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Intermittent hypercapnia enhances CO₂ responsiveness and overcomes serotonergic dysfunction

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Abstract

Serotonergic dysfunction compromises ventilatory chemosensitivity and may enhance vulnerability to pathologies such as the Sudden Infant Death Syndrome (SIDS). We have shown raphé contributions to central chemosensitivity involving serotonin (5-HT)- and γ -aminobutyric acid (GABA)-mediated mechanisms. We tested the hypothesis that mild intermittent hypercapnia (IHc) induces respiratory plasticity, due in part to strengthening of GABA mechanisms. Rat pups were IHc-pretreated (8 consecutive cycles; 5 min 5 % CO₂ - air, 10 min air) or constant normocapnia-pretreated as a control, each day for 5 consecutive days beginning at P12. We subsequently assessed CO₂ responsiveness using the *in situ* perfused brainstem preparation. Hypercapnic responses were determined with and without pharmacological manipulation. Results show IHc-pretreatment induces plasticity sufficient for responsiveness despite removal of otherwise critical ketanserin-sensitive mechanisms. Responsiveness following IHc-pretreatment was absent if ketanserin was combined with GABAergic antagonism, indicating that plasticity depends on GABAergic mechanisms. We propose that IHc-induced plasticity could reduce the severity of reflex dysfunctions underlying pathologies such as SIDS.

Keywords

respiration; chemosensitivity; SIDS; serotonin; GABA

1. Introduction

The brain's ability to detect and respond to changes in CO₂/pH (central chemosensitivity) is a fundamental homeostatic process and essential for life. Central chemoreception is likely best described as a complex system function that involves a limited but varied group of neuron types, brainstem sites, and multiple neurotransmitter mechanisms (Feldman et al.,

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2003; Nattie, 2009; Nattie and Li, 2009; Putnam et al., 2004). It may be that different mechanisms contribute to sensitivity under different conditions. Organization of chemosensitivity as a system function affords the potential for considerable plasticity. Not only could a specific mechanism play a differential role in overall system sensitivity under specific conditions, the involvement of multiple mechanisms may allow homeostatic regulation despite partial dysfunction, injury, or disease (Feldman et al., 2003). Thus, if one reflex mechanism is dysfunctional, alternative mechanisms may facilitate an appropriate response.

Medullary raphé neurons contribute to chemoresponsiveness. Located within the raphé are at least two neuronal subsets documented to possess intrinsic chemosensitivity *in vitro* (Corcoran et al., 2009; Richerson, 2004; Richerson et al., 2005; Wang et al., 1998); acidosis-stimulated serotonin(5-HT)-synthesizing neurons and acidosis-inhibited γ -aminobutyric acid(GABA)-synthesizing neurons. We have developed a model describing raphé contributions to central chemosensitivity wherein both 5-HT and GABA mechanisms modulate ventilation to maintain tissue CO_2/pH through synaptic activation and disinhibition, respectively (Corcoran et al., 2008; Richerson, 1995). We have demonstrated that these cell types retain chemosensitivity in the intact and unanesthetized brainstem (Corcoran et al., 2008, 2013; Iceman et al., 2013). Hypercapnia stimulates 5-HT neurons, contributing to excitation of ventilation. Hypercapnia also inhibits normally inhibitory GABA neurons, exciting ventilation through disinhibition. So, although the ventilatory homeostatic response to hypercapnia is normally mediated by 5-HT-synthesizing neurons (Corcoran et al., 2008), it may be possible that strengthening the response of GABA mechanisms could preserve responsiveness to a hypercapnic challenge despite a dysfunction in 5-HT mechanisms. We speculate that chemosensory plasticity may underlie our observations that, while ventilatory chemoresponsiveness is abolished by acute disruption of serotonergic function (Corcoran et al., 2013), genetically modified mice chronically lacking 5-HT neurons retain partial responsiveness (Hodges et al., 2008). Thus, although acute loss illustrates that serotonergic contributions are normally critical, plasticity may restore partial function under conditions of chronic abnormality.

We propose that chemosensory plasticity could be induced to precondition the system and enhance multiple chemosensory mechanisms. We tested the hypothesis that preconditioning with intermittent hypercapnia (IHc) during postnatal development enhances chemoresponsiveness to prevent the abolition of responses normally associated with acute interruption of 5-HT signaling. Based on our model of 5-HT- and GABA-mediated contributions, we hypothesized that plasticity results from enhancement of GABAergic chemosensory mechanisms. We chose an intermittent hypercapnic stimulus, as chronic hypercapnia is shown to attenuate subsequent hypercapnic sensitivity (Bavis et al., 2006; Dempsey and Forster, 1982; Kondo et al., 2000; Lai et al., 1981; Rezzonico et al., 1990; Rezzonico and Mortola, 1989; Schaefer et al., 1963).

Abnormal serotonergic brainstem mechanisms may contribute to infant vulnerability to Sudden Infant Death Syndrome (SIDS), and that vulnerability may result in part from chemosensory dysfunction (Cummings et al., 2009; Duncan et al., 2010; Hodges and Richerson 2010; Kinney et al., 2009; Paterson et al., 2006; Richerson, 2004). If induced

reflex plasticity is sufficient to overcome or reverse ventilatory chemosensitivity dysfunctions similar to those thought to contribute to SIDS, then interventions that induce plasticity could be therapeutic in augmenting chemoresponsiveness and decreasing infant vulnerability to SIDS.

