Published in final edited form as:

Eur J Neurosci. 2012 June ; 35(11): 1725–1737. doi:10.1111/j.1460-9568.2012.08091.x.

Metabotropic glutamate receptors (mGluR5) activate TRPC channels to improve the regularity of the respiratory rhythm generated by the pre-Bötzinger Complex in mice

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Abstract

Metabotropic glutamate receptors (mGluR) are hypothesized to play a key role in generating the central respiratory rhythm and other rhythmic activities driven by central pattern generators (e.g., locomotion). Yet, the functional role of mGluR in rhythmic respiratory activity and many motor patterns is very poorly understood. Here, we used mouse respiratory brain-slice preparations containing the pre-Bötzinger Complex (pre-BötC) to identify the role of group I mGluR (mGluR1 and mGluR5) in respiratory rhythm generation. We found that activation of mGluR1/5 is not required for the pre-BötC to generate a respiratory rhythm. However, our data suggest that mGluR1 and mGluR5 differentially modulate the respiratory rhythm. Blocking endogenous mGluR5 activity with MPEP decreases the inspiratory burst duration, burst area and frequency, while it increases the irregularity of the fictive eupneic inspiratory rhythm generated by the pre-BötC. In contrast, blocking mGluR1 reduces the frequency. Moreover, the mGluR1/5 agonist DHPG increases the frequency and decreases the irregularity of the respiratory rhythm. Based on prior studies, we hypothesized that mGluR-signaling decreases the irregularity of the respiratory rhythm by activating transient receptor potential canonical (TRPC) channels, that carry a non-specific cation current (ICAN). Indeed, DHPG application reduces cycle-by-cycle variability and subsequent application of the TRPC channel blocker SKF-96365 reverses this effect. Our data suggest that mGluR5 activation of ICAN-carrying TRPC channels plays an important role in governing the cycle-by-cycle variability of the respiratory rhythm. These data suggest that modulation of TRPC channels may correct irregular respiratory rhythms in some central neuronal diseases.

Keywords

breathing; transient receptor potential; central pattern generator; irregularity; rhythm generation

Introduction

Mammalian central pattern generators (CPGs) produce rhythmic activities that rely on synaptic interactions and intrinsic bursting properties (Smith *et al.*, 1991; Reikling & Feldman, 1998; Thoby-Brisson & Ramirez, 2001; Harris-Warrick, 2002; Kiehn & Kullander, 2004; Ramirez *et al.*, 2004; Grillner, 2006; Harris-Warrick, 2010). As part of the

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medullary ventral respiratory group (VRG) (Wenninger *et al.*, 2004; McKay *et al.*, 2005; McKay & Feldman, 2008), the pre-Bötzinger Complex (pre-BötC) generates the inspiratory rhythm, during normal quiet breathing, called eupnea (Tan *et al.*, 2008; Pagliardini *et al.*, 2011). Rhythmic inspiratory activity generated by pre-BötC neurons appears to be essential to sustain life, as lesioning the pre-BötC, or electrically silencing a critical subpopulation of pre-BötC neurons, can lead to irregular breathing and/or terminal apnea (Ramirez *et al.*, 1998; Gray *et al.*, 2001; Wenninger *et al.*, 2004; McKay *et al.*, 2005; McKay & Feldman, 2008; Tan *et al.*, 2008).

The pre-BötC contains largely glutamatergic inspiratory neurons (Guyenet *et al.*, 2002) that are synaptically synchronized to discharge bursts of action potentials that trigger inspiration. AMPA receptors appear to be required for inspiratory rhythm generation, as blocking AMPA receptors abolishes the respiratory rhythm generated by the pre-BötC, *in vitro* (Funk *et al.*, 1993; Koshiya & Smith, 1999; Thoby-Brisson & Ramirez, 2001; Ireland *et al.*, 2008). In addition, activating AMPA receptors depolarizes pre-BötC inspiratory neurons and increases respiratory rhythm frequency (Greer *et al.*, 1991; Funk *et al.*, 1993). In contrast, NMDA receptors do not appear to play an essential role in the inspiratory rhythm generated by the pre-BötC (Funk *et al.*, 1997). Nonetheless, ionotropic GluR (AMPA and NMDAR) and metabotropic GluR (mGluR) are hypothesized to cooperatively activate non-specific cation currents (ICAN) and generate depolarizing inspiratory drive potentials that trigger rhythmic bursts of action potentials in inspiratory pre-BötC neurons. This cooperative synaptic activation of an intrinsic burst-generating current (ICAN) forms the basis of the 'group pacemaker' burst generating mechanism that *may* underlie inspiratory rhythm generation in mammals (Pace *et al.*, 2007a; Del Negro *et al.*, 2010). Thus, ICAN is a predominant, intrinsic, inspiratory burst-generating current, that is coupled to mGluR (Pace *et al.*, 2007a) which could satisfy the 'group pacemaker' mechanism (Del Negro & Hayes, 2008). However, the functional role of mGluR activation in respiratory network activity is poorly understood (Lieske & Ramirez, 2006; Mironov, 2008). Here, we use brainstem respiratory slice preparations containing the pre-BötC network to identify the role of group I mGluR (mGluR1 and mGluR5) in the respiratory rhythm generated by the pre-BötC. Both mGluR1/5 are Gαq/11 protein-coupled receptors that can trigger signaling pathways that activate transient receptor protein canonical (TRPC3/7) channels which carry ICAN in pre-BötC neurons (Ben-Mabrouk & Tryba, 2010). Here, we demonstrate that mGluR5 activates TRPC channels to govern the cycle-by-cycle variability of the inspiratory rhythm generated by the pre-BötC network. Interestingly, mGluR1 modulates the frequency of the inspiratory rhythm, without affecting the regularity of the respiratory rhythm. Importantly, our data indicate that modulating mGluR5 activation can reduce irregular respiratory rhythms that can underlie morbidity and mortality associated with central respiratory disorders.

