exposure for both sensory and motor roots indicated by further spike activity during cuticle stimulation. In each preparation with exposure to Cd^{2+}, the RMP of the muscle fiber remained relatively unchanged; although in two out of five preps, the RMP fluctuated to a more negative value. The effect of Cd^{2+} on synaptic transmission at the NMJ showed that the EPSP gradually became smaller and faded out all together after 15 min (Fig. 10A–C). Representative traces of EPSP activity in saline, 1 mM cadmium after 5 min and after 15 min, showed that evoked EPSPs gradually become smaller ($P < 0.05$, non-parametric analysis, $n = 5$).

Heptanol is widely known to reversibly decrease membrane ionic currents in electrically coupled cells (Burt and Spray, '88; Rüdisüli and Weingart, '89). Percent changes with application of heptanol on the sensory root did not show any significant changes from saline, before or during cuticular stimulation (Fig. 9). While the sensory root did not show any significant differences from saline control with cuticle stimulation, there was an increase similar to saline indicating continued activity in the sensory root after 1 and 10 min of heptanol exposure. Interestingly, the motor root showed a significant decrease (−35%) in spike frequency with stimulation after 10 min of heptanol exposure (ANOVA; $^{***}P > 0.001$, Holm-Sidak post hoc analysis Fig. 9). Stimulation on the cuticle and monitoring on the motor root, showed there was a significant decrease in activity, indicating the inhibition of the neural circuit (ANOVA; $^{***}P > 0.001$, Holm-Sidak post hoc analysis; Fig. 9). Results indicate there was no statistical difference from basal rates with stimulation after 10 min in heptanol. There was a consistent, significant effect in five out of five preparations ($P < 0.05$, Wilcoxon non-parametric analysis).

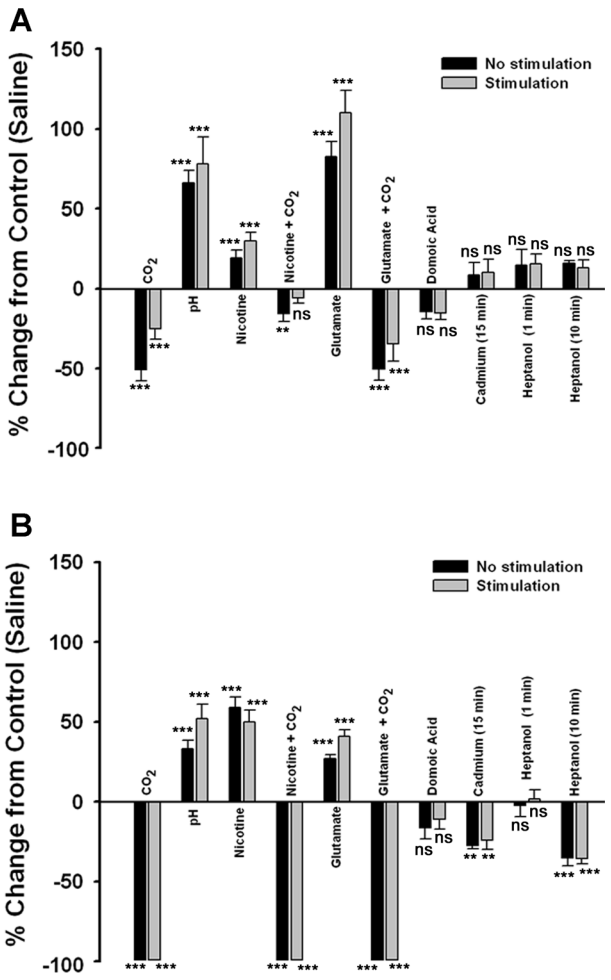

Figure 9. Influence of CO_2 and other compounds on a "sensory root-ganglia-motor root" neural circuit. Average percent change from saline control is shown for the (A) sensory root and (B) the motor root. Carbon dioxide exposure consistently shows a significant decrease in spike frequency in both the sensory and motor roots. The most dramatic effect is seen in the motor root where CO_2 causes complete cessation in the motor root even when combined with excitatory neurotransmitters. Before (black bars) and during cuticular stimulation (gray bars) were compared across conditions. The mean rate (±SEM) of spike frequency over multiple 30 msec time periods were assessed for the conditions. ANOVA $^{*}P > 0.05$, $^{**}P > 0.02$, and $^{***}P > 0.001$; ns, not significant.

