Adenosine 2A Receptor Inhibition Enhances Intermittent Hypoxia-Induced Diaphragm but Not Intercostal Long-Term Facilitation

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Abstract

Acute intermittent hypoxia (AIH) elicits diaphragm (Dia) and second external intercostal (T2 EIC) long-term facilitation (LTF) in normal unanesthetized rats. Although AIH-induced phrenic LTF is serotonin dependent, adenosine constrained in anesthetized rats, this has not been tested in unanesthetized animals. Cervical (C2) spinal hemisection (C2HS) abolishes phrenic LTF because of loss of serotonergic inputs 2 weeks post-injury, but LTF returns 8 weeks post-injury. We tested three hypotheses in unanesthetized rats: (1) systemic adenosine 2aA (A2A) receptor inhibition with intraperitoneal (IP) KW6002 enhances Dia and T2 EIC LTF in normal rats; (2) Dia and T2 EIC LTF are expressed after chronic (8 weeks), but not acute (1 week) C2HS; and (3) KW6002 enhances Dia and T2 EIC LTF after chronic (not acute) C2HS. Electromyography radiotelemetry was used to record Dia and T2 EIC activity during normoxia (21% O₂), before and after AIH (10, 5-min 10.5% O₂, 5-min intervals). In normal rats, KW6002 enhanced DiaLTF versus AIH alone ($33.1 \pm 4.6\%$ vs. $22.1 \pm 6.4\%$ baseline, respectively; $p < 0.001$), but had no effect on T2 EIC LTF ($p > 0.05$). Although Dia and T2 EIC LTF were not observed 2 weeks post-C2HS, LTF was observed in contralateral (uninjured) Dia and T2 EIC 8 weeks post-C2HS ($18.7 \pm 2.7\%$ and $34.9 \pm 4.9\%$ baseline, respectively; $p < 0.05$), with variable ipsilateral expression. KW6002 had no significant effects on contralateral Dia ($p = 0.447$) or T2 EIC LTF ($p = 0.796$). We conclude that moderate AIH induces Dia and T2 EIC LTF after chronic, but not acute cervical spinal injuries. A single A2A receptor antagonist dose enhances AIH-induced Dia LTF in normal rats, but this effect is not significant in chronic (8 weeks) C2HS unanesthetized rats.

Key words: cervical; diaphragm; intercostal muscle; intermittent hypoxia; long-term facilitation; respiratory plasticity; spinal cord injury

Introduction

PLASTICITY IS A FUNDAMENTAL PROPERTY of the respiratory motor control system.¹ For example, moderate acute intermittent hypoxia (AIH) elicits plasticity in the (spinal) phrenic motor system.^{2–4} AIH-induced phrenic motor plasticity is expressed as a persistent increase in phrenic motor output in anesthetized rats, an effect known as phrenic long-term facilitation (pLTF). Increased diaphragm (Dia) motor activity is also observed after AIH (i.e., Dia long-term facilitation, DiaLTF) in unanesthetized rats instrumented with Dia electromyography (EMG) telemeters.³ Similar facilitation is observed after AIH in inspiratory EMG activity from the second thoracic external intercostal (T2 EIC) muscle (T2 EIC LTF).⁵

We have come to realize that multiple, distinct cellular mechanisms give rise to similar AIH-induced spinal respiratory motor plasticity.⁶ For example, whereas pLTF is serotonin dependent

when AIH consists of mild to moderate hypoxic episodes, it converts to an adenosine dependent (serotonin independent) form of pLTF when AIH consists of severe hypoxic episodes.⁷ Although moderate AIH-induced pLTF requires spinal serotonin receptor activation,^{8–11} concurrent spinal adenosine 2A (A2A) receptor activation constrains pLTF.¹² Thus, both systemic and spinal A2A receptor antagonist administration enhances pLTF in uninjured, anesthetized rats.¹² It is not known if similar enhancement in DiaLTF or T2 EIC LTF with systemic A2A receptor inhibition occurs in normal, unanesthetized rats.

Accumulating evidence suggests that distinct mechanisms give rise to AIH-induced plasticity in rats with acute (< 4 weeks) versus chronic (> 8 weeks) spinal cord injuries (SCI). For example, spinal serotonergic innervation is transiently reduced after cervical (C2) spinal hemisection (C2HS),¹³ diminishing the capacity for serotonin-dependent pLTF at 2 weeks, but not 8 weeks post-injury.¹³ Interestingly, 1 week of daily AIH (10 episodes/day, 7 days)

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beginning 7 days post-C2HS restores breathing capacity in unanesthetized rats,¹⁴ suggesting a mechanism independent of serotonin receptor activation, or that repetitive AIH overcomes persistent deficits in serotonergic innervation.

The functional benefits of daily AIH shortly (~2 weeks) after C2HS may arise from alternate (possibly adenosinergic) mechanisms. We do not know if a single AIH presentation is sufficient to elicit Dia or T2 EIC LTF with acute (7 days) or chronic (8 weeks) SCI. It is of considerable interest to know whether moderate AIH-induced LTF is enhanced by systemic A2A receptor inhibition because that observation would have important therapeutic implications; A2A receptor inhibition may enhance the therapeutic benefits of repetitive AIH after chronic, cervical SCI.