Materials and Methods

In vitro respiratory brain slice preparations

All experiments conformed to the guiding principles for the care and use of animals approved by the National Institutes of Health (U.S.A.) and the Internal Animal Care and Use Committee at the Medical College of Wisconsin. All experiments used the transverse, rhythmic 600μm thick respiratory brain-slice obtained from the medulla of 8–11 day old (P8–P11) CD-1 outbred mice (Charles River Laboratories, Wilmington, MA). CD-1 mice were quickly decapitated at the C3/C4 spinal level and the brain-stem was dissected in ice cold artificial cerebral spinal fluid (ACSF) that was equilibrated with carbogen (95% O₂ and 5% CO₂, pH=7.4). The ACSF contained in mM: 118 NaCl, 3 KCl, 1.5 CaCl₂, 1 MgCl₂*6H₂O, 25 NaHCO₃, 1 NaH₂PO₄ and 30 D-glucose, equilibrated with carbogen (95% O₂ and 5% CO₂, pH = 7.4). All ACSF chemicals were obtained from Sigma (St. Louis, MO, U.S.A.). Rhythmic medullary brain slice preparations (600μm thick) containing the ventral

respiratory group (VRG), including the pre-BötC, were obtained by slicing the medulla using a microslicer (Leica, VT1000S, Nussloch, Germany) as described in detail elsewhere (Thoby-Brisson & Ramirez, 2001; Tryba *et al.*, 2003; Chevalier *et al.*, 2008). The medulla was serially sliced, from rostral to caudal, until the rostral boundary of the pre-BötC was identified by anatomical landmarks such as disappearance of the facial nucleus and appearance of the inferior olive, the nucleus ambiguus and the hypoglossal nucleus (Fig. 1). A single 600µm thick slice was then cut and used for study. Respiratory brain-slices were submerged in a recording chamber (6 mL) under circulating oxygenated ACSF (30°C; flow rate 17 ml/min, total circulating volume = 200mL). Circulating bath ACSF was saturated with carbogen (95% O₂ and 5% CO₂, pH = 7.4). Before commencing electrophysiological recordings, extracellular KCl was elevated from 3mM to 8mM over a span of 30 minutes to maintain rhythmic population activity (Tryba *et al.*, 2003). Note that raising ACSF [K⁺]_o does not artificially introduce intrinsic bursting properties; intrinsic bursting neurons show similar bursting properties in 3mM versus 8mM [K⁺]_o ACSF (Tryba *et al.*, 2003). Further, it should be noted that [K⁺]_o changes on a breath by breath basis, however these changes in [K⁺]_o do not obviously alter the form of respiratory activity generated, as both eupneic and gasping activities can be recorded in hypokalemic and hyperkalemic conditions *in situ* (St.-John *et al.*, 2005). As temperature can alter respiratory activity (Tryba & Ramirez, 2003; Tryba & Ramirez, 2004), bath temperature was monitored and maintained at 30°C ± 0.7°C using a Warner Instrument Corp. (Hamden, CT) TC-344B temperature regulator with an in-line solution heater (SH-27B); bath temperature at various locations within the bath was uniform.

Electrophysiology—Extracellular recordings were obtained with glass suction electrodes positioned on the slice surface in the ventral respiratory group (VRG) nearby, or on top of, the pre-BötC (Figs. 1A, 1B). The extracellular signals were amplified 10,000-fold and filtered between 0.25 and 1.5 kHz using a pre-amplifier (built by JFIE electronics at The University of Chicago, Chicago, IL, U.S.A.) and a Model P-55 A.C. amplifier (Grass Instruments Technologies, Astro-Med, Inc., West Warwick RI, U.S.A.). The multi-unit population activity was rectified and integrated with an electronic integrator with a time constant of 70ms (JFIE electronics, The University of Chicago, Chicago, IL, U.S.A.). The VRG population bursting is dominated by inspiratory neurons such that integrated VRG (∫VRG) activity is in-phase with integrated hypoglossal nerve (∫XII) activity (Tryba *et al.*, 2006; Chevalier *et al.*, 2008), which serves to open the upper airways during inspiration. Thus, ∫VRG population bursts serve as a marker of fictive inspiration (Fig. 1)(Tryba *et al.*, 2006; Chevalier *et al.*, 2008). The VRG population activity was rectified and integrated and the data were digitized with a Digidata acquisition system (Molecular Devices, CA), stored on an IBM compatible personal computer using Axoscope 10 (Molecular Devices, CA) software and analyzed off-line using customized analysis software written with Igor Pro (WaveMetrics, Lake Oswego, OR).