2. Methods

2.1. Experimental groups

All experiments were done in accordance with the guidelines of the “Guide for the Care and Use of Laboratory Animals” of the National Institutes of Health and were approved by the University of Alaska Fairbanks Institutional Animal Care and Use Committees. Nineteen naïve rat dams received normal rat chow and water *ad libitum*, and were bred with 19 males. Resulting pups also received food and water *ad libitum* and were housed and maintained in the UAF Animal Care Facility on a 12 h light/dark cycle, at a room temperature of 21 °C. A total of 112 animals from both sexes were used in these studies.

2.2. Gas pre-treatments

Rat pups were exposed to intermittent hypercapnia (IHc; 8 consecutive cycles of 5 min 5 % CO₂: balance air, followed by 10 min air) or constant normocapnia (Nc; Type 1-Grade D air only, as a treatment sham) each day for 5 consecutive days beginning at post-natal day 12 (P12). Entire litters were randomly assigned to either IHc-pretreatment or Nc-pretreatment. Because related litter-mates, rather than randomly assigned individuals, were used as experimental subjects, multiple litters (2 to 5) received each combination of gas and pharmacological treatments. During gas treatments, dams were separated from pups and the home cage was transported to a procedure room adjacent to the animal holding area. Cages were fitted with an airtight lid with a gas inflow and outflow. In this manner, litters (ranging from 3 to 15 pups) were exposed to room temperature inlet gas, supplied at 10 l/min through a countercurrent heat exchanger. The gas outlet was connected to 1 m of 5.5 mm internal diameter tubing to prevent room air infiltration without creating substantial positive pressure within the chamber. Nc- and IHc-pretreated animals received the same flow of gas. For the IHc protocol, a programmable digital timer and solenoid valve automatically switched inlet gases to produce 8 consecutive cycles of 5 min hypercapnia followed by 10 min normocapnia. In the Nc protocol the solenoid did not cycle. Valve cycling was monitored using a computerized data acquisition system (LabChart 7, ADInstruments). Pilot studies indicated that CO₂ levels in the enclosure equilibrated with inlet gas concentrations within 90 s. Chamber gas was not monitored during these treatments. After this exposure protocol, cages were removed from the enclosure, dams were returned to pups in the home cage, and cages were returned to the adjacent housing facility. In no cases did pup abandonment occur following these brief maternal separations, and patterns and durations of maternal separation were equal between IHc- and Nc-pretreatment groups. Procedures were repeated for the same durations on 5 consecutive days, at approximately the same time each day.

2.3. In situ assessment of chemoresponsiveness

At least 7 days following IHc- or Nc-pretreatments, hypercapnic ventilatory sensitivity was assessed using the *in situ* arterially perfused brainstem preparation as previously described

(Corcoran et al., 2013; Toppin et al., 2007, after Paton, 1996). Briefly, animals were anesthetized with isoflurane (5 %, vaporized in 100 % O₂) and pretreated with heparin sodium (500 units, I.P.). Animals were bisected subdiaphragmatically and submerged in an ice-chilled artificial cerebral spinal fluid (aCSF) containing (mM in H₂O): MgSO₄·7H₂O, 1; NaH₂PO₄·H₂O, 1.25; KCl, 4; NaHCO₃, 24; NaCl, 115; D-glucose, 10; CaCl₂·2H₂O, 2). The forebrain rostral to the colliculi was removed by aspiration, and fur, skin and viscera were removed. The diaphragm was separated from the body wall with care taken to ensure integrity of the phrenic nerve. The preparation was moved to the recording station where the descending aorta was cannulated using a double-lumen catheter (Ø 1.25 mm, Braintree Scientific) and perfused retrogradely from a reservoir containing 350 ml aCSF (with 13 g/l ficoll 70, Sigma, added as an osmotic agent). The perfusate was first equilibrated with 95 % O₂-5 % CO₂, warmed to 32 °C, and circulated through a bubble trap and particle filters (25 µm, 45 µm; Millipore) prior to entering the aorta. Perfusate passing through the animal was collected and recycled to the reservoir. The neuromuscular blocker gallamine triethiodide (20 mg/l) and vasopressin (5 µM) were added to the perfusate. The aortic perfusion pressure was adjusted to 70–80 mmHg using an adjustable perfusate bypass valve, and a bolus of sodium cyanide (50 µl 0.1 % solution) was injected into the perfusate line to transiently stimulate peripheral chemoreceptors (Dutschmann et al., 2000). After partial pneumectomy, the phrenic nerve was exposed and aspirated into a glass capillary suction electrode pulled to a diameter that ensured adequate seal on the nerve.

The signal was amplified (×10,000; DAM50, WPI) and filtered (band-pass 300 Hz – 1 kHz). Using a computerized data acquisition system (Powerlab, ADInstruments), data were digitized at 1 kSamples/s and digitally integrated through full wave rectification and 50 ms moving average on a duplicate channel. Phrenic burst frequency (bursts/min) was determined by counting the number of phrenic bursts occurring during 60 s of normocapnia immediately preceding the gas challenge, the final 60 s of hypercapnia, and 60 s during normocapnic recovery 5 min after a return to baseline. Phrenic burst amplitude was derived from the average integrated peak height of all bursts within these 60 s periods, expressed in arbitrary units within each preparation and as a proportional change within a preparation in response to the gas challenge. Neural minute ventilation was calculated as the product of burst frequency and amplitude, again expressed in arbitrary units and as a proportional change with treatment (Eldridge, 1971).