AIH-induced LTF in the phrenic motor system has been extensively studied in anesthetized and paralyzed rats;^{15,1} however, there are no reports concerning the impact of A2A antagonists on AIH-induced LTF in Dia or T2 EIC muscles in cervically hemisectioned, unanesthetized rats. Physiological measurements collected from unrestrained conscious rats better represent the normal state of an animal because they are unaffected by chemical (e.g., anesthesia) or psychological factors (e.g., stress).¹⁶ Radiotelemetry systems permit investigation of drug effects in freely moving animals, enabling physiological data collection in biologically relevant conditions.

The main purpose of this investigation is to study the effects of systemic A2A receptor inhibition on AIH-induced Dia and T2 EIC LTF in normal, unanesthetized rats, and rats with acute and chronic C2HS using EMG radiotelemetry. Specifically, we tested four hypotheses: (1) systemic A2A receptor inhibition with KW6002 enhances both Dia and T2 EIC LTF in normal, unanesthetized rats; (2) there is a greater spontaneous recovery in ipsilateral T2 EIC versus ipsilateral Dia with both acute and chronic C2HS; (3) rats with chronic (8 weeks), but not acute (1 week), C2HS express AIH-induced LTF in bilateral Dia and T2 EIC muscles; and (4) systemic A2A receptor inhibition enhances Dia and T2 EIC LTF in rats with chronic (not acute) C2HS.

Methods

Animals

All experiments were performed on 3–4 month old, male Sprague-Dawley rats (310–445 g, colony 211, Harlan, Indianapolis, IN). Animals were individually housed in a controlled environment (12-h light/dark cycle). The Animal Care and Use Committee at the School of Veterinary Medicine, University of Wisconsin, approved all experimental procedures in this study.

Experimental preparation

Surgical preparation. For telemetry implantation and C2HS procedures, a sterile operation was performed under anesthesia induced with isoflurane in 100% O₂. The rats were injected with buprenorphine (0.03 mg/kg), carprofen (Rimadyl, 5 mg/kg) and enrofloxacin (Baytril, 4 mg/kg) subcutaneously to minimize potential post-operative pain and infection. Body temperature was maintained at 36.5–37.5°C using a rectal probe and external heating pad. A cannula was inserted into the trachea, and the animals were artificially ventilated (tidal volume, 2.0–2.5 mL; Rodent Ventilator, model 683; Harvard Apparatus, South Natick, MA) with 1.5–2.5% isoflurane in 100% O₂ during the procedure. Effective anesthesia was judged by abolition of pedal withdrawal and corneal blink reflexes. Oxygen saturation was monitored by pulse oximetry (model 8600; Nonin Medical Inc. Plymouth, MN) during the surgical procedures.

At the end of the procedure, buprenorphine, carprofen, and enrofloxacin at the same dose rats received before the surgery were administered at 12 h intervals for 48 h post-surgery. Rats were vi-

sually monitored and weighed daily. In addition, in spinal cord injured rats, animal care included trimming nails after surgery, and cleaning fur, eyes, and mouth with warm water daily for 7 days to avoid accumulation of porphyrin. Rats had free access to pellets and high caloric nutritional gels inside their cages. In both telemetry implantation and SCI procedures, stainless steel staples were removed 7 days post-surgery. We report no post-surgery complications after telemetry implantation or SCI procedures.

Telemetry transmitter implantation. After anesthesia induction and pre-operative care (see above), the rat was placed in a supine position and the ventral surface of the abdominal muscles were exposed. A sterilized telemetry transmitter body (model 4ET-S1/2; Data Sciences International [DSI], St. Paul, MN) was inserted into the peritoneal cavity. The transmitter allowed simultaneous and continuous monitoring of electrical bio-potentials, body temperature, and general locomotor activity. In experiments with normal rats, two bio-potential channels were used to record EMG from right Dia and T2 EIC muscle. In SCI experiments, the four bio-potential channels were used to record EMG activity from bilateral Dia and second external intercostal (T2 EIC) muscles.

During bilateral Dia and T2 EIC implantations, both right and left hemi-Dias were exposed through a midline incision following the Alba line. In both hemi-Dias, two leads were implanted in the midcostal area using a 23-gauge syringe needle guide and tissue adhesive (Vetbond 1469SB; 3M Animal care product, St. Paul, MN) as reported previously.³ Next, right and left T2 EIC muscles were exposed through a 2.5 cm midsternum incision, starting in the upper edge of the sternum, followed by retraction of the pectoralis major and minor on the right and left side. The right and left T2 EIC muscles were implanted 1.0 cm right and left from the sternum, respectively, and the second interspace was identified.

Bio-potential lead pairs targeting EIC muscles were tunneled subcutaneously from the body of the transmitter in the peritoneal cavity. As in the Dia, all leads in T2 EIC were implanted using a 23-gauge syringe needle guide with tissue adhesive to keep the leads in place. Finally, abdominal muscles and pectoralis major were joined by midline suture (Polysorb 3.0). Skin was closed with wound staples.

C2HS. One week after telemetry implantation, C2HS were performed, consistent with previous studies.^{17,18} After anesthesia induction and pre-operative care (see above), the spinal cord was exposed at C2 via a dorsal laminectomy. The dura mater was cut and a left C2 hemisection was performed using a microscalpel followed by aspiration. Overlying muscles were sutured and the skin closed with stainless steel wound clips. Sham rats received laminectomy, but not C2HS.