After control recordings were made over a period of 10 mins, group I mGluR - selective agonist or antagonists (obtained from Tocris Biosciences, Ellisville, MO, USA) were bath-applied. The group I mGluR agonist, DHPG (3,5-Dihydroxyphenylglycine) was bath-applied at 20µM. The mGluR5-selective antagonist (Salah & Perkins, 2008), MPEP ((S)-(+)-α-Amino-4-carboxy-2-methylbenzeneacetic acid, Tocris Biosciences, Ellisville, MO, USA) was bath-applied (or microinjected) at 20µM, while the mGluR1 selective (Salah & Perkins, 2008) antagonist, LY 367385 (Tocris Biosciences), was bath-applied at 100µM. The transient receptor potential canonical (TRPC) channel antagonist, SKF-96365 (1-[2-(4-Methoxyphenyl)-2-[3-(4-methoxyphenyl)propoxy]ethyl-1H-imidazole hydrochloride (Tocris Biosciences) was bath-applied at 10µM. At the end of some experiments, the persistent sodium current (INaP) blocker, riluzole (10 µM, Sigma) was added to the perfusate. Riluzole has been widely used to block INaP in rhythmic neurons in networks

involved in rhythm generation for breathing, locomotion, slow-wave neocortical oscillations and epilepsy (Mao & Wang, 2001; Bazhenov *et al.*, 2004; Pena *et al.*, 2004; van Drongelen *et al.*, 2006; Zhong *et al.*, 2007; Ben-Mabrouk & Tryba, 2010; Tryba *et al.*, 2011). That said, it should be noted that riluzole can have a variety of effects in different systems, especially when used at concentrations greater than 20 μM (O'Neill *et al.*, 1997; Siniscalchi *et al.*, 1997; Stefani *et al.*, 1997; Zona *et al.*, 1998; Peña & Tapia, 2000; Jan *et al.*, 2002). For example, with an EC_{50} of 150 μM , riluzole causes a rapid and sustained increase in Ca^{2+} in human osteosarcoma cells (Jan *et al.*, 2002). Furthermore, at concentrations between 40 μM – 50 μM , riluzole accelerates calcium channel activation kinetics, without altering activation voltage-dependency (Huang *et al.*, 1997). Note that in this study riluzole 10 μM was applied within the range of concentrations (0.5 μM – 20 μM) that was previously shown to preferentially block INaP (Urbani & Belluzzi, 2000; Del Negro *et al.*, 2002).

Superfusion of MPEP into the pre-BötC of the ventral respiratory group (VRG)

We compared the effects of bath applying the mGluR5 antagonist (MPEP) versus superfusion of MPEP into the pre-BötC region, on the $\int$ VRG inspiratory rhythm. To do this, we unilaterally superfused MPEP (20 μM at 3–5 p.s.i.) into the pre-BötC region, using a micropressure ejection system (PV-820 Pneumatic PicoPump, World Precision Instruments, Sarasota, FL, U.S.A.). Pipettes (with 30–50 μm tip diameters) used for superfusion were fabricated from capillary glass and filled with either: 1) 8mM $[\text{K}^+]_o$ ACSF containing MPEP (20 μM); or, 2) 8mM $[\text{K}^+]_o$ ACSF. During the superfusion protocol, ejection pipettes were placed just above ($\sim 100 \mu\text{m}$) the surface of the tissue, adjacent to the VRG population electrode. Polystyrene fluorescent microspheres (0.2 μm yellow-green FluoSpheres, Invitrogen, Molecular Probes, Eugene, OR, U.S.A.) were included in superfusion solutions and used to visibly monitor the direction and flow of injected solutions.

Intracellular patch-clamp recordings of inspiratory pre-BötC neurons

Intracellular whole-cell current-clamp recordings were obtained with a MultiClamp 700B amplifier (Molecular Devices), applying the blind-patch technique to VRG neurons in brain-stem slice preparations (Thoby-Brisson & Ramirez, 2001). Patch electrodes were manufactured from filamented borosilicate glass tubes (Clark G150F-4; Warner Instruments Corp., Hamden, CT, USA) and filled with an intracellular solution containing (in mM): 140 K-gluconic acid, 1 $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$, 10 EGTA, 2 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 4 Na₂ATP and 10 HEPES. Only inspiratory VRG neurons active in-phase with the $\int$ VRG population burst were recorded in this current-clamp study. The discharge pattern of each cell type was first identified in the cell-attached mode and remained similar in whole-cell configuration (Pena *et al.*, 2004; Tryba *et al.*, 2008b). Experiments were then performed in the whole-cell patch-clamp mode. Membrane voltage (V_m) values were corrected for the liquid junction potential as calculated using pClamp 10 software (Molecular Devices).

Western blots of mGluR1/5 expression in the ventral respiratory group (VRG)

In order to determine the expression of group I mGluR (mGluR1 and mGluR5) in the VRG, we made rhythmic, 600 μm thick, ventral respiratory group (VRG)-island (Johnson *et al.*, 2001) preparations containing the pre-BötC (n=4 for mGluR5 expression, n=3 for mGluR1 expression). The VRG-island preparation (Johnson *et al.*, 2001) was made by taking a medullary brain-slice preparation (described above) and cutting a wedge-shaped piece of tissue containing the pre-BötC out of the transverse brain-slice using a sharp scalpel blade (Tryba *et al.*, 2008a; Ben-Mabrouk & Tryba, 2010). The extent of the VRG-island/pre-BötC to be removed was determined by delineating the VRG borders with population electrode mapping on the surface of the slice prior to cutting out the VRG-island (Tryba *et al.*, 2008a; Ben-Mabrouk & Tryba, 2010). We also isolated the cortex and cerebellum, as a positive control for group I mGluR expression. Tissues were homogenized in a 10mM potassium