2.4. Gas Challenges

Preparations were maintained on perfusate equilibrated with 95 % O₂-5 % CO₂ for at least 1 h. Equilibration mixtures were produced from O₂ and CO₂ using a precision gas mixer (GSM3, CWE) and verified with a CO₂ analyzer (CD-3A, Applied Electrochemistry). This baseline condition approximated normocapnic plasma *in vivo*; perfusate entered the aorta at P_{CO₂} and pH of approximately 33 mmHg and 7.4, respectively. For the gas challenge, gas equilibrating the perfusate was switched in sequence to 5 min periods of hypocapnia (96.5 % O₂-3.5 % CO₂) followed by 5 min periods of hypercapnia (91 % O₂-9 % CO₂) and subsequently returned to baseline normocapnia (95 % O₂-5 % CO₂). Lacking hemoglobin, solution hyperoxia (P_{O₂} ≈ 600mmHg) was necessary to maintain O₂ content sufficient to meet tissue metabolic demands. This unavoidable hyperoxia was constant under all

conditions. The hypercapnic challenge conditions approximated those occurring during a 4 % increase in inspired CO₂ (perfusate equilibrated with 91 % O₂ – 9 % CO₂; Pco₂ 60 mmHg; pH 7.2).

2.5. Pharmacological challenges

Preparations were tested either without further pharmacological manipulation, or with the addition of specific antagonists to disrupt particular neurotransmitter signaling mechanisms. Pharmacological agents were added after the 60 min normocapnic period, which was maintained for an additional 10 min prior to gas challenge. In one series, the selective 5-HT_{2A} receptor antagonist ketanserin tartrate (5 µM, Sigma) was administered to induce an acute serotonergic dysfunction and determine the influence of removing ketanserin-sensitive (5-HT_{2A} receptor-mediated) processes on subsequent responsiveness to the gas challenge. We have previously shown that a ketanserin-sensitive mechanism is critical for chemoresponsiveness in this system (Corcoran et al., 2013). In a second series, a combination of ketanserin along with the GABA_A and GABA_B receptor antagonists bicuculline (20 µM, Sigma) and saclofen (20 µM, abcam) were administered to produce combined serotonergic and GABAergic dysfunction and illustrated the impact of this combined dysfunction on responsiveness to the gas challenge. In a third series, bicuculline and saclofen alone were administered to assess the degree to which the response to the gas challenge was mediated by GABAergic mechanisms. Comparing the effects of these treatments demonstrated the chemoresponsiveness of this preparation, the dependence of responses on ketanserin-sensitive serotonergic mechanisms, and the degree to which responses that were retained subsequent to ketanserin exposure were dependent on GABAergic mechanisms not normally contributing to responsiveness.

2.6. Data and statistical analyses

Ventilatory parameters were quantified from the integrated neurograms. Frequency (f; the number of bursts per unit time), amplitude (the mean integrated peak amplitude of bursts), and a neural correlate of minute ventilation (NVE; frequency·amplitude) were calculated from periods of normocapnia or hypercapnia. Hypercapnic responsiveness was determined from ventilatory parameters recorded during baseline normocapnia and hypercapnia. This was quantified by deriving a relative hypercapnic response, calculated by expressing the difference in parameter values between normocapnia and hypercapnia, normalized as a ratio relative to values occurring with normocapnia prior to gas challenge (responsiveness = [value during hypercapnia – value during normocapnia]/value during normocapnia).

Data Parsing—We and others have demonstrated that the *in situ* rat brainstem preparation exhibits clear responsiveness to hypercapnia and provides an appropriate model for the study of mechanisms contributing to chemosensitivity (Corcoran et al., 2013; Day et al., 2007; Iceman et al., 2013; Massey et al., in review, Toppin et al., 2007). These prior studies have illustrated that the response is most clearly manifest in changes in burst frequency and the index of minute ventilation, NVE. Our pilot investigations suggested the possibility that preparations exhibiting a relatively high initial burst frequency have a lower apparent responsiveness to hypercapnia, which suggests a frequency limitation. The present study

was designed to investigate mechanisms contributing to responsiveness, necessitating a focus on responsive preparations free of potential confounding factors.

To remove the potential confounding influence of dampened chemoresponsiveness in preparations with elevated baseline burst frequencies, we subjected the data set to the following parsing protocol. In preparations exposed to neither pharmacological manipulation nor IHc-pretreatment, we plotted the relationship between hypercapnic responsiveness and initial burst frequency and determined a linear regression ($P < 0.001$; $R^2 = 0.74$) described by the equation hypercapnic responsiveness (HcR) = $192 - 3.45$ (initial frequency). The highest responsiveness observed in any of these preparations was an 84.6 % increase. From this regression, we determined the initial frequency associated with the half-maximum responsiveness. We then used this value as a threshold to identify preparations in which high initial burst frequency could confound expression of chemoresponsiveness. Preparations having a normocapnic initial burst frequency above this threshold (32 bursts/min) were omitted from all portions of analysis. When so parsed, 17 of 112 preparations (15 %) were removed from analyses. Roughly equal numbers of preparations were parsed from experimental groups such that a total of 95 preparations contributed to the analyzed data. All reported group mean values are those obtained after parsing.