Telemetry. For the AIH protocol (see below), rats were placed in custom-made Plexiglas chambers positioned on telemetry receivers (model RPC-2; DSI, St. Paul, MN). Signals from the implanted radiotelemetry transmitter were detected by the receivers and sent to a data exchange matrix (model ACQ-7700; DSI, St. Paul, MN). Channels of EMG (Dia and T2 EIC muscles), body temperature, and general locomotor activity were monitored in the unanesthetized, freely moving rats using a data acquisition system (PONEMAH Physiology Platform; DSI, St. Paul, MN). EMG analysis was performed with Neuroscore software (DSI, St. Paul, MN) as described below.

Drug preparation. KW6002 (Istradefylline, Sigma-Aldrich) is a selective A2A receptor (A2AR) antagonist with a molecular weight of 384 and a K_i of 29.6 nM in rats. It has a half-life of 110 min, 97% availability after intraperitoneal (IP) injection, and brain concentration of 500 uM after 4 h,¹⁹ making it a suitable drug for our prolonged, *in vivo* experiments. KW6002 was dissolved in dimethyl sulfoxide (DMSO) at 9.3 mg/mL, sonicated and stored at

4°C in a dark vial protected from light. The day of the experiment, the drug was administered via IP injection at a dose of 0.5 mg/kg.

Experimental groups. To investigate the effects of AIH and A2AR inhibition, rats ($n=28$) were randomly allocated into the following groups: (1) AIH+KW6002, $n=8$; (2) AIH+vehicle, $n=8$; (3) normoxia (Nx)+KW6002, $n=6$; (4) Nx+vehicle, $n=6$. Another cohort of rats with C2HS ($n=16$) was randomly assigned to the following groups 7 days post-surgery: (1) AIH+KW6002, $n=4$; (2) AIH+vehicle, $n=4$; (3) Nx+KW6002, $n=3$; (4) Nx+vehicle, $n=3$; (5) Sham, $n=2$. Finally, a third group of rats with chronic C2HS (8 weeks post-surgery; $n=16$) were assigned to the following groups: (1) AIH+KW6002, $n=4$; (2) AIH+vehicle, $n=4$; (3) Nx+KW6002, $n=3$; (4) Nx+vehicle, $n=3$; (5) Sham, $n=2$.

Sham rats were exposed to Nx and did not receive IP injections. Neither acute nor chronic C2HS rats received any treatments before beginning experiments. Allocation of groups was performed after telemetry implantation. For the C2HS and administration of treatment, the investigator was blind. For the final analysis, the investigator was not blind.

AIH protocol. Normoxic (21% O₂) and hypoxic (10.5% O₂) conditions were established in custom-made chambers (Plexiglas cylinder, 12×4 inches ID; 1 rat per chamber) by mixing O₂ and N₂ via a custom-made computer-controlled system to obtain the desired inspired oxygen concentrations. Within the chambers, CO₂ and O₂ levels were continuously monitored (O₂ Analyzer, model 17S18; CO₂ Analyzer, model 17S15; VacuMed Inc, Ventura, CA). Gas flowed through the chamber at a rate of 4 L/min, keeping chamber CO₂ concentration well below 0.5% at all times. Of the change in O₂ levels within the chamber during AIH protocols, 95% was achieved in 25±5 sec.

In normal rats, experiments were performed 7 days after telemetry implantation. In C2HS rats, EMG amplitude was recorded during baseline conditions (normoxia) for 20 min 1 day after C2HS to assure complete hemisection. Acute and chronic C2HS experiments were performed 7 days and 8 weeks post-C2HS, respectively. At 8:00 AM on the experimental day, rats were placed in the chamber above the signal receivers for 2 h of acclimation, followed by 1 h of baseline recording. Next, rats received an IP injection of either A2AR antagonist (KW6002) or vehicle (DMSO). Once all rats were in the chambers, experimental groups received AIH (10, 5-min episodes of 10.5% O₂ interspersed with 5-min 21% O₂ intervals). Control and sham rats also received time control (TC) exposures (time matched continuous Nx). Finally, 1 h post-treatment, recordings were made in all groups during normoxia. Chamber temperature was 22.5–24.5°C during protocols.

Tissue processing. To verify the extent of C2HS, spinal cords were removed immediately after completion of experiments, immersed in paraformaldehyde (4%, overnight at 4°C), and cryoprotected in increasing concentrations of sucrose (20–30%). Tissues were frozen in isopentane at –45°C and stored at –80°C. Longitudinal sections of the spinal cord (C1 to C6, 30 μm thick) were stained with cresyl violet and examined histologically using a light microscope to reconstruct the injury in a transverse plane.^{20,21} NIH ImageJ software (National Institute of Health; <http://rsb.info.nih.gov/ij/>) was used to measure and compare the extent of hemisections among groups.

Data analyses

EMG signals were analyzed with Neuroscore software. Raw signals were filtered (100–624 Hz), rectified, integrated (100 msec) and averaged for each muscle. EMG values during active locomotor and grooming activity were excluded from analysis. Mean peak amplitude, respiratory frequency, and calculated minute activity (amplitude×frequency) were averaged in all rats before and after AIH. Data obtained during the pre-treatment period (1 h) were

regarded as baseline values. For experiments assessing motor activity in Dia and T2 EIC muscles after C2HS, values were normalized to pre-injury baseline values.

Statistical comparisons were made for time (pre- and post-treatment) and treatment groups (see experimental groups, above) using a two-way, repeated measures analysis of variance (ANOVA) with Fisher LSD *post hoc* tests (Sigma-Stat version 2.03, Systat Software Inc, San Jose, CA). Differences indicated as statistically significant were $p<0.05$. All values are expressed as means±standard error of the mean (SEM).

