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Adenosine 2A Receptor Inhibition Protects Phrenic Motor Neurons from Cell Death Induced by Protein Synthesis Inhibition

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Abstract

Respiratory motor neuron survival is critical for maintenance of adequate ventilation and airway clearance, preventing dependence to mechanical ventilation and respiratory tract infections. Phrenic motor neurons are highly vulnerable in rodent models of motor neuron disease versus accessory inspiratory motor pools (e.g. intercostals, scalenus). Thus, strategies that promote phrenic motor neuron survival when faced with disease and/or toxic insults are needed to help preserve breathing ability, airway defense and ventilator independence. Adenosine 2A receptors (A_{2A}) are emerging as a potential target to promote neuroprotection, although their activation can have both beneficial and pathogenic effects. Since the role of A_{2A} receptors in the phrenic motor neuron survival/death is not known, we tested the hypothesis that A_{2A} receptor antagonism promotes phrenic motor neuron survival and preserves diaphragm function when faced with toxic, neurodegenerative insults that lead to phrenic motor neuron death. We utilized a novel neurotoxic model of respiratory motor neuron death recently developed in our laboratory: intrapleural injections of cholera toxin B subunit (CtB) conjugated to the ribosomal toxin, saporin (CtB-Saporin). We demonstrate that intrapleural CtB-Saporin causes: 1) profound phrenic motor neuron death (~5% survival); 2) ~7-fold increase in phrenic motor neuron A_{2A} receptor expression prior to cell death; and 3) diaphragm muscle paralysis (inactive in most rats; ~7% residual diaphragm EMG amplitude during room air breathing). The A_{2A} receptor antagonist istradefylline given after CtB-Saporin: 1) reduced phrenic motor neuron death (~20% survival) and 2) preserved diaphragm EMG activity (~46%). Thus, A_{2A} receptors contribute to neurotoxic phrenic motor neuron death, an effect mitigated by A_{2A} receptor antagonism.

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Keywords

Adenosine; ADORA2A; A_{2A} receptor; Neuroprotection; Phrenic motor neuron survival; Apoptosis; p38

Introduction

Respiratory motor neuron loss contributes to respiratory insufficiency, ventilator dependence, and death in clinical disorders such as ALS, cervical spinal cord injury, and infectious or toxic neuropathies (Nogués and Benarroch, 2008; Johnson and Mitchell, 2013; Seven and Mitchell, 2019). Since the diaphragm is the major inspiratory muscle, improved understanding of factors exacerbating/ameliorating phrenic motor neuron death is essential in our effort to design new therapies that promote phrenic motor neuron survival to preserve breathing function.

Mechanisms of neuroprotection are often studied on neuronal cultures and/or *in vivo* rodent models (Pedata et al., 2005; Melani et al., 2006; Mojsilovic-Petrovic et al., 2006; Jeanneteau et al., 2008; Paterniti et al., 2011). However, differential susceptibilities of discrete motor pools are seldom considered in these investigations (Lladó et al., 2006; Nichols et al., 2013; Seven et al., 2018c), despite the fact that motor neurons are diverse in their susceptibility to insults such as trauma, inflammation, infection and/or neurotoxins. For example, in the SOD1^{G93A} rat model of ALS, phrenic motor neuron death (~80%) is more pronounced than intercostal (~50%) or hypoglossal (~0%) motor pools (Nichols et al., 2013; Seven et al., 2018c). Unknown differences in protein expression or signaling cascades likely determine differential motor neuron susceptibility to insults between motor pools. In particular, phrenic motor neurons present unique gene expression patterns versus motor neurons innervating limbs (Machado et al., 2014), and differ in cell signaling versus motor neurons innervating the intercostals (Navarrete-Opazo and Mitchell, 2014; Navarrete-Opazo et al., 2014). These differences may play a role in differential phrenic motor neuron susceptibility to death. Thus, neuroprotective therapies specialized to phrenic motor neuron survival should be investigated.

Adenosine receptors are emerging as potential targets for therapeutic interventions to promote neuroprotection. For example, adenosine 2A (A_{2A}) receptor antagonism slows excitotoxic death of embryonic motor neurons *in vitro* (Mojsilovic-Petrovic et al., 2006), and elicits neuroprotective and anti-inflammatory effects in the face of certain challenges *in vivo* (Melani et al., 2006; Yu et al., 2008; Orr et al., 2018).