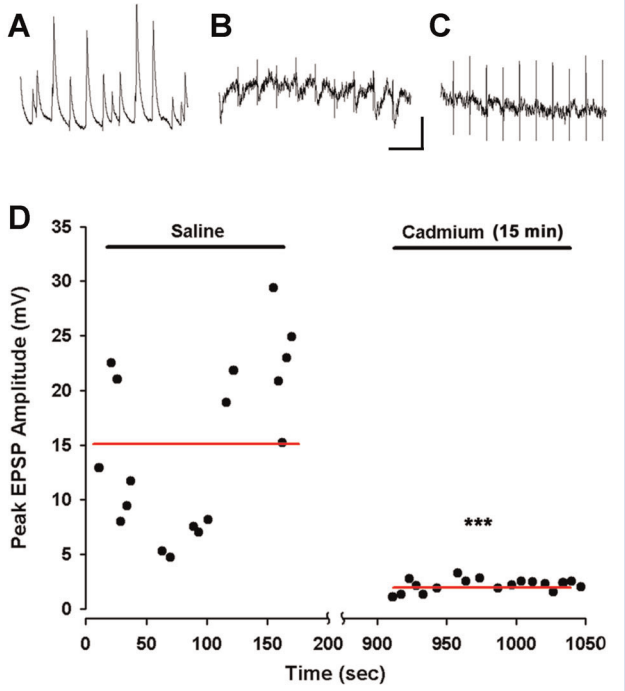

Figure 10. Cadmium exposure at the NMJ resulted in the EPSPs to became smaller and undetectable after 15 min. Representative traces of EPSP activity in saline (A), 1 mM cadmium after 5 min (B) and after 15 min (C). The evoked EPSPs become so small that basically they could not be discerned from the noise in the recording (C and D; $P < 0.05$, non-parametric analysis, $n = 5$). The vertical scale shown below the trace in panel B is 1 mV for trace A and 0.1 mV for traces B and C. The time scale is 50 msec for traces A–C.

As a composite, results are presented in Figure 9 and in Table 2 to summarize the significance of each component discussed and presented.

DISCUSSION

Some of the mechanisms, at a neuronal circuit level, which could account for the decreased behavioral responses induced by high levels of environmental CO_2 (as presented in Bierbower and Cooper, 2010) are presented in this study. Since rising CO_2 also resulted in a reduction of pH_o, this variable needed to be addressed throughout these studies. The presence of CO_2 blocked the muscle from being receptive to evoked, as well as exogenous glutamate; thus, a paralytic effect is proposed for CO_2's action. However, the intrinsic spontaneous and sensory evoked activity in the sensory root and motor neurons is also reduced in the presence of CO_2, so CO_2 also has some level of anesthetic effect. We propose that the reduction in motor neuron activity could arise due to blockage of electrical synapses, as well as some of the glutamatergic central drive. Agonists and antagonists applied to various synaptic inputs theoretically possible in the VNC, supported the idea that there is electrical as well as chemical drive within the circuit that can modulate intrinsic as well as sensory evoked activity in the motor neurons. The exact wiring diagram of the sensory-CNS-motor circuit remains unclear at this time due to the numerous possibilities. The conformation of any proposed work requires

Table 2. The composite of responses on sensory and motor roots due to the various agents which were exposed to the neuronal circuit.

Chemical	Sensory	Motor
Effects in neural activity of the superficial flexor neural circuit		
CO_2	↓	↓
pH	↑	↑
Nicotine	↑	↑
Nicotine + CO_2	NS	↓
Glutamate	↑	↑
Glutamate + CO_2	↓	↓
Domoic acid	NS	NS
Cadmium (15 min)	NS	↓
Heptanol (10 min)	NS	↓

The responses are reported for the change in activity during sensory stimulation. Arrows indicate direction of statistical significance from basal activity. NS indicates a non-significant effect.

detailed anatomic mapping and neurophysiological studies of individual neurons. For the purposes of this study, we have documented that CO_2 has actions in the periphery, as well as in the CNS to account for the behavior responses; thus, gap junctions as well as glutamatergic synapses are potential targets. Numerous further detailed studies are required to dissect the circuit to know where precisely these synapses are occurring within the driven circuit used herein, as well as what drives the spontaneous activity of the superficial flexor motor neurons.