Results

Enhanced Dia long-term facilitation with A2A receptor inhibition in normal rats

AIH caused a sustained increase in Dia and T2 EIC EMG amplitude for at least 60 min post-AIH (22.1±6.4% and 42.6±3.5% baseline at 60 min post-AIH, respectively, $p<0.001$; Fig. 1A, 1B), confirming previous reports of AIH-induced Dia and T2 EIC LTF.⁵ Systemic A2AR inhibition (KW6002) significantly increased DiaLTF (AIH+KW6002: 33.1±4.6% vs. AIH+vehicle: 22.1±6.4% baseline; $p<0.001$; Fig. 1A, 1D), demonstrating that A2A receptors constrain DiaLTF in normal, unanesthetized rats. A2A receptor inhibition, however, did not increase T2 EIC LTF (AIH+KW6002: 44.1±3.6% vs. AIH+vehicle: 42.6±3.5% baseline, $p=0.238$; Fig. 1B, 1D).

Respiratory frequency was not statistically different between AIH plus KW6002 and AIH plus vehicle-treated rats (18.7±2.5% vs. 15.3±2.7% baseline, $p=0.365$; Fig. 1C). Finally, LTF in Dia minute activity was significantly greater in AIH plus KW6002 versus AIH plus vehicle-treated rats (61.9±3.9% vs. 40.4±7.4% above baseline, $p<0.001$; Fig. 2A). In time controls rats, no differences in EMG amplitude, frequency, or minute activity were observed in Dia or T2 EIC muscles (Fig. 1A, 1B, 1C).

Similar cervical C2HS among groups

We confirmed C2HS with histology and electrophysiology. Reconstructions of cervical hemisections in perfused tissues demonstrated that similar areas (as a percent of total spinal cross-sectional area) were injured in all groups (AIH+vehicle: 49.3±2.9%, AIH+KW6002: 47.7±1.3%, Nx+vehicle: 50.2±1.7%, Nx+KW6002: 45.3±3.0%, $p=0.241$). EMG recordings 1 day post-surgery confirmed a lack of activity ipsilateral to injury. For all groups, the ipsilateral (injured) Dia had greatly reduced EMG signals versus pre-injury values (AIH+vehicle: 9.6±2.4%; AIH+KW6002: 7.1±1.2%; Nx+vehicle: 8.3±2.1%; Nx+KW6002: 8.0±2.0% of pre-injury values, $p<0.001$). Similar reduced EMG activity was found in the ipsilateral T2 EIC muscle after hemisection (AIH+vehicle: 7.7±2.7%, AIH+KW6002: 11.4±1.3%, Nx+vehicle: 8.4±2.8%, Nx+KW6002: 8.7±1.0% of pre-injury values, $p<0.001$).

Considering that these experiments were performed in freely moving, unanesthetized rats, the presence of some small EMG signal after hemisection may reflect contamination from nearby muscles, as described in similar studies.²² Interestingly, EMG amplitude increased in both contralateral Dia (110.8±1.4% of pre-injury values) and T2 EIC muscles (116.2±0.9% of pre-injury values) 1 day post-injury in all groups, showing that compensatory mechanisms start early after C2HS.

Contralateral versus ipsilateral spontaneous motor recovery in acute C2HS unanesthetized rats

Seven days post-C2HS, rats were assigned to experimental groups (see Methods). In all groups, contralateral (uninjured) Dia

FIG. 1. Summary of changes in diaphragm (Dia) (A) and second external intercostal (T2 EIC) muscle (B) integrated burst amplitude, and respiratory frequency (C) in unanesthetized, normal rats; variables are expressed as a percent change from baseline averaged between 0 and 60 min after acute intermittent hypoxia (AIH) or normoxia (Nx). Representative integrated electromyography (EMG) activity of AIH plus KW6002-treated group in Dia (D, upper trace) and T2 EIC muscle (D, lower trace). A significant increase in EMG integrated burst amplitude was observed in Dia (A) and T2 EIC muscle (B) 0 to 60 min post-AIH plus vehicle (V, dimethyl sulfoxide), but not in control rats (NX + KW6002 and NX + V), indicative of robust Dia and T2 EIC long-term facilitation (DiaLTF and T2 EIC LTF, respectively). A significantly greater DiaLTF was observed in rats treated with AIH plus KW6002 versus AIH plus vehicle (i.e., enhanced DiaLTF) (A and D). Similar enhancement was not observed in T2 EIC muscle (B and D). Respiratory frequency was significantly increased in AIH plus KW6002 and AIH plus vehicle-treated rats 0–60 min post-AIH compared with control rats. Values are means $\pm$ 1 standard error of the mean. *Significantly different from time control rats; †significantly different from baseline; # significantly different from AIH plus vehicle-treated group; $p < 0.001$.

baseline values (before AIH/Nx) had significantly greater EMG amplitudes versus Sham and pre-injury values (AIH + vehicle: $121.7 \pm 9.4\%$; AIH + KW6002: $123.2 \pm 7.9\%$; Nx + vehicle: $120.5 \pm 20.1\%$; Nx + KW6002: $119.8 \pm 6.2\%$; Sham: $100.9 \pm 3.1\%$ of pre-injury values, $p < 0.05$; Fig. 3C), demonstrating compensa-

tory motor activity in the uninjured hemi-Dia. Some small spontaneous recovery was observed in ipsilateral (injured) Dia, which was not different among groups (AIH + vehicle: $19.6 \pm 5.3\%$; AIH + KW6002: $16.0 \pm 2.8\%$; Nx + vehicle: $16.9 \pm 5.8\%$; Nx + KW6002: $15.7 \pm 2.2\%$ of pre-injury values; $p > 0.05$; Figs. 3A, 4A).