One-way repeated-measures analysis of variance (ANOVA) was used to compare parameter values before and during hypercapnia for each drug treatment (no drugs; ketanserin alone; bicuculline + saclofen; ketanserin + bicuculline + saclofen), which quantified hypercapnic responsiveness for ventilatory parameters under each drug treatment. One-way ANOVA was used to compare the effect of drug treatment on the relative hypercapnic response in each parameter. Values reported in the text are mean $\pm$ standard error.

3. Results

3.1. Burst discharge recordings

All our recorded phrenic neurograms displayed a “eupneic” pattern (Figs. 1, 2) characteristic of this preparation (Eldridge, 1971; St.-John and Paton, 2000), and displayed this pattern throughout normocapnia, hypercapnia, and recovery from normocapnia and drug treatment. A potential exception was during combined ketanserin, bicuculline and saclofen treatment, where bursts appeared prolonged.

Preparations without pharmacological manipulation derived from pups that received Nc-pretreatments (Nc-preparations) had a mean normocapnic burst frequency of 20.7 ± 1.26 bursts/min. This mean normocapnic burst frequencies of all treatment groups were not different from this value. Burst amplitude was expressed in arbitrary units that were consistent between treatments within a preparation, but may have differed between preparations. NVE was also expressed in arbitrary units.

3.2. Response to hypercapnia

In Nc-preparations, hypercapnic responsiveness of f and NVE were 22 ± 5.0 % (Fig. 3) and 27 ± 14 % (Fig. 4), respectively. One-way repeated-measures ANOVA indicated that these hypercapnia-induced changes were significant ($P < 0.001$ for f ; $P < 0.05$ for NVE). No

significant responsiveness was resolved for burst amplitude alone ($P = 0.62$). In Nc-preparations that received bicuculline and saclofen, hypercapnic responsiveness of f , amplitude and NVE were $31 \pm 9\%$ ($P < 0.05$), $24 \pm 7\%$ ($P < 0.05$) and $61 \pm 9\%$ ($P < 0.05$) respectively. This hypercapnic responsiveness was no different from that of Nc-preparations without pharmacological manipulations (f , $P = 0.43$; amplitude, $P = 0.62$; NVE, $P = 0.28$). Nc-preparations treated with ketanserin had no significant response to hypercapnia ($f = 3.4 \pm 7.5\%$, $P = 0.61$, Fig. 3; NVE = $5.6 \pm 11.0\%$, $P = 0.58$; Fig. 4). We were able to statistically resolve the difference between the frequency component of the hypercapnic response in Nc-preparations (which presented chemoresponsiveness), and similar preparations treated with ketanserin (in which responsiveness was absent; $P < 0.05$, Fig. 3). Although Nc-preparations also demonstrated an NVE component to the hypercapnic response, absent in such preparations treated with ketanserin, we could not resolve a difference between these groups ($P = 0.14$, Fig. 4).

Preparations derived from IHc-pretreated pups (IHc-preparations) showed hypercapnic responsiveness of f and NVE ($38 \pm 11\%$, $P < 0.001$, Fig. 3; and $130 \pm 58\%$, $P < 0.001$, Fig. 4; respectively), and again no responsiveness in burst amplitude alone ($P = 0.68$). For NVE, hypercapnic responsiveness was significantly greater in IHc-preparations than Nc-preparations ($P = 0.05$, Fig. 4).

Unlike Nc-preparations, IHc-preparations did show responsiveness of f and NVE to hypercapnia under ketanserin treatment ($f = 28 \pm 3\%$, $P < 0.001$, Fig. 3; NVE: $29 \pm 5\%$, $P < 0.001$, Fig. 4). This responsiveness was attenuated relative to that of IHc-preparations without ketanserin treatment (NVE, $P < 0.05$, Fig. 4) but was no different than that of Nc-preparations without ketanserin (frequency, $P = 0.25$; NVE, $P = 0.90$).

In Nc-preparations, the GABA_A and GABA_B receptor antagonists bicuculline and saclofen had no influence on chemoresponsiveness ($n = 4$), which was not different than that of Nc-preparations not exposed to pharmacological manipulations. As ketanserin treatment alone abolished chemoresponsiveness in these preparations, bicuculline and saclofen were not co-applied with ketanserin. When bicuculline, saclofen, and ketanserin were co-applied in IHc-preparations, hypercapnic responsiveness was absent. The hypercapnic response of IHc-preparations treated with bicuculline, saclofen, and ketanserin was not statistically different from that of Nc-preparations treated with ketanserin only (frequency, $P < 0.28$, Fig. 3; NVE: $P < 0.47$, Fig. 4) but was much less than that of IHc-preparations treated with ketanserin only (frequency, $P < 0.001$, Fig. 3; NVE: $P < 0.01$, Fig. 4).

4. Discussion

Our study assessed preconditioning with intermittent hypercapnia during postnatal development as a modulator of chemoresponsiveness. We tested the hypothesis that such IHc preconditioning would sufficiently enhance chemoresponsiveness to overcome acute pharmacologically-induced serotonergic dysfunction, previously shown to compromise/abolish chemoresponsiveness. Furthermore, we tested the hypothesis that GABA-mediated mechanisms underlie the responsiveness present in IHc-preparations retained when ketanserin-sensitive contributions are removed by pharmacological blockade.