phosphate buffer, pH=7.7, containing a protease inhibitor cocktail CompleteMini tablet 400 (0.36mg/mL) (Roche Applied Science, Indianapolis, IN, USA) added and dissolved at time of use. The lysates were collected after centrifugation (at 4°C, 4600 r.p.m., for 15 mins.) and subjected to electrophoresis using a 10% Tris-HCl Ready Gel (Bio-Rad Labs, Hercules, CA, USA). Each gel lane was loaded with 15µg/well total protein, based on A280 protein concentration. Following electrophoresis, proteins were then transferred *via* standard Western blot to a nitrocellulose membrane (Bio-Rad Labs, USA). The membranes were blocked overnight at +4°C with 2% non-fat dried milk (NFDM) (Bio-Rad Labs, Hercules, CA, USA) and 2% BSA (Sigma Aldrich, Milwaukee, WI, USA) in Tris buffered saline, pH = 7.5, containing 0.1% Tween-20 (TBS-T) buffer and immuno-blotted for 2h at room temperature with either anti-mGluR5 antibody (1:400) (Abcam, Cambridge, MA, USA), anti-mGluR1 antibody (1:800) (Alomone labs, Jerusalem, Israel), or anti-GAPDH antibody (1:1000, Abcam, USA) in 2% non-fat dried milk in TBS-T buffer. The secondary antibody, goat anti-rabbit-HRP (1:10,000) (Santa Cruz, CA, USA) containing 2% BSA was incubated in TBS-T buffer for 1h at room temperature. Membranes were developed using enhanced chemiluminescence (Pierce Super-signal West Pico, Thermo Fisher Scientific, Pittsburgh, PA, USA) on X-ray film (Phenix Research Products, Candler, NC, USA).

Data analysis and statistics

To measure $\int$ VRG network or inspiratory neuron bursting regularity, we calculated an irregularity score by applying a formula for consecutive cycle length values: $S_n = 100 * \frac{ABS(P_n - P_{n-1})}{P_{n-1}}$, where S_n = score of the n th cycle, P_n being its period, P_{n-1} the period of the preceding burst and ABS the absolute value (Barthe & Clarac, 1997; Telgkamp *et al.*, 2002; Viemari *et al.*, 2005; Chevalier *et al.*, 2008; Ben-Mabrouk & Tryba, 2010). Regular rhythms have lower irregularity scores and less cycle-by-cycle variability than irregular rhythms, which have higher irregularity scores and more cycle-by-cycle variability (Telgkamp *et al.*, 2002; Chevalier *et al.*, 2008; Ben-Mabrouk & Tryba, 2010). For comparison, we calculated the irregularity score of the $\int$ VRG and inspiratory neurons (over a period of 5mins) in control and following experimental manipulation. The $\int$ VRG and inspiratory neuron burst area were measured as the area between the baseline and the burst envelope, while frequency was calculated based on the burst peak intervals. To minimize the potential influence of baseline fluctuations and differences in burst peak trajectories, the VRG and inspiratory neuron burst duration was calculated as the duration of the population burst at half-maximal burst amplitude. The inspiratory drive potential burst area or population burst area is calculated as the product of voltage (V) * dt (V*dt) under the inspiratory burst (see also, Pace *et al.*, 2007b). Data were analyzed off-line using custom written analysis tools within Igor Pro software (WaveMetrics, Lake Oswego, OR) and statistical analysis was performed on raw data with GraphPad Prism 5.03 (GraphPad Software, LaJolla, CA, USA) or SigmaPlot 11.0 software (SPSS, ASC GmbH, Erkrath, Germany). All quantitative data are given in the text in the form of mean $\pm$ standard error mean (S.E.M.), significance was assessed with paired Student's t-test and significance was assumed for values $p < 0.05$. Regularity measures and burst areas are measured in arbitrary units throughout. Percent changes in measured parameters are represented in the figures.

Results

Exogenous activation of group I mGluR (mGluR1/5) enhances inspiratory rhythm activity via TRPC3/7

Metabotropic glutamate receptor (mGluR) activation is thought to play a role in respiratory rhythm generation within the parafacial respiratory group/retrotrapezoid nucleus (pFRG/RTN) and pre-BötC, as distinct mGluR subtypes differentially modulate the excitability of respiratory motoneurons and interneurons. Both subtypes of group I mGluR (mGluR1 and

5) are proposed to contribute to endogenous inspiratory drive potentials in pre-BötC neurons (Pace & Del Negro, 2008). However, using immunoreactivity, it has been suggested that mGluR1, but not mGluR5 are expressed in the ventrolateral medulla (Hay et al., 1999). To further confirm the expression of group I mGluR (1 and 5) in the region of the medulla containing the inspiratory network, we made western blots prepared from rhythmic VRG-Island preparations (Johnson *et al.*, 2001; Ben-Mabrouk & Tryba, 2010) from postnatal day 8 to 10 (P8–P10) mice (Figs. 1B₁–1B₂); these data verified the expression of mGluR5 (Fig. 1C₁) and mGluR1 (Fig. 1C₂) in the VRG region containing the pre-BötC. As positive controls for the antibodies used, western blots included cortical and cerebellar tissues isolated from the same mice as VRG-islands (Figs. 1C₁–1C₂).