Although A_{2A} receptors may contribute to death in some populations of CNS neurons, their role on phrenic motor neuron cell death is not known. Thus, we tested the hypothesis that A_{2A} receptor antagonism promotes phrenic motor neuron survival and improves diaphragm function following a neurotoxic insult that causes phrenic motor neuron death. We utilized a novel and selective neuro-toxicological model of protein translation inhibition-induced neuronal loss via intrapleural injections of cholera toxin B subunit (CtB) conjugated to the ribosomal toxin saporin (CtB-Saporin). Intrapleural CtB-Saporin injections cause dose-dependent phrenic motor neuron death in the mid-cervical spinal cord and intercostal motor

neuron death in the thoracic spinal cord (Nichols et al., 2015). Here, we utilized immunohistochemistry to assess phrenic motor neuron survival, A_{2A} receptor expression and phosphorylation state of candidate downstream signaling proteins, as well as electrophysiology to assess diaphragm activity. We found that: 1) intrapleural CtB-Saporin injections upregulate A_{2A} receptor expression in phrenic motor neurons prior to their death, and 2) A_{2A} receptor antagonist administration reduces phrenic motor neuron loss, at least partially preserving diaphragm EMG activity.

Methods

Animals

Experiments were conducted on 32 adult, male, Sprague-Dawley rats weighing ~350g (Envigo, Indianapolis, IN, USA). Experimental procedures were approved by the University of Florida Institutional Animal Care and Use Committee and were conducted in accordance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals, and the *NIH Guide for the Care and Use of Laboratory Animals* (2011). Rats were fed *ad libitum*, monitored daily for their body weights and behavior, and were exposed to 12-hour light-dark cycle. Intrapleural injections and terminal procedures were conducted under anesthesia, whereas intraperitoneal injections were performed while awake. Toe pinch and corneal reflexes were absent during anesthesia; blood pressure and heart rate responses to the toe pinch and were also absent during terminal EMG studies. All rats were sacrificed via transcardial perfusion.

Experimental Design

A total of 32 rats were studied. Different sets of rats were used for electrophysiology and phrenic motor neuron count (Day 8, $n = 21$), and protein analysis experiments to quantify protein expression prior to total phrenic motor neuron loss (Day 5, $n = 11$, see Figure 1). In electrophysiological experiments, we tested the hypothesis that A_{2A} receptor antagonist enhances phrenic motor neuron survival and preserves diaphragm activity following CtB-Saporin injections. In immunohistochemistry experiments, we tested the hypothesis that A_{2A} receptor expression is upregulated in CtB-identified phrenic motor neurons following CtB-Saporin injections prior to complete cell death.

Drugs

CtB-Saporin (50 µg, Catalog No: IT-14, Advanced Targeting Systems, San Diego, CA) was dissolved in 80 µl phosphate buffered saline (PBS) and administered intrapleurally to target phrenic motor neurons (25 µg per side) using 50 µl Hamilton syringes having semi-blunt needles of 23-gauge and 6 mm length through the fifth intercostal space anterior axillary line (Mantilla et al., 2009). Additionally, 50 µg CtB (Catalog No: 227039, EMD Millipore, Billerica, MA, USA) was dissolved in 25 µl sterile water and injected intrapleurally (12.5 µl on each side) to label the spared phrenic motor neurons.

Rats were administered with A_{2A} receptor antagonist starting 36 hours after CtB-Saporin injections, until 6 hours before perfusion at day 5 or 24 hours before EMG recordings (to minimize acute effects of the drug on recordings) and subsequent perfusion. KW6002

(Istradefylline, Sigma-Aldrich) is a selective, blood-brain-barrier-penetrable A_{2A} receptor antagonist (Yang et al., 2007). KW6002 was dissolved in DMSO (37°C) at 9.3 mg/ml concentration, sonicated, aliquoted and stored at 4°C in dark. Prior to use, KW6002 was re-warmed and administered intraperitoneally twice daily at a dose of 0.5 mg/kg (1.0 mg/kg per day total). The volume of DMSO administered to the vehicle rats was also normalized to body weight (~17–20 µl DMSO per injection, ~50 µl/kg).