Our results confirm that the rat *in situ* perfused brainstem preparation does exhibit chemoresponsiveness (Day et al., 2007; St.-John and Paton, 2000; Figs. 1, 2). Serotonergic dysfunction was induced using the potent 5-HT_{2A} receptor antagonist ketanserin. We have previously shown that acute disruption of ketanserin-sensitive mechanisms abolishes chemoresponsiveness in the rat *in situ* brainstem preparation and conclude that serotonergic neurotransmission is critical for chemosensitivity (Awouters, 1985; Corcoran et al., 2013). We confirm these findings as ketanserin treatment alone abolished responsiveness Nc-preparations. Baseline burst frequency was consistent between untreated preparations and those treated with ketanserin. Baseline burst amplitudes were dependent on recording parameters within a preparation, and were not compared between preparations. We add to the previous finding that bicuculline- and saclofen-sensitive GABA mechanisms do not contribute to the response to hypercapnia as antagonism of such mechanisms did not result in an alteration of the response to hypercapnia in Nc-preparations.

Despite the apparent critical nature of 5-HT mediated mechanisms confirmed by these findings, central chemosensitivity is best characterized as a complex system function that potentially involves multiple neuron types, brainstem sites, and neurotransmitter mechanisms (Feldman et al., 2003; Nattie, 2009; Nattie and Li, 2009; Putnam et al., 2004). The possibility that diverse mechanisms contribute to such a system function allows for plasticity, providing homeostatic regulation despite dysfunction in any one mechanism (Feldman et al., 2003).

Although our results confirm that serotonergic dysfunction greatly disrupts central chemosensitivity, we aimed to illustrate that plasticity induced through IHc-pretreatment could overcome the chemosensory impairment associated with acute (pharmacological) 5-HT dysfunction. We propose that chemosensory plasticity can be induced to precondition the system and enhance multiple chemosensory mechanisms. We aimed to induce plasticity within the chemosensory system with IHc-pretreatment and thereby facilitate the recruitment of non-5-HT chemosensory mechanisms; we aimed to invoke chemoresponsiveness when 5-HT-mediated mechanisms were rendered dysfunctional.

Under normocapnic conditions absent any 5-HT dysfunction, neuroventilatory parameters in IHc-preparations were comparable to those of Nc-preparations. Pretreating with IHc, however, greatly augmented subsequent chemoresponsiveness. In these preparations, hypercapnia produced 38 ± 11 % and 130 ± 58 % increases in burst frequency and NVE, respectively. When compared to Nc-preparations, chemoresponsiveness was increased 73 % for frequency and 381 % for NVE responses for IHc- preparations.

Ours is the first reported observation that IHc-pretreatment can influence subsequent chemoresponsiveness. In these experiments, IHc-pretreatments were initiated on P12 and continued daily through P16. Protocols were designed to administer cycles of mild hypercapnia to pups, without altering maternal conditions and while limiting maternal separation. This developmental period was chosen to follow the apparent critical period proximal to P12 where rat chemosensitivity is altered (Putnam et al., 2005; Wong-Riley and Liu, 2005, 2008), and the developmental period beyond which chemosensitive 5-HT neurons have been identified *in vitro* (Wang and Richerson, 1999; Wu, 2008). Prior attempts

to influence hypercapnic responsiveness showed no influence when a comparable IHc protocol was conducted between P7 and P14 (Steggerda et al., 2009). It is likely that the developmental timing and/or nature of the IHc stimulus influences resultant plasticity.

Importantly, our central finding indicates that the plasticity induced by IHc-pretreatment is capable of overcoming the profound chemoresponsive dysfunction produced by acute ketanserin treatment. Neuroventilatory frequency during normocapnia was no different in ketanserin-treated preparations, regardless of IHc- or Nc-pretreatment. Ketanserin abolished chemoresponsiveness in Nc-pretreated preparations. Chemoresponsiveness persisted, however, in ketanserin-treated IHc-preparations. The $28 \pm 3\%$ and $29 \pm 5\%$ frequency and NVE responses were no different than those observed in ketanserin free Nc- preparations. We and others have interpreted the loss of chemosensitivity with ketanserin to illustrate critical 5-HT_{2A} receptor-mediated mechanisms. We attribute the retention of relatively normal chemoresponsiveness, overcoming the expected ketanserin-mediated abolishment of chemoresponsiveness, to plasticity imparted by IHc-pretreatment. We conclude that IHc-pretreatment induced a ketanserin-insensitive (non-5-HT_{2A}-mediated) mechanism not normally critical to chemosensitivity. This plasticity appears sufficient to overcome what is normally a complete failure in chemosensitivity associated with acute ketanserin-sensitive serotonergic dysfunction.

Our observations indicate that plasticity initiated with IHc-pretreatment resulted in chemoresponsiveness mediated by mechanisms other than the ketanserin-sensitive mechanisms normally critical for responsiveness. As such, we investigated whether IHc-induced plasticity was enhancing an alternate non-ketanserin-sensitive 5-HT-mediated mechanism, or a mechanism involving signaling molecules independent of 5-HT.