While mGluR1 and mGluR5 are expressed in the VRG, the functional role of mGluR in respiratory rhythm generation is poorly understood. Here, we used the medullary respiratory brain-slice preparation from mice containing the pre-BötC (Fig. 1) to test the role of mGluR activation in pre-BötC respiratory rhythm generation. Fictive inspiratory bursts were recorded from respiratory brain-slice preparations under control conditions (ACSF; Fig. 2A₁) and following subsequent bath-application of the group I mGluR (mGluR1/5) agonist, DHPG (20 μM; Fig. 2A₂). DHPG decreases the irregularity of the $\int$ VRG inspiratory rhythm [the irregularity score was 0.33 ± 0.02 before and 0.27 ± 0.03 after DHPG, $n=12$, $t=3.46$, $df=9$, $p=0.007$, paired t-test]. DHPG also decreases the burst area [from 0.62 ± 0.15 to 0.50 ± 0.13 , $n=12$, $t=2.93$, $df=11$, $p=0.014$, paired t-test], burst duration [from $0.45s \pm 0.03s$ to $0.38s \pm 0.02s$, $n=12$, $t=3.88$, $df=11$, $p=0.003$, paired t-test] and it increases the rhythm frequency [from $0.18Hz \pm 0.03 Hz$ and $0.31 Hz \pm 0.06 Hz$ $n=12$, $t=3.17$, $df=11$, $p=0.009$, paired t-test] (Fig. 2A₃). A similar DHPG-mediated enhancement in the $\int$ VRG inspiratory rhythmic activity has also been shown by Mironov (Mironov, 2008).

Group I mGluR (mGluR1/5) -mediated signaling activates ion channels carrying a Ca^{2+} -activated non-selective cation current (ICAN) (Dong *et al.*, 2009). ICAN is proposed to play a major role in $\int$ VRG inspiratory rhythm generation in the pre-BötC, as it underlies the majority of the intrinsic membrane depolarization contributing to inspiratory drive potentials in pre-BötC neurons (Pace *et al.*, 2007a) and can underlie intrinsic bursting activity (Pena *et al.*, 2004; Pace *et al.*, 2007a; Ben-Mabrouk & Tryba, 2010). Group I mGluR-mediated signaling activates ICAN carrying TRP channels (Mironov, 2008), including TRPC3/7 (Berg *et al.*, 2007). In particular, TRPC3/7 channels are expressed in the pre-BötC (Ben-Mabrouk & Tryba, 2010) wherein their activation can underlie intrinsic bursting and they can enhance the regularity of fictive eupneic inspiratory bursts generated by the pre-BötC (Ben-Mabrouk & Tryba, 2010). Thus, after activating group I mGluR (1/5) with DHPG, which decreases irregular $\int$ VRG inspiratory rhythm (Figs. 2A₂, 2A₃), we tested whether subsequent bath-application of the TRPC channel antagonist, SKF-96365 would reverse the DHPG-enhancement of rhythm regularity (Figs. 2B₁, 2B₂). In the presence of DHPG, subsequent co-application of SKF-96365 (10 μM) increases the irregularity of the $\int$ VRG inspiratory rhythm [from 0.28 ± 0.02 to 0.33 ± 0.01 , $n=5$, $t=4.38$, $df=3$, $p=0.022$, paired t-test] without altering the burst area [0.32 ± 0.04 before and 0.29 ± 0.01 after SKF, $n=5$, $t=0.62$, $df=3$, $p=0.58$, paired t-test], burst duration [$0.36s \pm 0.04s$ before and $0.35s \pm 0.04s$ after SKF, $n=5$, $t=2.44$, $df=3$, $p=0.093$, paired t-test] or burst frequency [$0.51Hz \pm 0.13Hz$ before and $0.51Hz \pm 0.16Hz$ after SKF, $n=5$, $t=0.041$, $df=3$, $p=0.97$, paired t-test] (Fig. 2B₂).

The $\int$ VRG inspiratory rhythm, generated by the pre-BötC can be eliminated after bath-applying both an ICAN antagonist and subsequent co-application of persistent sodium current (INaP) antagonist, such as riluzole, or low-concentrations of TTX (Pena *et al.*, 2004; Del Negro *et al.*, 2005; Ben-Mabrouk & Tryba, 2010). Thus, after blocking ICAN-carrying TRPC channels with SKF-96365, we tested whether the remaining $\int$ VRG inspiratory rhythm is riluzole-sensitive. After bath-application of SKF-96365, additionally blocking

INaP with riluzole (10 μ M) completely abolished the $\int$ VRG inspiratory rhythm (Fig. 2C; n=5/5 slice preparations). Taken together, the above data suggest that group I mGluR activates ICAN-carrying TRPC3/7 channels to improve the regularity of the $\int$ VRG inspiratory rhythm.