FIG. 2. Changes in minute activity (amplitude $\times$ frequency) in diaphragm (Dia) (A) and second external intercostal (T2 EIC) muscles (B) in unanesthetized, normal rats; variables are expressed as a percent change from baseline averaged between 0 and 60 min after acute intermittent hypoxia (AIH) or normoxia (Nx). Note significant increases in minute activity in both Dia and T2 EIC muscle after AIH plus vehicle (vehicle, dimethyl sulfoxide). A2A antagonist (KW6002) significantly enhances Dia (A) but not T2 EIC (B) minute activity long-term facilitation. Values are means $\pm$ 1 standard error of the mean. *Significantly different from controls (NX + V, NX + KW6002); †significantly different from baseline; #significantly different from AIH plus vehicle; $p < 0.001$.

FIG. 3. Representative motor electromyography (EMG) activity of right/left diaphragm (R/L Dia) and second thoracic external intercostal muscle (R/L T2 EIC) and changes in contralateral (uninjured) Dia (C) and T2 EIC (D) EMG amplitude (expressed as percent change of pre-injury values) 7 days post-injury. At this time point, raw recordings show a remarkable spontaneous recovery in left (injured) T2 EIC muscle but only modest in the Dia (A). In contralateral Dia (C), all groups show significant increases in EMG burst amplitude (above 100%) during pre-treatment (normoxia [NX], grey bars) compared with pre-injury and Sham values, demonstrating a degree of spontaneous compensatory motor activity, which is not observed in contralateral T2 EIC muscle (D). Unlike normal rats (Fig. 1) and chronic C2 hemisectioned rats (Fig. 5), AIH alone or combined with KW6002 does not elicit long-term facilitation (LTF) in Dia and T2 EIC muscle (0 to 60 min post-AIH/NX, black bars; B, C, D). AIH, acute intermittent hypoxia; KW, KW6002; V, vehicle (dimethyl sulfoxide). Values are means $\pm$ 1 standard error of the mean. *Significantly different from pre-injury values; #significantly different from Sham; $p < 0.05$.

Unlike the contralateral Dia, uninjured T2 EIC muscle showed no differences in baseline EMG amplitude versus pre-injury values and Sham rats in any group (AIH + vehicle: $104.6 \pm 3.8\%$; AIH + KW6002: $102.9 \pm 2.5\%$; Nx + vehicle, $101.3 \pm 4.5\%$; Nx + KW6002: $100.5 \pm 3.7\%$; Sham: $102.8 \pm 5.7\%$ of pre-injury values; $p > 0.05$; Fig. 3D). In contrast, the ipsilateral T2 EIC muscle shows a remarkable increase in EMG amplitude 7 days post-injury with no significant differences among groups (AIH + vehicle: $80.9 \pm 4.7\%$; AIH + KW6002: $78.9 \pm 1.0\%$; Nx + vehicle: $83.4 \pm 3.2\%$; Nx + KW6002: $85.0 \pm 5.2\%$ of pre-injury values; $p > 0.05$; Figs. 3A, 4B), consistent with previous studies in anesthetized rats.²³

The spontaneous, compensatory plasticity of uninjured T2 EIC muscle 1 day post-hemisection returned completely to baseline 7 days post-injury, coinciding with the remarkable spontaneous recovery of the injured T2 EIC muscle. The presence of early spontaneous compensatory activity in contralateral Dia and T2 EIC muscle activity after C2HS suggests that contralateral respiratory

motor neurons might be recruited to increase motor output as a compensation for lost activity ipsilateral to injury. Once ipsilateral activity returns to normal (as in T2 EIC), these compensatory mechanisms are lost, and activity returns to pre-injury values.

Spontaneous contralateral versus ipsilateral motor recovery in chronic C2HS rats

Eight weeks post-C2HS, a different cohort of rats assigned to four groups (see Methods) was studied. Similar to acute spinal injury, contralateral (uninjured) Dia baseline shows EMG amplitudes elevated above pre-injury values or Sham rats (AIH + vehicle: $119.7 \pm 7.8\%$; AIH + KW6002: $122.7 \pm 3.4\%$; Nx + vehicle, $120.7 \pm 9.3\%$; Nx + KW6002: $122.8 \pm 6.5\%$; Sham: $99.5 \pm 1.6\%$ of pre-injury values; $p < 0.05$; Fig. 5C), demonstrating slightly greater compensation versus acute (7 days) spinal injury. Contralateral T2 EIC muscle in all groups showed no differences in baseline EMG

FIG. 4. Changes in ipsilateral (injured) diaphragm (Dia, **A**) and second external intercostal (T2 EIC, **B**) electromyography (EMG) amplitude expressed as percent change of pre-injury values for pre-treatment (during baseline, grey bars) and post-treatment (0 to 60 min post-acute intermittent hypoxia/normoxia (AIH/NX, black bars) at 7 days post-injury. Note: (1) In **A**, all experimental groups exhibit a small degree of spontaneous recovery in ipsilateral Dia EMG peak amplitude; (2) in **B**, all groups show remarkable spontaneous recovery of ipsilateral T2 EIC EMG peak amplitude, although these values remain significantly below Sham rats; and (3) no AIH-induced long-term facilitation was observed 7 days post-injury in either left Dia or T2 EIC muscle. KW, KW6002; V, vehicle (dimethyl sulfoxide). Values are means $\pm$ 1 standard error of the mean. *Significantly different from pre-injury values; #significantly different from Sham; $p < 0.05$.