EMG Recordings

Rats were anesthetized with isoflurane (3% induction, 2–2.3% maintenance), then converted to urethane anesthesia (1.5–1.6 g/kg) via tail vein intravenous fluid delivery (R-99, Razel Scientific Instruments, Saint Albans, Vermont). To maintain fluid and acid-base balance, Lactated Ringer's Solution was administered via tail vein as necessary. Right femoral artery was catheterized to measure arterial pressure and to collect arterial blood sample to measure PO₂, PCO₂, pH, and standard base excess (ABL90 Flex, Radiometer, Copenhagen, Denmark). Body temperature was maintained at 37.5 ± 0.5 °C with a custom-built heating table and rectal thermistor (Physitemp, model 700 1H).

Teflon-coated stainless-steel multistrand electrodes (AS631, Cooner Wire, Chatsworth, CA) were uninsulated for a length of ~2 mm under a dissecting scope on graph paper. A pair of electrodes were implanted in parallel to the muscle fibers to the mid-costal region of diaphragm muscle following laparotomy. To eliminate potential influences of upper airway muscles, the trachea was cannulated with tubing matching upper airway volume. Experiments were performed during spontaneous breathing with an inspired gas composition regulated to match desired blood-gas values (Table 1). Mechanical ventilation was not performed at any point during the experimental protocols. Following surgical procedures, isoflurane was cleared from the tissues by waiting at least 1 hour or the total isoflurane exposure period, whichever is longer.

EMG signals were differentially amplified (1000x) and bandpass filtered (10–1000 Hz) using a biopotential amplifier (Model 1800, A-M Systems, Carlsborg, WA, USA) prior to digitization at 10 kHz (Powerlab, AD Instruments, Colorado, United States). Since root-mean-squared (RMS) EMG correlates with force generated by various muscles including diaphragm muscle (Fuglevand et al., 1993; Lawrence and De Luca, 1983; Mantilla et al., 2010), it was used to estimate EMG amplitude using a 50-ms sliding window.

Spontaneous EMG signals were recorded in supine position at 4 different diaphragm activation levels: (1) Eupnea (i.e. PO₂>85 mmHg and PCO₂ ~45 mmHg). To attain these blood-gas values, inspired O₂ was slightly supplemented (FiO₂ = 21–26%) (2) Maximum chemoreceptor stimulation (MCS; FiO₂ = 10.5%, FiCO₂ = 7%). (3) Augmented breaths/sighs during MCS (RMS EMG amplitude > 2 x Eupneic value, presenting post-sigh apnea). Augmented breaths elicit near-maximal respiratory muscle activation (Seven et al., 2013; Seven et al., 2014; Seven et al., 2018c).

Immunohistochemistry and Image Acquisition

Immunohistochemistry was performed to count the phrenic motor neurons at day 8 and to evaluate protein expression and phosphorylation at day 5. Protein analyses were performed

at day 5, because vehicle-treated CtB-Saporin group had nearly no surviving phrenic motor neurons by the 8th day. All rats were perfused transcardially using 0.01 M PBS followed by 4% paraformaldehyde (PFA) in 0.01 M PBS at the pH of 7.4 and 4°C. Rats perfused at day 8, had received their last A_{2A} antagonist injection ~24 h ago. On the other hand, rats perfused at day 5, had received their last A_{2A} antagonist injection ~4 h ago. Spinal cords were harvested, post-fixed at 4°C in 4% PFA overnight, and cryo-protected in 20% sucrose for 1 day and 30% sucrose for 3 days until sinking. C3, C4, and C5 spinal segments were sectioned at the transverse plane at 40 µm thickness using a freezing microtome (SM2010R, Leica, Buffalo Grove, IL, USA). Sections were stored in antifreeze solution (30% glycerol + 30% ethylene glycol in 0.01 M PBS at the pH of 7.4). All sections were uniformly sampled throughout C3–5 spinal segments for each protein of interest, washed (3x) in 0.01 M PBS, and blocked in the blocking solution (5% normal donkey serum, 0.5% bovine serum albumin, and 0.1% Triton X-100 in PBS) for 1 h. Sections were incubated in primary antibodies and 2.5% normal donkey serum, 0.25% bovine serum albumin, and 0.1% Triton X-100 in PBS at 4°C for 2 days. Then, sections were washed in PBS and incubated with their respective secondary antibodies at room temperature for 2 h. After the final washes, sections were mounted on charged slides with hard-set anti-fade solution (Vector Labs, Burlingame, CA, USA). Slides were imaged at 20x magnification (Numerical aperture: 0.75) with a fluorescence microscope (BZ-X710, Keyence Co., Osaka, Japan). The same exposure times and excitation intensities were used for all groups.