Endogenous activation of mGluR5 improves inspiratory rhythm regularity

The activation of mGluR has been proposed to play a major role in the generation of the inspiratory rhythm by the pre-BötC (Del Negro & Hayes, 2008; Del Negro *et al.*, 2010). However, bath-application of MPEP, a specific antagonist of mGluR5, did not block the respiratory rhythm generated by the pre-BötC, even after 30min of perfusion (n=8/8, Figs. 3A₁; 3A₂). Additionally blocking mGluR1, with a specific antagonist, LY367385, also did not abolish the inspiratory rhythm (n=8/8, Fig. 3A₃). However, blocking both mGluR5 and mGluR1 increased the irregularity of the inspiratory rhythm [0.30 ± 0.04 before versus 0.38 ± 0.06 after MPEP + LY, n=8, t=2.56, df=6, p=0.043, paired t-test] and decreased the burst frequency [0.25 ± 0.06 before versus 0.22 ± 0.06 after blocking both mGluR1/5, n=8, t=3.10, df=6, p=0.021, paired t-test] (Fig. 3A₄). After blocking mGluR5, additionally blocking mGluR1 did not alter the burst area [$0.35 \pm 0.07 \int V*dt$ before versus $0.34 \pm 0.08 \int V*dt$ after blocking mGluR1/5, n=8, t=0.22, df=7, p=0.83, paired t-test] or the burst duration [$0.36s \pm 0.04s$ before versus $0.35s \pm 0.03s$ after blocking both mGluR1/5, n=8, t=1.24, df=7, p=0.25, paired t-test] (Fig. 3A₄). After blocking group I mGluR 1/5, subsequent additional blockade of the persistent sodium current (INaP) with riluzole completely abolished the $\int$ VRG inspiratory rhythm, for at least 20mins (n=8/8) (Fig. 3B). These data suggest that blocking group I mGluR modulates, but does not abolish, the ongoing inspiratory rhythmic activity generated by the pre-BötC and suggest that mGluR1/5 activation is not required to sustain the inspiratory rhythmic activity. Thus, we next sought to identify the specific contribution of endogenous activation of mGluR5 and mGluR1 to the pre-BötC inspiratory rhythm.

To examine how endogenous activation of mGluR5 influences eupneic inspiratory network activity, after recording the $\int$ VRG inspiratory rhythm under control conditions (ACSF; Fig. 4A₁), endogenous mGluR5 activity was blocked by bath-application of the mGluR5-selective antagonist, MPEP (20 μ M) for 10mins (Fig. 4A₂). Blocking mGluR5 does not eliminate the inspiratory rhythm generated by the VRG (n=15/15) (Fig. 4A₂). However, MPEP application increases the irregularity of inspiratory bursting during fictive eupnea [0.29 ± 0.03 before versus 0.36 ± 0.03 after MPEP, n=15, t=2.98, df=14, p=0.01, paired t-test] and decreases the burst area [$0.32 \pm 0.05 \int V*dt$ before versus $0.29 \pm 0.05 \int V*dt$ after MPEP, n=15, t=2.5, df=11, p=0.03, paired t-test], decreases the burst duration [$0.37s \pm 0.02s$ before versus $0.35s \pm 0.02s$ after MPEP, n=15, t=3.86, df=14, p=0.002, paired t-test] and reduces the rhythm frequency [$0.26Hz \pm 0.03Hz$ before versus $0.22Hz \pm 0.02Hz$ after MPEP, n=15, t=4.9, df=14, p=0.0002, paired t-test] (Fig. 4A₃). Further, after blocking endogenous activation of mGluR5 with MPEP, subsequent co-application of the INaP antagonist, riluzole eliminated the inspiratory rhythm generated by the pre-BötC (n=7/7 slice preparations tested; Figs. 4B).

Bath-application of agonists and/or antagonists can affect cells throughout the respiratory brain-slice preparation. Thus, we next tested the effect of superfusion of MPEP (20 μ M) into the pre-BötC region, on the $\int$ VRG inspiratory rhythm (Fig. 5A). Like bath-application of MPEP (Fig. 4A₃), unilateral superfusion of MPEP within the pre-BötC region (Fig. 5A₂; 5A₃) increases the inspiratory rhythm irregularity [0.11 ± 0.025 before versus 0.31 ± 0.078 after MPEP, n=4 slices, t=3.26, df=20, p=0.005, paired-t-test] and decreases inspiratory burst area (Fig. 5A₃) [$13.2 \pm 0.98 \int V*dt$ before versus $5.7 \pm 0.79 \int V*dt$ after MPEP, n=4, t=8.59, df=28, p<0.001, paired t-test], the inspiratory burst duration (Fig. 5A₃) [$0.79s \pm 0.023s$ before versus $0.69s \pm 0.032s$ after MPEP, n=4, t=3.25, df=23, p=0.004, paired t-test]

and inspiratory rhythm frequency (Fig. 5A₃) [$0.34\text{Hz} \pm 0.025\text{Hz}$ before versus $0.24\text{Hz} \pm 0.027\text{Hz}$ after MPEP, $n=4$, $t=10.8$, $df=21$, $p<0.001$, paired t-test]. In contrast, unilateral superfusion of ACSF (Figs. 5B₁; 5B₂ vehicle control) did not alter the inspiratory rhythm irregularity [0.15 ± 0.025 before versus 0.13 ± 0.016 after ACSF, $t=0.62$, $df=19$, $p=0.59$, paired t-test], frequency [$0.18\text{Hz} \pm 0.008\text{Hz}$ before versus $0.19\text{Hz} \pm 0.0075\text{Hz}$ after ACSF, $t=0.52$, $df=20$, $p=0.61$, paired t-test], burst area [$4.54 \pm 1.46 \int V*dt$ before versus $4.24 \pm 1.25 \int V*dt$ after ACSF, $t=0.73$, $df=17$, $p=0.473$, paired t-test], or burst duration [$0.72\text{s} \pm 0.018\text{s}$ before versus $0.74\text{s} \pm 0.017\text{s}$ after ACSF, $t=0.66$, $df=20$, $p=0.52$, paired t-test] (Fig. 5B₃). These data indicate that MPEP penetrated the tissue enough to significantly alter the respiratory rhythm, whereas application of the control solution (no MPEP) did not alter the rhythm.