FIG. 5. Representative raw (**A**) and integrated (**B**) traces of right/left diaphragm (R/L Dia) and second thoracic external intercostal muscle (R/L T2 EIC) and changes in contralateral (uninjured) Dia (**C**) and T2 EIC (**D**) electromyography (EMG) amplitude (expressed as percent change of pre-injury values) at 8 weeks post-injury. In contralateral Dia (**C**), all groups show significant increases (above 100%) in EMG amplitude during pre-treatment (normoxia [NX], grey bars) compared with pre-injury and Sham values demonstrating spontaneous compensatory motor activity of Dia on the intact side. Similar increases were not observed in T2 EIC muscle activity (**D**). Raw trace (**A**) illustrates complete ipsilateral T2 EIC spontaneous recovery, but only modest spontaneous recovery of ipsilateral Dia activity. Unlike acute C2 hemisected rats (Fig. 3), at this time point, acute intermittent hypoxia (AIH)-induced long-term facilitation (LTF) is restored in contralateral Dia (i.e., DiaLTF; **B, C**) and T2 EIC muscle (i.e., T2 EIC LTF; **B, D**), as in normal unanesthetized rats (Fig. 1). Although A2A receptor inhibition with KW6002 tends to increase DiaLTF, this effect was not statistically significant. A2A inhibition had no apparent effect on T2 EIC LTF. KW, KW6002; V, vehicle (dimethyl sulfoxide). Values are means $\pm$ 1 standard error of the mean. *Significantly different from pre-injury values; #significantly different from Sham; $\neq$ significantly different from pre-treatment values; $p < 0.05$.

FIG. 6. Changes in ipsilateral (injured) diaphragm (Dia, **A**) and second external intercostal (T2 EIC, **B**) electromyography (EMG) amplitude expressed as percent change of pre-injury values for pre-treatment (baseline, grey bars) and post-treatment (after acute intermittent hypoxia/normoxia [AIH/NX], black bars) at 8 weeks post-injury. Note: (1) in (A), all experimental groups show modest spontaneous recovery of ipsilateral Dia activity versus pre-injury values and sham; (2) in (B), complete spontaneous recovery of ipsilateral T2 EIC activity is observed at this time post-injury; (3) AIH-induced Dia long-term facilitation was not observed in either ipsilateral Dia or T2 EIC activity (see text). KW, KW6002; V, vehicle (dimethyl sulfoxide). Values are means $\pm$ 1 standard error of the mean. *Significantly different from pre-injury values; #significantly different from Sham; $p < 0.001$.

amplitude versus pre-injury values or Sham rats (AIH + vehicle: $104.2 \pm 4.9\%$; AIH + KW6002: $102.7 \pm 6.5\%$; Nx + vehicle: $102.5 \pm 5.8\%$; Nx + KW6002: $100.4 \pm 4.5\%$; Sham: $102.8 \pm 5.7\%$ of pre-injury values; $p > 0.05$; Fig. 5D).

In all experimental groups, ipsilateral (injured) Dia exhibited a modest spontaneous recovery of EMG amplitude (AIH + vehicle: $22.4 \pm 6.5\%$; AIH + KW6002: $24.4 \pm 2.1\%$; Nx + vehicle: $19.5 \pm 1.3\%$; Nx + KW6002: $18.3 \pm 2.3\%$ of pre-injury values; $p > 0.05$; Fig. 5A, 6A). In contrast, ipsilateral (injured) T2 EIC muscles showed complete recovery of activity with no significant differences among groups (AIH + vehicle: $95.2 \pm 4.4\%$; AIH + KW6002: $99.8 \pm 3.0\%$; Nx + vehicle: $99.5 \pm 5.2\%$; Nx + KW6002: $99.8 \pm 6.4\%$; Sham: $103.5 \pm 3.4\%$ of pre-injury values; $p > 0.05$; Fig. 5A, 6B), consistent with previous studies.²³

LTF in chronic, not acute C2HS unanesthetized rats

After acute C2HS (7 days), we compared Dia and T2 EIC EMG amplitude before and after AIH (Fig. 3B). AIH, alone or combined with A2A receptor inhibition, had no lasting impact on EMG amplitude in either Dia or T2 EIC muscles (Fig. 3, 4). Thus, acute spine injured rats do not exhibit Dia or T2 EIC LTF.

In chronic (8 weeks) C2HS rats, AIH elicits a significant increase in EMG amplitude in both contralateral Dia and T2 EIC muscles ($18.7 \pm 2.7\%$ and $34.9 \pm 4.9\%$ above baseline, respectively; i.e., Dia and T2 EIC LTF; $p < 0.05$; Fig. 5, 6). AIH plus A2A inhibition tended to elicit greater LTF in contralateral (uninjured) Dia EMG amplitude, although this difference was not statistically significant versus AIH plus vehicle-treated rats ($23.2 \pm 7.0\%$ vs. $18.7 \pm 2.7\%$ above baseline, respectively; $p = 0.447$; Fig. 5C). A2A receptor inhibition had no effect on contralateral T2 EIC EMG amplitude (36.1 ± 2.1 vs. $34.9 \pm 4.3\%$ above baseline; $p = 0.796$; Fig. 5D). There was highly variable expression of LTF in ipsilateral (injured) Dia and T2 EIC.