Neuromuscular Junction

A reduction in the receptivity to glutamate suggests an action at the receptor level. The mechanism of action could be packaging of the vesicles, but receptivity in our study was independently checked by exogenous application of glutamate on the muscle in the presence of the compound of interest. The results we obtained at the crayfish opener and superficial flexor muscles, mimic the ones documented earlier from our lab at the NMJ of larval *Drosophila* (Badre et al., 2005). The opener and superficial flexor muscles both showed a decrease in the amplitude of the evoked multiquatal events and the EPSPs became smaller, as well as, the muscle being unresponsive to the application of glutamate. Thus, a reasonable explanation is that the glutamate receptors are being blocked/inactivated by the presence of CO_2. Given that CO_2 can diffuse into the muscle, and thus decreases pH rapidly by the action of carbonic anhydrase, we should consider that protons may have an action on the cytoplasmic side of glutamate ionotropic channel. The lower extracellular pH saline that we used to control for the drop in pH of saline bubbled with CO_2, can not rule out the effects of pH on the inner side of the glutamate ligand-gated ion channel. Possibly, CO_2 blocks the receptor directly by being trapped within the channel pore since it can not diffuse through the protein structure as it can through the bilipid membrane. As a side note, there is a precedence that Rh proteins, commonly associated with human blood typing, may indeed serve as a CO_2 channel (Endeward et al., 2008). However, no investigations we are aware of, have addressed the presence of Rh proteins in crustaceans. Future studies, need to examine if the muscle shows a drop in intracellular pH when exposed to CO_2. This needs to be examined independently of CO_2, to learn if the low pH_i, by itself, is the key contributor to the decreased glutamate sensitivity. Interestingly, the NMDA receptor of vertebrates is pH sensitive on the extracellular side due to protonation (Giffard et al., '90; Tang et al., '90; Traynelis and Cull-Candy, '90; Tombaugh and Sapolsky, '93; Low et al., 2003). At the crayfish and *Drosophila* NMJ, an extracellular pH of 5.0 does reduce the EPSP amplitude; however, glutamate receptors are still responsive to exogenous glutamate (Badre et al., 2005).

Ventral Nerve Cord: CNS

Since the postsynaptic glutamate receptors are blocked or made unresponsive by CO_2, either directly or indirectly, the activity of the motor neurons may seem to be irrelevant to investigate in relation to the mechanism of the paralytic behavior. However, to understand the full breadth of the effects of CO_2, studying the effects on the neural circuit is important. In addition, it was established that in the larval *Drosophila*, the motor nerve roots from the CNS remained active in the presence of CO_2, so for comparative studies in detailing the mechanisms of action on the whole animal behavior, it is of interest to know if the crayfish showed a similar response to CO_2. Much to our surprise, the CO_2 containing saline completely blocked spontaneous motor nerve root activity in the pure motor third root as well as evoked activity through sensory stimulation via second nerve root. To solve the conundrum if CO_2 blocks electrical conductance along an axon or the ability of the motor neurons to generate an action potential, we turned to the large diameter excitatory motor nerve axon of the opener muscle in the walking leg. Generation, as well as the shape of the action potential, was not substantially altered by CO_2. Since we do not expect the motor nerve axons innervating the superficial flexor muscle to be any different in response to CO_2 from the axon to the opener muscle, the site of action of CO_2 in the VNC must be at the level of neural drive to the motor neurons within the ganglion and not transmission along an axon.

The source of spontaneous activity of the third motor root has been a topic of sincere investigation since the 1960s when Eckert ('61) examined if the tonic firing muscle receptor organ (MRO) within the same or neighboring segment could account for the spontaneous motor drive. In these earlier studies, it became apparent that the activity was driven within the VNC, possibly from higher centers (Eckert, '61; Kennedy and Takeda, '65a, b; Strawn et al., 2000). Since the presence of CO_2 stops the spontaneous activity, one can assume somewhere in the drive

to the motor neurons there might be a glutamatergic excitatory drive where the receptors are similar in properties to those at the NMJ which are blocked or have a decreased sensitivity to glutamate in the presence of CO_2. Another possibility is the presence of gap junctions directly on these motor neurons and that CO_2/pH_i blocks these junctions causing the reduced activity. The presence of CO_2 has been shown to block gap junction in the LG axons of the VNC in crayfish (Arellano et al., '90; Peracchia, '90). It has not been established yet, if the lateral or medial giant axons play a role in the spontaneous activity of the third motor nerve root. As for the evoked drive of the motor neurons through sensory stimulation (i.e., brushing of the cuticle), this enhances the nerve activity; but when CO_2 is present the drive is significantly depressed to the point of no activity present. Thus, the paralytic effect at the NMJ appears to be insignificant, as it cannot be driven anyways by this particular circuit, since it is blocked centrally. By definition, this could be referred to as an anesthetic action of CO_2, since the "sensory to motor" activity is stopped within the CNS. The possibilities to explain the potential sites of action for CO_2 blocking the evoked drive are numerous, given that we do not know the extent of interneuronal connections via chemical and/or electrical synapses. Comparative studies in the command of the motor roots in the larval *Drosophila* brain indicate that the drive on the motor neurons is different since the spontaneous, as well as the evoked, drive was not blocked by CO_2 (Badre et al., 2005).