Blocking mGluR1 reduces the frequency of the inspiratory rhythm

To examine the contribution of mGluR1 to the inspiratory rhythm, we selectively blocked mGluR1 by bath-application of LY367385 ($100\mu\text{M}$) during fictive eupneic inspiratory activity generated by the pre-BötC. Blocking mGluR1 did not abolish the $\int\text{VRG}$ inspiratory rhythm ($n=7/7$) (Figs. 6A₁; 6A₂). However, it decreased the $\int\text{VRG}$ inspiratory rhythm frequency [$0.29 \pm 0.03\text{Hz}$ before versus $0.22 \pm 0.05\text{Hz}$ after LY367385, $n=7$, $t=4.50$, $df=6$, $p=0.004$, paired t-test], without altering the inspiratory rhythm regularity [0.37 ± 0.06 before versus 0.37 ± 0.07 after LY367385, $n=7$, $t=0.080$, $df=6$, $p=0.94$, paired t-test], the burst area [$0.41 \pm 0.01 \int V*dt$ before versus $0.37 \pm 0.08 \int V*dt$ after LY367385, $n=7$, $t=0.73$, $df=6$, $p=0.49$, paired t-test] or the burst duration [$0.41\text{s} \pm 0.04\text{s}$ before versus $0.41\text{s} \pm 0.03\text{s}$ after LY367385, $t=0.14$, $df=6$, $p=0.50$ paired t-test] (Fig. 6A₃).

Endogenous mGluR5 activation increases the regularity and frequency of inspiratory neuronal rhythmic activity

We also studied the effect of blocking group I mGluR (mGluR5 and mGluR1) on inspiratory neurons during fictive eupneic activity. During fictive inspiratory rhythmic activity (Fig. 7A₁), we first bath-applied $20\mu\text{M}$ MPEP for 20 min (Figs. 7A₂; 7A₃). Blocking endogenous activation of mGluR5 with MPEP increases inspiratory neuron irregularity [0.45 ± 0.11 before versus 0.50 ± 0.09 after MPEP, $n=9$, $t=3.870$, $df=8$, $p=0.005$, paired-t-test] and decreases inspiratory neuron frequency [$0.17\text{Hz} \pm 0.02\text{Hz}$ before and $0.13\text{Hz} \pm 0.02\text{Hz}$ after MPEP, $n=9$, $t=4.34$, $df=8$, $p=0.003$, paired t-test] (Fig. 7A₃) and reduces the inspiratory neuron burst area [$8.16 \pm 2.88 \int V*dt$ before versus $4.93 \pm 1.81 \int V*dt$ after MPEP, $n=9$, $t=2.78$, $df=8$, $p=0.02$, paired t-test; Fig. 7A₃], but did not significantly alter inspiratory neuron burst duration [$0.38\text{s} \pm 0.06\text{s}$ before versus $0.33\text{s} \pm 0.05\text{s}$ after MPEP, $n=9$, $t=1.90$, $df=8$, $p=0.094$, paired t-test] (Fig. 7A₃).

These data suggest that endogenous activation of mGluR5 enhances inspiratory rhythm regularity and increases the rhythm frequency. After 20 minutes in MPEP, in a subset of $n=7$ experiments, we subsequently blocked mGluR1 by additional co-application of LY367385 (Fig. 7A₄). LY367385 did not further disturb inspiratory neuron regularity [0.37 ± 0.04 before versus 0.39 ± 0.07 after LY367385, $t=0.19$, $df=6$, $p=0.85$ paired t-test], alter inspiratory neuron burst area [$5.15 \pm 2.31 \int V*dt$ before versus $4.84 \pm 2.38 \int V*dt$ after LY367385, $n=7$, $t=1.27$, $df=6$, $p=0.25$ paired t-test], or change inspiratory neuron burst duration [$0.29\text{s} \pm 0.06\text{s}$ before versus $0.24\text{s} \pm 0.05\text{s}$ after LY367385, $n=7$, $t=0.54$, $df=6$, $p=0.61$, paired t-test] or inspiratory neuron burst frequency [$0.15\text{Hz} \pm 0.02\text{Hz}$ and $0.15\text{Hz} \pm 0.02\text{Hz}$ before versus after LY367385, $t=0.013$, $df=6$, $p=0.99$, paired t-test] (Fig. 7A₅). Neither blocking mGluR5 with MPEP (Fig. 7A₂, $n=7$ slice preparations), or additionally blocking mGluR1 with LY367385 eliminated inspiratory neuron bursting activity (Fig. 7A₄). However, subsequent co-application of riluzole stopped the network and inspiratory

neuron bursting activity (Fig. 7B, $n = 7$ slices preparations; in $n = 7/7$, and no rhythmic activity was recorded for > 17 min).