Phrenic Motor Neuron Counts.—Twelve sections per rat were systematically sampled from the C3–C5 spinal segments. CtB-labeled phrenic motor neurons were immunostained using goat anti-CtB primary antibody (1:2500, catalogue no: 227040, Millipore, Billerica, MA, USA) and anti-goat secondary antibody (1:1000, Alexa Fluor 488; Thermo Fisher Scientific, Waltham, MA). Imaging was performed with a GFP filter (BZ-X, model no: OP-87763) with exposure time of 1/35 s. CtB-positive motor neurons with intact nuclei were counted; phrenic motor neuron number was averaged per slice/ side.

Protein Expression, Phosphorylation and Localization.—Six sections per animal were systematically sampled from C3–C5 spinal segment and double-labeled for CtB and the protein of interest. CtB-labeled phrenic motor neurons were immunostained using goat anti-CtB primary antibody (1:2500, catalogue no: 227040, Millipore, Billerica, MA, USA) and anti-goat secondary antibody (1:1000, Alexa Fluor 488, Thermo Fisher). Other proteins were immunostained with the following primary antibodies: mouse anti-A_{2A} receptor (1:500, 05–717, Millipore, Billerica, MA, USA), rabbit anti-phospho-p38 MAPK (1:500, 4370, Cell Signaling, Danvers, MA, USA), rabbit anti-phospho-ERK1/2 (1:500, 4370, Cell Signaling, Danvers, MA, USA), rabbit anti-phospho-JNK (1:250, 4668, Cell Signaling, Danvers, MA, USA); and the anti-mouse and anti-rabbit secondary antibodies (1:500, Alexa Fluor 594, Thermo Fisher). The exposure time for A_{2A} receptor labeling was 1/5 s. For the other proteins, sectioning module of the Keyence Imaging Software was used with the exposure times of 1/5, 1/4, and 1/3 s for phospho -ERK1/2, phospho-p38 MAPK, and phospho-JNK, respectively.

Image Analysis

Fluorescence images for A_{2A} receptors, p-p38 MAPK, and pJNK were analyzed based on colocalization with the CtB-labelled phrenic motor neuron somata. A histogram was created for each image channel containing CtB immunostaining (green). For each CtB image, a threshold was set at a binarized pixel intensity value corresponding to a fixed percentile (P_{99.2}) for all rats and images to account for changes in signal and background intensities. Using binarized images, CtB+ areas greater than 200 μm^2 were used to mask the other channel to assess immunofluorescence intensity of the protein of interest.

Statistics

EMG recordings were averaged within each experimental condition to represent the value for each condition for each rat. Statistical analyses were performed using ANOVA. When the outcome of ANOVA was significant, Tukey-Kramer honestly significant difference test was used to assess pairwise differences *post hoc*. Data was presented as mean $\pm$ standard error of the mean. JMP 14.0 software was used for statistical calculations.

Results

Increased A_{2A} receptor expression following CtB-Saporin injections

A_{2A} receptors were observed in CtB-labelled phrenic motor neurons and other neurons in the spinal cord (Figure 2A–I) in agreement with our earlier report (Seven et al., 2018a). Extra-neuronal A_{2A} receptor expression was uncommon. Five days after intrapleural CtB-Saporin injections, A_{2A} receptors were upregulated by ~7-fold within identified phrenic motor neurons (3). This increase temporally precedes the 8-day time-point when most phrenic motor neurons die. Furthermore, A_{2A} receptors were translocated to the nucleus following CtB-Saporin injections (the mechanism is currently under investigation). With A_{2A} antagonist administration, no changes were observed in A_{2A} receptor expression or localization within phrenic motor neurons.