We have suggested that interactions between chemosensory mechanisms mediated either by 5-HT- or GABA-synthesizing neurons contribute to chemosensitivity (Iceman et al., 2010; Richerson et al., 2001, 2005). As such, we predicted that the ketanserin-insensitive chemoresponsiveness induced by IHc-pretreatment was due to an enhancement of GABA-mediated mechanisms (Corcoran et al., 2008; Richerson, 1995). To determine the contribution of GABA mechanisms, we tested the combined influences of GABA_A and GABA_B receptor antagonists bicuculline and saclofen. Bicuculline and saclofen treatment had no influence on chemoresponsiveness in Nc-preparations, indicating that GABA_{A/B} receptor-mediated processes are not normally critical to chemoresponsiveness. As ketanserin alone abolished chemoresponsiveness in Nc-preparations, the influence of combined ketanserin, bicuculline, and saclofen treatment was not assessed in these preparations.

We assessed if the ketanserin-insensitive responsiveness present in IHc-preparations was mediated by bicuculline/saclofen-sensitive mechanisms. Although IHc-preparations maintained chemoresponsiveness when treated with ketanserin alone, responsiveness was absent in preparations with combined ketanserin, bicuculline, and saclofen treatment. This result suggests that the plasticity induced by IHc-pretreatment, responsible for ketanserin-insensitive responsiveness, was produced through GABA_{A/B} receptor-mediated processes not normally critical for chemoresponsiveness. As bicuculline and saclofen were

administered simultaneously, future work is necessary to determine individual influences of GABA_A and GABA_B receptors in the plasticity induced by IHc-pretreatment.

5. Conclusion

Our findings are consistent with considerations of central chemosensitivity as a system function potentially involving multiple neuron types and neurotransmitter mechanisms. We propose that 5-HT and GABA mechanisms contribute to chemosensitivity. We confirm the finding that, in otherwise untreated preparations, ketanserin-sensitive mechanisms are critical for chemoresponsiveness, suggesting contributions mediated by 5-HT neurons are both necessary and sufficient for chemoresponsiveness under normal circumstances. We show that IHc-pretreatment induces plasticity such that chemoresponsiveness is maintained despite removal of otherwise critical ketanserin-sensitive mechanisms. In addition, we provide evidence to suggest that this plasticity may be dependent upon GABA mechanisms not normally critical for chemoresponsiveness.

We show that IHc-induced plasticity is sufficient to overcome profound chemosensory dysfunctions produced by acute pharmacological disruption of critical ketanserin-sensitive 5-HT mechanisms. Serotonergic dysfunction likely contributes to a number of pathologies, including the Sudden Infant Death Syndrome, by compromising homeostatic reflexes such as hypercapnic chemoresponsiveness. It may be that IHc-induced plasticity could enhance homeostatic reflex efficacy and potentially reduce vulnerabilities to conditions such as SIDS. A number of critical factors remain unknown. These include: identification of GABAergic mechanisms contributing to chemosensory plasticity; changes in 5-HT-mediated mechanisms with IHc-pretreatment; the optimal pattern of IHc; potential developmental sensitivities to IHc-induced plasticity; the longevity of this plasticity and the degree to which IHc-pretreatment could overcome chronic serotonergic dysfunctions similar to those associated with vulnerability to SIDS or other pathologies. The clear potential to induce chemosensory plasticity, however, provides possible targets for therapeutic intervention to reverse or offset chemosensory dysfunction.

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References

- Awouters F. The pharmacology of ketanserin, the first selective serotonin S₂-antagonist. *Drug De Res.* 1985; 6:263–300.
- Bavis RW, Johnson RA, Ording KM, Otis JP, Mitchell GS. Respiratory plasticity after perinatal hypercapnia in rats. *Respir Physiol Neurobiol.* 2006; 153:78–91. [PubMed: 16338177]
- Corcoran AE, Hodges MR, Wu Y, Wang W, Wylie CJ, Deneris ES, Richerson GB. Medullary serotonin neurons and central CO₂ chemoreception. *Respir Physiol Neurobiol.* 2009; 168:49–58. [PubMed: 19394450]
- Corcoran AE, Richerson GB, Harris MB. Both serotonergic and GABAergic neurons contribute to central chemosensitivity in a perfused rat brainstem. *Soc Neuroscience abstracts.* 2008 Online.
- Corcoran AE, Richerson GB, Harris MB. Serotonergic mechanisms are necessary for central respiratory chemoresponsiveness in situ. *Respir Physiol Neurobiol.* 2013; 186:214–220. [PubMed: 23454177]