Anatomical dissection of neuronal circuits is a tedious undertaking and even after knowing the anatomical pathways, one still needs to understand the physiology of the connections to explain the various contributions (Kennedy and Takeda, '65a,b). The application of antagonist or agonist to potentially drive or reduce activity in the third root can help one to understand if particular receptor subtypes are used within this system. In this regard, we applied nicotine to drive a nicotinic receptor subtype, as the sensory input to the CNS of many invertebrates are cholinergic (olfaction input in *Drosophila*, Silbering et al., 2008; mechanosensory afferents in cricket—Yono and Aonuma, 2008; escape circuit in *Drosophila*—Fayyazuddin et al., 2006; Aplysia—Susswein et al., '96). The present findings indicate that nicotine enhanced the motor drive, which suggest a nicotinic Ach receptor present somewhere in the circuit.

To examine if quisqualate subtype of glutamate receptors had a role in this motor circuit, we examined the effects of domoic acid. This compound blocks the quisqualate glutamate receptor subtype in *Drosophila* (Lee et al., 2009) and crayfish have these same pharmacological profiled receptors at their NMJs (Shinozaki and Shibuya, '74; Shinozaki and Ishida, '81). In this crayfish CNS circuit, we showed that domoic acid did not really alter the spontaneous drive; however, glutamate did which suggests that indeed there is likely a glutamatergic input within the synapses that drive the motor neurons. This scenario accounts for the lack of enhanced activity during sensory stimulation in the presence of domoic acid. Thus, it would seem reasonable if glutamate itself is applied that at least a transient alteration in activity in the motor root would occur. One might expect a rapid enhanced effect and then potentially a reduced rate below basal activity in the motor root as glutamate receptors in *Drosophila* and crayfish are known to desensitize rapidly to glutamate (Dudel et al., '92). We observed a substantial increase in the activity in the motor root without sensory stimulation upon exogenous application of glutamate. Upon sensory stimulation, a slight increase in the activity is observed, so likely there might be a non-glutamatergic drive present as we would expect the exogenously applied glutamate to saturate any additional sensory driven by a glutamate contribution. It is curious to note that the activity did not increase and then quickly decrease due to desensitization of the activated glutamate receptors.

As for the contributions of gap junctions being used in the spontaneous drive as well as the evoked sensory to motor neuron, we used heptanol since it is a well-established blocker of gap junctions (Johnston et al., '80). It was surprising to observe that heptanol decreased the spontaneous drive on the motor neurons. This suggests that possible background input from the lateral or medial giants in driving the motor neuron in the third root, as they do have electrical input on other motor neurons, but it could also be likely that the drive is through other interneurons communicating via gap junctions. At least it is now established that sensory drive to the motor neurons is not exclusively via gap junctions but some direct input could be possible. Given that we also observed with the presence of Cd^{2+} that the motor root activity is not completely blocked supports the notion that electrical input is also a contributing factor in regulating activating the third root. When it becomes known which neurons specifically are contributing to the electrical junctional input on these motor neurons, then similar detailed studies into pH altered sensitivity of Ca^{2+} ions on gap junctional proteins could be studied for this circuit. However, the detailed studies of the LG axons of the crayfish VNC (Arellano et al., '90; Peracchia, '90) serve as a good model of the likely mechanistic explanation of how CO_2 blocks gap junctions in other neuronal types. Back filling of the third motor root with Lucifer yellow or other compounds permeable through gap junctions (Payton et al., '69) would address the question if gap junctions occur directly on the superficial flexor motor neurons.

In constructing a model to explain the observed results, we do know that the presence of CO_2 completely attenuates the spontaneous activity in the third root as well as evoked sensory activity. Also, Cd^{2+} or heptanol by themselves do not entirely block the spontaneous or evoked activity of these motor neurons, although there is a strong reduction by each compound individually. Thus, it would appear there are parallel inputs and not series inputs of chemical and electrical synapses in the circuitry from the sensory drive as well as the yet unknown drive responsible for the spontaneous activity. It would appear that CO_2 is able to block both the chemical, likely glutamatergic, and electrical synapses.