A_{2A} receptor inhibition protects phrenic motor neurons from death

Only 5.4 $\pm$ 2.9% of CtB-labelled phrenic motor neurons survived one week after intrapleural injection of 50 μg CtB-Saporin; no CtB-labelling was observed in 4 of 7 rats treated with CtB-Saporin. Punctate CtB staining is sometimes observed in phagocytic bodies at 1-week. These results were confirmed by the absence of neuronal nucleus (and peri-nucleus) marker (NeuN) staining and neuron-specific membrane marker (KCC2: Chloride potassium symporter 5) in the region of the phrenic motor nucleus in CtB-Saporin injected rats (unpublished observations). Selective A_{2A} receptor antagonism protected phrenic motor neurons from toxic cell death; phrenic motor neuron survival was significantly increased (19.6 $\pm$ 3.3%, $p < 0.0001$).

A_{2A} receptor inhibition preserves diaphragm activity after toxic insult

Viability of motor neuron soma may not imply phrenic motor neuron functionality. Thus, we evaluated diaphragm muscle activity (EMG) during different levels of diaphragm muscle effort. Representative diaphragm EMG traces (Figure 5) show diaphragm EMG activation

profiles comparable to our previous report (Seven et al., 2018c). Rat diaphragm muscle utilizes approximately a fifth of its functional reserve during normal breathing. With hypoxia and hypercapnia, diaphragm EMG amplitude is augmented, although this activation is well below maximal activation. Near-maximal diaphragm effort is observed during airway protective reflexes, such as augmented breaths, sneezing and coughing.

Consistent with the motor neuron numbers, diaphragm EMG activity was abolished at all levels of effort in most CtB-Saporin injected rats, and was blunted in the rest. A_{2A} receptor antagonist partially rescued diaphragm EMG activity across all effort levels ($p < 0.01$). During normoxic breathing, diaphragm EMG activity was ~7% of control following CtB-Saporin ($p < 0.001$). However, ~46% of diaphragm activity during normoxic breathing was observed in rats that had received the A_{2A} antagonist ($p < 0.01$ vs. CtB-Saporin + Vehicle). Similar effects were observed across all effort levels. During maximum chemoreceptor stimulation, diaphragm EMG activity was ~19% vs. ~65% of control in CtB-Saporin + vehicle ($p < 0.001$) and CtB-Saporin + A_{2A} antagonist treated rats, respectively ($p < 0.01$). Augmented breaths during maximum chemoreceptor stimulation generated diaphragm EMG activity ~14% vs. ~51% of control in rats that had received CtB-Saporin + vehicle ($p < 0.001$) and CtB-Saporin + A_{2A} antagonist, respectively ($p < 0.01$). Lastly, normoxic EMG activity was normalized to augmented breath amplitude since this normalization reveals the diaphragm functional reserve. In this analysis, normalized EMG values were assumed to be zero in rats with no detectable EMG activity across all conditions tested (i.e. there was no functional reserve in these rats). Normalized eupneic amplitude dropped from 27% (Control) to 4% following CtB-Saporin injections ($p < 0.001$), suggesting the diaphragmatic functional reserve was eliminated. CtB-Saporin + A_{2A} antagonist treatment restored the normalized EMG value ($p = 0.82$ vs. control, $p < 0.05$ vs. CtB-Saporin + Vehicle), demonstrating improved functional reserve.

A_{2A} Receptor Inhibition Reduces CtB-Saporin-Induced p38 MAPK Phosphorylation

Levels of phospho-p38 MAPK were significantly higher in phrenic motor neurons 5 days post-CtB-Saporin injections versus control ($p < 0.05$). With A_{2A} receptor antagonism, phospho-p38 MAPK levels were reduced versus CtB-Saporin+Vehicle treated rats ($p < 0.05$), although it was still higher than in controls ($p < 0.05$).

Phosphorylated extracellular signal-regulated kinase (phospho-ERK) is observed as boutons surrounding phrenic motor neurons (Dale et al., 2012). Following CtB-Saporin injections, phospho-ERK immunoreactivity is significantly increased ($p < 0.05$). A_{2A} receptor antagonism did not change phospho-ERK immunoreactivity ($p > 0.05$).