- Cummings KJ, Commons KG, Fan KC, Li A, Nattie EE. Severe spontaneous bradycardia associated with respiratory disruptions in rat pups with fewer brain stem 5-HT neurons. *AJP-Regul Integr Comp Physiol*. 2009; 296:1783–1796.
- Day TA, Wilson RJ. Brainstem PCO₂ modulates phrenic responses to specific carotid body hypoxia in an in situ dual perfused rat preparation. *J Physiol*. 2007; 578(3):843–857. [PubMed: 17082232]
- Dempsey JA, Forster HV. Mediation of ventilatory adaptations. *Physiol Rev*. 1982; 62:262–346. [PubMed: 6798585]
- Duncan JR, Paterson DS, Hoffman JM, Mokler DJ, Borenstein NS, Billiveau RA, Krous HF, Haas EA, Stanley C, Nattie EE, Trachtenberg FL, Kinney HC. Brainstem serotonergic deficiency in sudden infant death syndrome. *JAMA*. 2010; 303:430–437. [PubMed: 20124538]
- Dutschmann M, Wilson RJ, Paton JFR. Respiratory activity in neonatal rats. *Auton Neurosci-Basic*. 2000; 84:19–29.
- Eldridge FL. The relationship between phrenic nerve activity and ventilation. *Am J Physiol*. 1971; 221:535–543. [PubMed: 5560304]
- Feldman JL, Mitchell GS, Nattie EE. Breathing: Rhythmicity, Plasticity, Chemosensitivity. *Annu Rev Neurosci*. 2003; 26:239–266. [PubMed: 12598679]
- Hodges MR, Richerson GB. The role of medullary serotonin (5-HT) neurons in respiratory control: contributions to eupneic ventilation, CO₂ chemoreception, and thermoregulation. *J Appl Physiol*. 2010; 108:1425–1432. [PubMed: 20133432]
- Hodges MR, Tattersall G, Harris MB, McEvoy S, Richerson D, Deneris ES, Johnson RL, Chen ZF, Richerson GB. Defects in breathing and thermoregulation in mice with near-complete absence of central serotonin neurons. *J Neurosci*. 2008; 28:2495–2505. [PubMed: 18322094]
- Iceman, KE.; Richerson, GB.; Harris, MB. Identification of chemosensitive and insensitive serotonergic and GABAergic neurons in rat medullary raphé nuclei. Program No. 188.4. 2010 Neuroscience Meeting Planner; San Diego, CA: Society for Neuroscience; 2010. Online
- Iceman KE, Richerson GB, Harris MB. Medullary serotonin neurons are CO₂-sensitive *in situ*. *J Neurophysiol*. 2013; 110:2536–2544. [PubMed: 24047906]
- Iceman KE, Richerson GB, Harris MB. GABAergic neurons in the medullary raphé possess network independent chemosensitivity *in situ*. *FASEB J*. 2012; 26:894.13. [PubMed: 22051730]
- Kinney HC, Richerson GB, Dymecki SM, Darnall RA, Nattie EE. The brainstem and serotonin in the sudden infant death syndrome. *Annu Rev Pathol*. 2009; 4:517–550. [PubMed: 19400695]
- Kondo T, Kumagai M, Ohta Y, Bishop B. Ventilatory responses to hypercapnia and hypoxia following chronic hypercapnia in the rat. *Respir Physiol*. 2000; 122:35–43. [PubMed: 10936599]
- Lai Y-L, Lamm WJE, Hildebrandt J. Ventilation during prolonged hypercapnia in the rat. *J Appl Physiol*. 1981; 51:78–83. [PubMed: 6790501]
- Massey CA, Iceman KE, Johansen SL, Wu Y, Harris MB, Richerson GB. Isourane abolishes pH chemosensitivity of serotonin neurons and markedly diminishes the hypercapnic ventilatory response. In review.
- Nattie EE. CO₂, brainstem chemoreceptors and breathing. *Prog Neurobiol*. 2009; 59:299–331. [PubMed: 10501632]
- Nattie EE, Li A. Central chemoreception is a complex system function that involves multiple brain stem sites. *J Appl Physiol*. 2009; 106:1464–1466. [PubMed: 18467549]
- Paterson DS, Trachtenberg FL, Thompson EG, Belliveau RA, Beggs AH, Darnall R, Chadwick AE, Krous HF, Kinney HC. Multiple serotonergic brainstem abnormalities in sudden infant death syndrome. *JAMA*. 2006; 296:2124–2132. [PubMed: 17077377]
- Paton JFR. A working heart-brainstem preparation of the mouse. *J Neurosci Methods*. 1996; 65:63–68. [PubMed: 8815310]
- Putnam RW, Conrad SC, Gdovin MJ, Erlichman JS, Leiter JC. Neonatal maturation of the hypercapnic ventilatory response and central neural CO₂ chemosensitivity. *Respir. Physiol Neurobiol*. 2005; 149:165–179.
- Putnam RW, Filosa JA, Ritucci NA. Cellular mechanisms involved in CO₂ and acid signaling in chemosensitive neurons. *Am J Physiol Cell Physiol*. 2004; 287:1493–1526.