Overall, there was no significant increase of ipsilateral Dia EMG amplitude after AIH versus controls (AIH + vehicle: $6.9 \pm 6.1\%$; AIH + KW6002: $9.4 \pm 7.9\%$; Nx + vehicle: $7.9 \pm 2.3\%$; Nx + KW6002: $3.0 \pm 2.3\%$ above baseline; $p = 0.914$; Fig. 6A). Two rats from the AIH plus vehicle and one from the AIH plus KW6002-treated group, however, showed a clear increase in Dia EMG amplitude (i.e., DiaLTF) (15.8% , 13.8% and 26.1% above baseline,

respectively). Similarly, ipsilateral T2 EIC showed no significant increase in EMG amplitude 0 to 60 min post-AIH versus controls (AIH + vehicle: $11.2 \pm 14.6\%$; AIH + KW6002: $4.1 \pm 2.1\%$; Nx + vehicle: $2.9 \pm 0.8\%$; Nx + KW6002: $3.0 \pm 1.3\%$ above baseline; $p = 0.604$; Fig. 6B), with the exception of one rat from the AIH plus vehicle-treated group (49.2% above baseline in that rat; Fig. 5B).

Discussion

This study provides novel information regarding the impact of combined AIH and A2A receptor inhibition on Dia and T2 EIC LTF in normal unanesthetized rats. Further, we demonstrate the impact of acute and chronic spinal injury on ipsilateral and contralateral Dia and T2 EIC LTF. Last, we tested the impact of A2A receptor inhibition on plasticity post-spinal injury in unanesthetized rats.

We report five major findings: (1) A2A receptor inhibition has heterogeneous effects on AIH-induced respiratory muscle LTF in normal rats, enhancing Dia but not T2 EIC LTF; (2) spontaneous recovery of ipsilateral T2 EIC muscle activity below a C2HS is robust in comparison with the very modest recovery observed in ipsilateral Dia activity; (3) whereas persistent spontaneous increases in motor activity are observed in Dia contralateral to injury, only transient increases in motor activity were observed in contralateral T2 EIC; (4) AIH-dependent Dia and T2 EIC LTF occurs after chronic (8 weeks), but not acute (1 week) C2HS; and (5) LTF in either muscle is not enhanced by A2A receptor inhibition.

Collectively, these findings advance our understanding concerning heterogeneity in the capacity for AIH-induced LTF in different respiratory muscles, differential susceptibility of respiratory motor plasticity to adenosinergic inhibition, the shifting balance of muscle contributions to breathing with time post-spinal injury, and time-dependent shifts in the capacity for AIH-induced respiratory motor plasticity after cervical SCI.

A2A receptor inhibition enhances DIA but not T2 EIC LTF

Enhanced DiaLTF after KW6002 pre-treatment demonstrates that the A2A receptor constraint of pLTF after moderate AIH in

anesthetized rats^{6,12} is also expressed in DiaLTF in unanesthetized rats. On the other hand, the lack of enhanced AIH-induced T2 EIC LTF with A2A receptor inhibition demonstrates that this motor pool is not modulated by the same inhibitory, adenosinergic interaction. Thus, as with phrenic versus hypoglossal LTF,²⁴ there are both similarities and differences in factors modulating AIH-induced LTF among inspiratory motor pools.

A2A receptor inhibition tended to increase (vs. decrease) DiaLTF in rats with chronic C2HS, suggesting that serotonin-dependent LTF gradually recovers with time post-C2HS¹³; with additional time, we suggest that A2A receptor activation may constrain (vs. induce) AIH-induced plasticity. On the other hand, A2A receptor inhibition had no impact on the expression of AIH-induced DiaLTF with acute C2HS, most likely because serotonergic innervation of the phrenic motor nucleus is greatly reduced at this time.¹³ In fact, at this early time post-injury, we suggest that A2A receptor inhibition may limit the potential for alternative, adenosine-dependent pathways to respiratory motor plasticity.^{7,25}

With this idea in mind, it is possible that the plasticity promoting actions of combinatorial actions of intermittent hypoxia and A2A receptor inhibition in normal or chronic SCI rats may actually undermine intermittent hypoxia-induced plasticity in rats with acute SCI. Something as simple as drinking coffee (a well-known A2A receptor antagonist) may have major, but different consequences with time post-spinal injury.

Our working model of AIH-induced pLTF is that episodic hypoxia activates raphe serotonergic neurons that project to phrenic motor nuclei. Spinal serotonin release during hypoxic episodes activates Gq protein-coupled serotonin type 2 receptors on or near phrenic motor neurons, initiating intracellular cascades that underlie pLTF.⁶ On the other hand, A2A receptors, which are Gs protein coupled receptors, also give rise to phrenic motor facilitation (pMF) by a distinct cellular mechanism.⁶ AIH-induced pLTF (serotonin-dependent) and A2A receptor induced pMF interact via cross-talk inhibition¹² by a protein kinase A dependent mechanism.²⁶

Thus, we hypothesize that, although Gq protein-coupled serotonin receptors and Gs protein-coupled adenosine receptors are both activated during moderate hypoxia, the Gq protein-coupled receptor signaling predominates. When cross-talk inhibition from A2A receptors is relieved, the constraint to pLTF is removed and pLTF is enhanced. This same interaction between serotonin-dependent and adenosine-dependent mechanisms does not appear to be a general feature of inspiratory intercostal muscle activity, at least with the moderate AIH protocol used in this study.