Phosphorylated c-Jun N-terminal kinase (phospho-JNK) was not observed in phrenic motor neurons until terminal stages of cell death (i.e. phagocytic clearance).

Discussion

Given the vital role of phrenic motor neurons in maintaining proper breathing, understanding mechanisms of phrenic motor neuron death with injury, disease or toxic insults is of considerable importance. Our central hypothesis is that, with disease or injury,

increased A_{2A} receptor expression and signaling accelerates phrenic motor neuron death, diminishing diaphragm function. Thus, following a neurotoxic insult that normally causes phrenic motor neuron death, A_{2A} receptor inhibition would preserve both phrenic motor neuron survival and diaphragm function. Consistent with our hypothesis, following intrapleural CtB-Saporin injections, we observed: 1) A_{2A} receptors are significantly upregulated prior to phrenic motor neuron cell death; 2) A_{2A} receptor signaling hastens CtB-Saporin-induced phrenic motor neuron death; and 3) A_{2A} receptor antagonism improves phrenic motor neuron survival and diaphragm function. These findings indicate that A_{2A} receptors play a key role in early processes initiating phrenic motor neuron dysfunction and death.

Adenosine is present in both intracellular and extracellular CNS compartments at low concentrations under physiological conditions (Zetterström et al., 1982; Dale and Frenguelli, 2009). With challenges such as increased activity, hypoxia, injury and inflammation, adenosine rapidly accumulates in the extracellular space, maintaining homeostasis by modulating synaptic plasticity, regulating energy balance, reducing oxygen demand, inducing vasodilation, orchestrating inflammatory responses and other functions mostly due to its actions on adenosine receptors (Winn et al., 1980; Fredholm et al., 1999; Fredholm and Lindström, 1999; Berman et al., 2000; McAdoo et al., 2000; Dale and Frenguelli, 2009). However, when the challenge becomes too severe to be contained, adenosine can switch from protective to detrimental actions (Gomes et al., 2011), likely depending on receptor expression levels, the specific cell-type expressing adenosine receptors, and the severity and type of insult.

A_{2A} receptors are implicated in phrenic motor neuron plasticity, increasing phrenic activity at a constant level of chemoreflex activation (Golder et al., 2008; Seven et al., 2018b) or undermining other forms (e.g. serotonin-dependent) of phrenic motor plasticity (Hoffman et al., 2010). Thus, adenosine receptors play a key role in regulating important forms of respiratory motor plasticity that are presumably beneficial in mounting a response to compensate for injury or disease.

Conversely, A_{2A} receptors are suspected to play key roles in excitotoxic toxicity (e.g. glutamate and kainate), leading to or accelerating neuronal death (Mojsilovic-Petrovic et al., 2006; Gomes et al., 2011). Thus, A_{2A} receptor activation can trigger neurodegeneration when extracellular glutamate levels are high (Mojsilovic-Petrovic et al., 2006). This is particularly important since increased excitatory glutamatergic drive to phrenic motor neurons is a compensatory strategy of the respiratory control system in protecting against respiratory pathology (Johnson and Mitchell, 2013; Seven and Mitchell, 2019). Thus, A_{2A} receptors are integral regulators of both compensatory and pathogenic responses when neurons are challenged by injury and/or disease.

Earlier work concerning A_{2A} receptor involvement in neuronal death/survival utilized neuronal cultures to study the relevant signaling mechanisms and *in vivo* rodent studies to test efficacies of potential therapeutic approaches. Unfortunately, there is little information concerning susceptibilities of discrete motor neuron pools to toxic or other challenges. Differences are often reported in motor neuron death in rodent models of ALS, such as

SOD1^{G93A} over-expressing rodents. Using this same ALS rat model, we reported differential susceptibility of respiratory motor neurons with phrenic > intercostal > hypoglossal motor neurons (Nichols et al., 2013; Seven et al., 2018c). Furthermore, there are differences in response to A_{2A} receptor antagonism in the ability to elicit respiratory motor plasticity by exposing normal rats to modest intermittent hypoxia (Navarette-Opazo et al., 2014). Here, we demonstrate that A_{2A} receptors have a neuroprotective effect on phrenic motor neurons under toxic insult from CtB-Saporin. Our results parallel previous work in primary motor neuron cultures showing A_{2A} receptors accelerate motor neuron death from excitotoxic insult (Mojsilovic-Petrovic et al., 2006). The similarity in experimental outcomes using two different toxic insults suggests a common mechanism whereby A_{2A} receptors promote motor neuron loss under stress from, for example, toxins.