Sensory Input

The second root is primarily composed of 100s of afferent sensory inputs which monitor stimuli from the cuticular surface in the corresponding segment; however, there are a few motor neurons to the superficial and deep extensor muscles, as well as to the efferent control of the MROs (Sohn et al., 2000). In addition, the MRO sensory afferents are present in this nerve root. In the experiments with exposure to glutamate, Cd^{2+}, heptanol and domoic acid, it is not known what contribution they had on specific primary sensory neurons cuticular mechanosensory or MRO for the basal activity. However, during brush stimulation of the cuticle, the pronounced increase in the number and frequency of small sized spikes within the extracellular recording would indicate that primarily sensory neurons were recruited. A reduction in spike activity only occurred during exposure to CO_2 and only nicotine and glutamate significantly increased activity in the second root. The other agents (domoic acid, low pH, Cd^{2+}, heptanol) did not produce a significant effect on the sensory activity. The mechanistic action of CO_2 on these primary sensory neurons has not been established. We do not know if the transduction process itself is altered. Since these primary cuticular sensory axons are very small in diameter and difficult to be isolated individually, we could not directly assess if the action potential in these neurons is impacted by CO_2. However, we did address this possibility by examining the amplitude and shape of the action potential of the excitatory opener motor neuron in the walking leg since it is easily assessable and intracellular recordings are attainable (Cooper and Cooper, 2009). We assume the fundamental biophysical properties of the opener axon are similar enough to the primary sensory axons. Since CO_2 did not affect the action potential characteristics or conduction in the motor neurons, it is unlikely CO_2 has an action on conduction in the sensory neurons. It is possible that CO_2 is targeting the transduction process. This remains to be examined in more detail. Possibly examining the large sensory endings and somata of the MROs would shed some light on this issue. The large cell bodies of the MROs can be impaled with microelectrodes such that the membrane resistance, threshold and graded potentials can be examined before and during exposure to saline containing CO_2 as has been accomplished for addressing the action of 5-HT on these primary sensory neurons (Cooper et al., 2003a, b). As to why nicotine and glutamate increase activity in the second root, there might be some action on primary sensory neurons, but mechanisms remain to be elucidated. There is no precedence that we are aware of for these agents to have a direct action on sensory neurons. It is likely that these compounds are activating the efferent motor neurons within the VNC that have their axons present in this second root. The degree of impact from pharmacological agents is known to be related to the strength of synaptic efficacy (Cooper et al., 2003a, b; Sparks et al., 2003; Wu and Cooper, 2013). So, depending on the degree of sensory activation the effects could be minimal or substantial.

Model

A wiring circuit diagram of the potential neuronal pathways may appear to be premature given that the identity of only a few neurons in the circuit are known; however, it provides a framework to visualize where future research can be specifically directed to identify each component within the sensory-ganglion-motor path. In addition, the results in this study indicate those both chemical and electrical synapses are driving the third root to the superficial muscles and may account for the intrinsic activity (Evoy et al., '67). It may be possible that these motor neurons are coupled with the LG axons as there are motor neurons coupled with the LG in the third root which project to the deep flexor muscles for the rapid tail flips (Furshpan and Potter, '59). The superficial muscles would likely contract in synchrony, albeit slower, with the phasic flexors to help coordinate the articulating membrane between the segments. There is noted background activity in LG axons, but as far as we are aware no study has correlated it with the spontaneous activity in the superficial flexor motor neurons. Since heptanol blocks all the evoked activity in the motor root, one postulation is that the electrical connections from the LG or other interneurons to the motor neurons are blocked (Fig. 11). In a minimalistic view of the circuit, a sensory input could proceed directly onto the motor neuron by a mono-synaptic chemical input and some interneuron via an electrical input (Fig. 11A). This over simplistic view would account for the responses by Cd^{2+} and heptanol blocking of chemical and electrical inputs respectively, but not the actions observed for glutamate and nicotine. Building a postulated model allows one to design an Occam's razor approach to the circuit until otherwise disproven (Fig. 11B). It is known that the LG does contain vesicles along with gap junctions at synapses onto the fast flexor motor neurons (Leitch et al., '92), thus a possibility for a similar type of input on the slow flexor motor neurons is not too radical of a postulation. Given the few pharmacological trials and that it is likely the sensory input is Ach, we postulate a simple circuit with interneurons that would account for the glutamatergic system we measured along with potential electrical connections. The electrical input is likely not a series element within the sensory input, as even with heptanol present, the sensory to motor root can still be driven although with a reduced efficacy (Fig. 11).