- Rezzonico R, Gleed RD, Mortola JP. Respiratory mechanics in adult rats hypercapnic in the neonatal period. *J Appl Physiol.* 1990; 68:2274–2279. [PubMed: 2384407]
- Rezzonico R, Mortola JP. Respiratory adaptation to chronic hypercapnia in newborn rats. *J Appl Physiol.* 1989; 67:311–315. [PubMed: 2759958]
- Richerson GB. Response to CO₂ of neurons in the rostral ventral medulla in vitro. *J Neurophysiol.* 1995; 73:933–944. [PubMed: 7608778]
- Richerson GB. Serotonergic neurons as carbon dioxide sensors that maintain pH homeostasis. *Nat Rev Neurosci.* 2004; 5:449–461. [PubMed: 15152195]
- Richerson GB, Wang W, Hodges MR, Dohle CI, Diez-Sampedro A. Homing in on the specific phenotype(s) of central respiratory chemoreceptors. *Exp Physiol.* 2005; 90:259–266. [PubMed: 15728134]
- Richerson GB, Wang W, Tiwari J, Bradley SR. Chemosensitivity of serotonergic neurons in the rostral ventral medulla. *Respir Physiol Neurobiol.* 2001; 129:175–189.
- Schaefer KE, Hastings BJ, Carey CR, Nichols G Jr. Respiratory acclimatization to carbon dioxide. *J Appl Physiol.* 1963; 18:1071–1078. [PubMed: 14080722]
- St-John WM, Paton JFR. Characterizations of eupnea, apneusis and gasping in a perfused rat preparation. *Resp Phys.* 2000; 123:201–213.
- Steggerda JA, Mayer CA, Martin RJ, Wilson CG. Effect of Intermittent Hypercapnia on Respiratory Control in Rat Pups. *Neonatology.* 2009; 238:1–7.
- Toppin VA, Harris MB, Kober AM, Leiter JC, St-John WM. Persistence of eupnea and gasping following blockade of both serotonin type 1 and 2 receptors in the in situ juvenile rat preparation. *J Appl Physiol.* 2007; 103:220–227. [PubMed: 17412795]
- Wang W, Pizzonia JH, Richerson GB. Chemosensitivity of rat medullary raphe neurones in primary tissue culture. *J Physiol.* 1998; 511(2):433–450. [PubMed: 9706021]
- Wang W, Richerson GB. Development of chemosensitivity of rat medullary raphe neurons. *Neurosci.* 1999; 90:1001–1011.
- Wong-Riley MTT, Liu Q. Neurochemical development of brain stem nuclei involved in the control of respiration. *Respir Physiol Neurobiol.* 2005; 149:83–98. [PubMed: 16203213]
- Wong-Riley MTT, Liu Q. Neurochemical and physiological correlates of a critical period of respiratory development in the rat. *Respir Physiol Neurobiol.* 2008; 164:28–37. [PubMed: 18524695]
- Wu Y, Hodges MR, Richerson GB. Stimulation by hypercapnic acidosis in mouse 5-HT neurons in vitro is enhanced by age and increased temperature. *Society for Neuroscience abstracts.* 2008; 2008 Online.
- Zhang L, Wilson CG, Liu S, Haxhiu MA, Martin RJ. Hypercapnia-induced activation of brainstem GABAergic neurons during early development. *Respir Physiol Neurobiol.* 2003; 136:25–37. [PubMed: 12809796]

Highlights

- We confirm that chemoresponsiveness depends on ketanserin-sensitive 5-HT mechanisms
- Intermittent hypercapnia pretreatment maintains chemoresponsiveness despite removal of critical 5-HT mechanisms
- Intermittent hypercapnia enhancement of chemoresponsiveness is dependent on GABA mechanisms

Nc (air only) normocapnia **Nc (air only) hypercapnia** **Fig. 1.**

Shown are typical eupneic raw (above) and integrated (below) phrenic neurogram recording with ventilatory bursts representing the neural correlate of ventilation (Eldridge, 1971; St.-John and Paton, 2000). Single raw neurogram bursts are also shown with an expanded timescale. When the *in situ* preparation was exposed to perfusate containing 9 % CO₂ (4 % hypercapnic challenge; bottom panel) there was an increase in both frequency and NVE (frequency*amplitude) of ventilatory events compared to 5 % CO₂ exposure (normocapnia; top panel). Both panels are representative records of the last minute of the indicated CO₂ treatment. Records from a preparation derived from a pup that received intermittent air exposure (sham treatment) and no pharmacological manipulations are shown.

Fig. 2.

Shown are integrated phrenic neurogram recordings illustrating hypercapnic responses in a normocapnic pretreated preparation without pharmacological manipulation (A) and the absence of a response with ketanserin treatment (B). Similar recordings illustrate the enhanced hypercapnic response in an IHC pretreated preparation without pharmacological treatment (C) and preserved responses in an IHC preparation treated with ketanserin (D). Shown also are recordings from an IHC preparation lacking a hypercapnic response under combined ketanserin, bicuculline and saclofen treatment (E). There was an apparent increase in burst duration, not present with ketanserin treatment but observed when ketanserin was combined with bicuculline and saclofen. This observation may suggest a role of GABA mediated inhibition in burst generation, although such influences were not characterized in the current study.

Fig. 3.

Hypercapnia increased ventilatory frequency, normalized to normocapnia, in preparations derived from pups that received IHc air-control exposures without drugs (no fill bar, $n = 20$), those that received IHc and no drugs (horizontal stripe bar, $n = 16$) and those that received IHc and ketanserin (vertical stripe bar, $n = 27$). Significant increase from normocapnia to hypercapnia (one-way repeated-measures ANOVA): *** $P < 0.001$. Between groups comparison of difference in hypercapnic sensitivities determined using one-way ANOVA: † $P < 0.05$, ††† $P < 0.001$.

Fig. 4.

Hypercapnia increased ventilatory NVE, normalized to normocapnia, in preparations derived from pups that received IHc air-control exposures without drugs (no fill bar), those that received IHc and no drugs (horizontal stripe bar) and those that received IHc and ketanserin (vertical stripe bar). Significant increase from normocapnia to hypercapnia (1-way repeated-measures ANOVA): * $P < 0.05$, *** $P < 0.001$. Between groups comparison of difference in hypercapnic sensitivities determined using 1-way ANOVA: † $P < 0.05$, †† $P < 0.01$.