Two relevant factors in the process whereby A_{2A} receptors potentially accelerate phrenic motor neuron death are increased extracellular adenosine concentration and increased A_{2A} receptor expression by phrenic motor neurons. Here, we show that A_{2A} receptor expression is upregulated by CtB-Saporin in phrenic motor neurons *per se*. A_{2A} receptor expression is regulated by different mechanisms. For example, A_{2A} receptors are selectively upregulated at high extracellular adenosine concentrations due to direct A_{2A} receptor activation in mast cells (Sereda et al., 2011). Thus, increased extracellular adenosine levels may activate A_{2A} receptors, thereby enhancing their expression. Greater understanding of factors regulating A_{2A} receptor activation and expression in phrenic motor neurons is needed to understand their precise roles in neuroprotection and neuroplasticity.

After neurotoxic insults, A_{2A} receptors can activate several intracellular cascades, including downstream signaling via p38, ERK and JNK MAP kinases. Activation of these kinases contributes to neuronal death with a variety of lethal triggers. For example, p38 plays a key role in neuronal cell death from excessive reactive oxygen species formation (Kralova et al., 2008). Increased phospho-p38 immunoreactivity with CtB-Saporin points to potential involvement of the MAP kinase in toxic death, paralleling earlier observations in rodent ALS models (Bhinge et al., 2017). The observed increase in phospho-ERK is similar to observations in a rat model of ALS (Satriotomo et al., 2012; Satriotomo et al., 2016), although it could either contribute to accelerated cell death or compensatory responses that promote neuroprotection. The lack of observed changes in phospho-JNK provides little evidence for any role in CtB-Saporin/A_{2A} receptor enhanced phrenic motor neuron death. Although the roles of p38 and ERK MAP kinases require additional investigation, our finding that CtB-Saporin increases phosphorylation (and activation) of p38 MAP kinases prior to phrenic motor neuron death, and that A_{2A} receptor antagonism reverses this effect with associated neuroprotection leads us to suspect the p38 MAP kinase pathway is critical in the mechanism of accelerated cell death. Further investigation are needed to confirm the causality of p38 MAP kinase activation-phrenic motor neuron cell death relationship.

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Highlights

- Toxic phrenic motor neuron death can be caused by selective protein synthesis inhibition induced by intrapleural cholera-toxin beta subunit conjugated saporin (CtB-Sap) administration.
- Intrapleural CtB-Sap causes upregulation of neuronal A_{2A} receptors prior to phrenic motor neuron death.
- A_{2A} receptor inhibition protects phrenic motor neurons from death and preserves diaphragm activity.

Figure 1:

Timeline for experimental procedures: 36 hours after bilateral intrapleural CtB-Saporin injections (Total dose: 50 μ g/rat), rats were treated with A_{2A} receptor antagonist (KW6002; also known as Istradefylline) twice daily, attaining a total dose of 1 mg/kg/day. Terminal EMG measurements and phrenic motor neuron counts were performed on day 8 (24 h after the last A_{2A} receptor antagonist injection). Because almost all phrenic motor neurons died by day 8, an earlier time-point when phrenic motor neurons were still viable was selected for immunohistochemical evaluation of protein expression and localization. Thus, for molecular analyses, rats were sacrificed and perfused at Day 5 post-CtB-Saporin injection.

Figure 2:

Adenosine 2A (A_{2A}) receptor expression in phrenic motor neurons 5 days after intrapleural cholera toxin B (CtB)-Saporin injections (i.e. *before* phrenic motor neuron loss). A-I: Representative images (20x) for CtB (green) and A_{2A} receptors (red) at Day 5. J: A_{2A} receptor fluorescence intensity is increased following intrapleural CtB-Saporin injections *versus* control ($p < 0.01$; red asterisks). Mean $\pm$ 1 SEM. Scale bar = 40 μ m.