Future Research

Knowing if the observed actions of CO_2 are due to a lower pH, which then inhibits gap junctions in the crayfish giant lateral interneuron resulting in the decreased third motor root activity, would help to delineate if the giant axons has a contribution to the drive on these motor neurons and if it is through gap junctions. The effects of CO_2 reducing intracellular pH in these tissues are likely to account for the observed physiological effect in cessation of the activity and reduced sensory-driven responses. Future studies, such as possible cellular adaptations to chronically raised pCO_2 and/or reduced pH, would be feasible in these readily

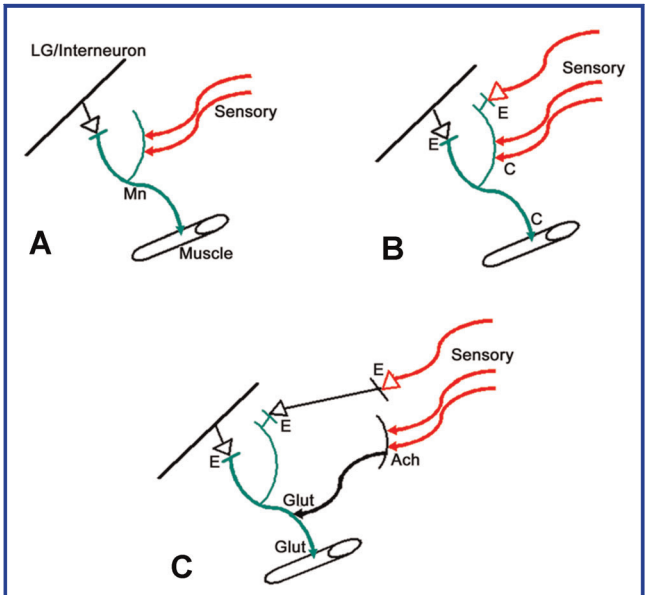

Figure 11. Putative neural circuit for sensory-ganglia-third root of the tonic superficial flexor motor neurons in the abdomen of the crayfish. A: A simplistic model with sensory neurons directly innervating the motor neurons via chemical communication. In this model, electrical drive is from the lateral giant (LG) or another interneuron. The spontaneous activity of the motor neurons (Mn) could be accounted for by basal activity in these inputs in a synergistic or additive fashion. B: Sensory inputs may also contribute by direct electrical inputs in addition to chemical synapses. This could account for the reduced sensory input when gap junctions are inhibited, independent of the interneurons. C: Given the pharmacological observations, the circuitry likely contains interneurons between sensory and motor that are glutamatergic with cholinergic sensory drive. Gap junctions also appear to be present in the circuit at some point in the sensory to motor evoked circuit. It is important to note that no anatomic data are yet provided for these hypothetical circuits and that these are likely oversimplified (i.e., inhibitory inputs are not provided). (Gap junctions are denoted as electrical, E or as a diode ⟶|; chemical-C; Ach-acetylcholine; Glut-glutamate).

accessible preparations. Re-examination of the spontaneous third root activity after chronic low-level pCO_2 exposure or if the sensory-CNS-muscle circuit is still sensitivity to additional application of CO_2, would be of interest for investigating the potential cellular plasticity and related behaviors for compensation to various environments. Physiological and behavioral acclimation to new environments is in part related to species survival (Bierbower et al., 2013). Squid axons that were acutely and chronically exposed to CO_2 revealed that the axons had ability for buffering pH_i with various ion exchange mechanisms (Boron and DeWeer, '76). A phenomenon not well understood in humans, particularly people inflicted with chronic obstructive pulmonary disease (COPD). In this condition, people become unresponsive to chronic increased levels of pCO_2 in the blood. Such people become more responsive to hypoxia (low pO_2) for respiratory drive than to elevated pCO_2 (Raurich et al., 2009; Zapata et al., 2009; Samolski et al., 2010). So, it is potentially feasible that long-term exposure of low CO_2 may result in some compensatory cellular mechanisms within the crayfish to allow it to survive and be responsive to additional alterations in CO_2, but this remains to be examined.

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