Ipsilateral spontaneous motor recovery after C2HS

Spontaneous recovery was robust in ipsilateral (injured/left) T2 EIC muscle activity versus the ipsilateral Dia, possibly reflecting greater crossed spinal polysynaptic pathways involving interneurons.^{23,27,28} As spontaneous ipsilateral recovery occurred, contralateral T2 EIC activity returned to normal, possibly in direct response to greater output from the injured side. Spontaneous recovery of Dia activity ipsilateral to injury was considerably less robust, and contralateral activity remained elevated. Collectively, these observations are consistent with the idea that (unknown) fundamental mechanisms shift the relative contributions of different respiratory muscles to preserve ventilation to the greatest extent possible when the system is challenged by selective injury.

The amplitude and post-injury onset of the crossed phrenic phenomenon is variable in published reports.^{21,29–31} Here, we found that the average ipsilateral Dia amplitude was similar among

groups, ranging between 18–22% of pre-injury values in both acute and chronic C2HS rats, consistent with studies in anesthetized rats.^{29,31} We did not explore spontaneous plasticity in the same animal over time to determine whether this recovery is time dependent between 1 and 8 weeks post-injury. Unlike Dia activity on the injured side, we found robust spontaneous recovery of left T2 EIC EMG activity after C2HS. Although there are no other studies in unanesthetized rats, time-dependent restoration of inspiratory intercostal activity has been reported in anesthetized rats. Two weeks post-injury, inspiratory intercostal EMG activity ipsilateral to injury was similar to the age-matched, uninjured controls, demonstrating complete functional recovery.²³

Activation of cross-synaptic inputs may explain spontaneous recovery in ipsilateral Dia and T2 EIC. The crossed phrenic phenomenon has been attributed to activation of a monosynaptic, bulbospinal pathway crossing the spinal midline caudal to C2.³² Although anatomically present, this pathway is not normally functional³²; however, the pathway can be revealed within minutes by depolarizing phrenic motor neurons pharmacologically.³³ There are also latent, crossed-spinal pathways to intercostal motor neurons and their interneurons. Anatomical³⁴ and neurophysiological²⁷ data suggest that thoracic interneurons relay respiratory synaptic drive across the spinal midline. Thus, spontaneous recovery of T2 EIC activity ipsilateral to C2HS may occur via similar plasticity in a more extensive “crossed spinal” pathway versus that innervating phrenic motor neurons. Greater spontaneous T2 EIC versus Dia recovery suggests that inspiratory intercostal muscles make relatively greater contributions to breathing after cervical SCI.

Contralateral (uninjured) spontaneous recovery after C2HS

Respiratory compensation for cervical SCI may involve recruitment of respiratory muscles with spared innervation. After C2HS, descending innervation of the contralateral respiratory muscles is spared, leaving them with considerable potential to offset loss of function in muscles ipsilateral to injury. One factor that may shift the balance of contributions from ipsilateral to contralateral respiratory muscles is the loss of inhibitory sensory inputs onto uninjured (e.g., contralateral) respiratory motoneurons.^{35–37} Similar mechanisms may also (at least partially) restore the contributions of affected (ipsilateral) motor pools.

In the present study, spontaneous compensation occurs mainly in the contralateral (uninjured/right) Dia, with a similar (but transient) increase in contralateral T2 EIC muscle activity. Although the mechanisms giving rise to these shifts are not known, they may involve: (1) loss of tonic inhibitory constraints from sensory inputs arising from the phrenic nerve/Dia ipsilateral to injury³⁸; and/or (2) changes in the neurochemical environment surrounding contralateral motor neurons from injury (e.g., microglial changes, changes in serotonergic modulation, etc.).

LTF occurs with chronic, not acute C2HS

Rats with acute C2HS do not express AIH-induced Dia or T2 EIC LTF, whereas robust right Dia and T2 EIC LTF was observed in chronically hemisected unanesthetized rats (although with considerable variability in LTF magnitude). To our knowledge, this is the first report of bilateral Dia and T2 EIC EMG measurements after acute and chronic spinal injury in unanesthetized rats. Disruption of descending serotonergic inputs to spinal respiratory motor nuclei¹³ and inadequate levels of hypoxemia to achieve serotonin-independent, adenosine-induced LTF⁷ may explain

differences between and acute and chronic spinal injuries. Mechanisms explaining time-dependent differences in the expression of plasticity after SCI remain to be investigated.

Conclusion

There is a need for new approaches, such as pharmacological interventions or AIH, to alleviate respiratory deficits after cervical SCI and in other clinical disorders that compromise breathing capacity.³⁹ AIH elicits phrenic motoneuron plasticity, strengthening activity in Dia and inspiratory intercostal muscles in rats with chronic (not acute) SCI. Thus, with chronic SCI, AIH provides an opportunity for at least transient restoration of lost breathing capacity. The potential of using repetitive AIH exposures to achieve greater and more enduring functional recovery has been recently demonstrated.¹⁴ Because systemic A2A receptor inhibition enhances DiaLTF, combined AIH and A2A receptor antagonists may amplify the therapeutic efficacy of repetitive AIH, particularly in rats with chronic injuries. Paradoxically, A2A receptor antagonists may actually be counterproductive when administered too soon after injury.

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