Figure 3:

Phrenic motor neuron counts at Day 8 post-intraleural cholera toxin (CtB-Saporin) injections (50 μ g/rat total). A-C: Representative images (20x) for CtB-labelled phrenic motor neurons. D: CtB-Saporin causes almost complete loss of phrenic motor neurons ($p < 0.01$ vs control; asterisk). Adenosine 2A (A_{2A}) receptor antagonist (Istradefylline; KW6002) administration following intraleural CtB-Saporin injections improves phrenic motor neuron survival versus CtB-Saporin injected + vehicle treated rats ($p < 0.05$; #). Mean $\pm$ 1 SEM. Scale bar = 40 μ m.

Figure 4:

Representative diaphragm EMG recordings during eupnea, maximum chemoreceptor stimulation (10.5% O₂ + 7% CO₂), and spontaneous deep breaths (sighs) 7 days after CtB (control) and CtB-Saporin injections. Intrapleural CtB-Saporin injections abolished diaphragm EMG activity across all behaviors in 4 of 7 rats. Twice daily treatment of A_{2A} receptor antagonist partially preserved the diaphragm EMG activity.

Figure 5:

Diaphragm EMG activity 8 days after intrapleural cholera toxin B-Saporin (CtB-Saporin) injections (50 μ g). Diaphragm EMG amplitude is estimated via root-mean-squared (RMS) calculation over a 50 -ms window. Diaphragm EMG was nearly abolished following CtB-Saporin injections during (A) eupnea, (B) hypoxia (10.5%)-hypercapnia (7%), and (C) augmented breaths ($p < 0.01$). Adenosine 2A (A_{2A}) receptor antagonist (KW6002) following intrapleural CtB-Saporin injections increases preserved diaphragm EMG activity compared to vehicle treatment ($p < 0.05$ during eupnea and augmented breath). (D) EMG activity was normalized to augmented breaths since this normalization reveals the functional reserve of the diaphragm muscle. Normalized EMG value was assumed to be zero in the rats with no EMG activity across all conditions tested, since there was no functional reserve in these rats. Diaphragmatic functional reserve was obliterated following intrapleural CtB-Saporin injections ($p < 0.001$). A_{2A} antagonist treatment restored normalized EMG value ($p = 0.82$ compared to control, $p < 0.05$ compared to CtB-Saporin + Vehicle), suggesting restored functional reserve. Mean $\pm$ SEM.

Figure 6:

Phosphorylation of p38 MAPK within the phrenic motor neurons 5 days after intrapleural cholera toxin B-Saporin (CtB-Saporin) injections. A-I. Representative images (20x) for CtB (green) and phospho-p38 MAPK (red) at Day 5. J. Signal-to-background ratio of phospho-p38 fluorescence intensity is increased following intrapleural CtB-Saporin injections compared to control ($p < 0.001$). A2A antagonist treatment reduced phosphor-p38 MAPK levels compared to CtB-Saporin+Vehicle group ($p < 0.05$); however, was still higher than control group ($p < 0.05$). Mean $\pm$ SEM. SEM in the control group represents the variability in the raw values normalized to averaged mean value. Scale bar = 40 μ m.

Table 1:

Physiological variables at baseline and maximal chemoreceptor stimulation in control, vehicle-treated CtB-Saporin injected and istradefylline-treated CtB-Saporin animals 24 h following the last treatment

	Condition	Control	CtB-Saporin + Vehicle	CtB-Saporin + KW6002
PaCO ₂ (mmHg)	Baseline	43.9±0.5	44.7±0.6	44.7±1.3
	MCS	58.6±1.2	59.8±1.3	57.0±1.5
sBE (m Eq/L)	Baseline	2.4±1.0	2.5±1.2	2.0±1.1
	MCS	3.5±0.9	2.3±1.4	4.3±1.0
PaO ₂ (mmHg)	Baseline	92.1 ±1.6	93.7±1.3	97.9±3.3
	MCS	46.3±1.8	41.4±2.0	45.3±2.2
Temperature	Baseline	37.3±0.2	37.4±0.2	37.5±0.2
	MCS	36.9±0.1	36.8±0.1	37.5±0.1
MAP (mmHg)	Baseline	94.8±5.2	96.2±7.3	96.6±5.8