Discussion

mGluR5 signaling improves inspiratory rhythm regularity via TRPC channel activation

The cooperative synaptic activation of ICAN has formed the basis of the 'group pacemaker' burst generating mechanism, that *may* underlie inspiratory rhythm generation in mammals (Pace *et al.*, 2007a; Del Negro *et al.*, 2010). The activation of group I mGluR receptors in the recruitment of ICAN (Pace *et al.*, 2007a) could possibly satisfy the 'group pacemaker' mechanism (Del Negro & Hayes, 2008). However, our data suggest that activation of group I mGluR (1 and/or 5) receptors is not essential to generate the fictive eupneic inspiratory rhythm, *in vitro*. For example, after simultaneously blocking both mGluR1 and mGluR5, the inspiratory rhythm continues. These data are expected – given that multiple parallel synaptic pathways can activate non-specific cation currents (ICAN) (e.g., Substance P and mGluR signaling (Ben-Mabrouk and Tryba, 2010; Pace *et al.*, 2007a; Dong *et al.*, 2009)), and prior research suggests that blocking ICAN alone is not sufficient to eliminate the fictive inspiratory rhythm generated *in vitro* (Pena *et al.*, 2004; Ben-Mabrouk & Tryba, 2010). However, blocking both ICAN and the persistent sodium current (INaP) abolishes the inspiratory rhythm (Pena *et al.*, 2004; Ben-Mabrouk & Tryba, 2010). Thus, after blockade of group I mGluR, to reduce ICAN, we observed that subsequent additional blockade of INaP abolished the inspiratory rhythm *in vitro*. These data support the hypothesis that both INaP and ICAN play an important role in the generation of the inspiratory rhythm, although they (INaP and ICAN) appear to contribute in different ways. For example, both ICAN and INaP dependent intrinsically bursting neurons have been proposed to play a major role in inspiratory rhythm generated by the pre-BötC during eupneic-like activity *in vitro* (Pena *et al.*, 2004), while INaP-dependent intrinsic bursting neurons are proposed to play a more important role in the generation of fictive gasping during severe hypoxia (Pena *et al.*, 2004; Tryba *et al.*, 2006; Peña & Aguileta, 2007; Viemari *et al.*, 2011). Additionally, during fictive eupnea, INaP promotes intraburst spiking in regular-firing (non-intrinsically bursting) pre-BötC inspiratory neurons, but does not significantly contribute to the depolarization that underlies inspiratory bursts (the inspiratory drive potential) (Pace *et al.*, 2007b). In contrast to INaP, ICAN appears to be largely synaptically recruited during ongoing bursting, where ICAN significantly contributes to inspiratory drive potentials (Pace *et al.*, 2007a) to reliably trigger bursting in inspiratory pre-BötC neurons. Thus, synaptic recruitment of ICAN appears to ensure that rhythmic bursting occurs at regular intervals – as blocking ICAN results in irregular inspiratory activity (see also (Ben-Mabrouk & Tryba, 2010)).

Both ionotropic glutamate receptors and mGluR are proposed to periodically recruit the post-synaptic, burst-generating conductance ICAN, that produces robust inspiratory bursts (Pace *et al.*, 2007a). It is estimated that group I mGluRs are responsible for approximately 40–50% of the inspiratory drive potentials that trigger bursts of action potentials during endogenous rhythmic activity *in vitro* (Pace *et al.*, 2007a). However, the role of group I mGluR in respiratory network activity and function is poorly understood. Our data suggest that there is differential regulation of inspiratory network activity by mGluR1 and mGluR5. Blocking endogenous activation of mGluR1 (*via* LY-367385) suppresses the $\int$ VRG inspiratory rhythm frequency, without significantly altering the area, duration, or regularity. Thus, our data suggest that the frequency and regularity of CPG network activity can be independently modulated. Further, in contrast to mGluR1 modulation, blocking endogenous mGluR5 activity has more profound effects, as it results in a slower, shorter duration and more irregular inspiratory rhythm. Further, our data suggest mGluR5 activation is likely to play a critical role in regulating the breath-to-breath variability of the respiratory cycle.

Increasing evidence suggests that TRP channels play an important role in the generation of rhythmic activity in several systems, including the interstitial cells of Cajal in the gastrointestinal tract (Kim *et al.*, 2006), the sinoatrial node of the heart (Ju & Allen, 2007), dopaminergic neurons of the substantia nigra (Mrejeru *et al.*, 2011) and inspiratory ICAN-dependent intrinsic bursting neurons (Ben-Mabrouk & Tryba, 2010). Activation of TRP-channels appears to underlie ICAN-dependent bursting activity in pre-BötC neurons and can govern the regularity of fictive eupnea (Ben-Mabrouk & Tryba, 2010). Here, our data suggest that activation of mGluR group I, improves the regularity of the fictive eupneic inspiratory rhythm *via* activation of TRPC channels. Activation of mGluR5 triggers the opening of ICAN-carrying TRPC3/7 channels (Berg *et al.*, 2007) that are expressed in the VRG, which can reduce irregular respiratory activity generated by the pre-BötC (Ben-Mabrouk & Tryba, 2010). Accordingly, blockade of mGluR5 (with MPEP) reduces the ICAN-dependent component of inspiratory drive potentials (Pace *et al.*, 2007a) and (here) results in irregular fictive eupneic inspiratory rhythms. In contrast, blocking endogenous mGluR1 activation (with LY367385) reduces the frequency of inspiratory activity, but has no significant effect on regularity of fictive eupneic activity. Taken together, these data suggest that modulating mGluR5 signaling can reduce irregular respiratory rhythms generated by the pre-BötC *via* activation of TRPC3/7 channels.

Accumulating data suggests that TRP channel activation underlies ICAN (Ben-Mabrouk & Tryba, 2010) and that TRP channels contribute the majority of the intrinsic membrane depolarization during inspiratory drive potentials (Pace & Del Negro, 2008) in non-pacemaker inspiratory pre-BötC bursts, as well as ICAN-dependent intrinsically bursting neurons (Ben-Mabrouk & Tryba, 2010). In short, our data suggest that activation of ICAN-carrying TRP channels can govern the regularity of pre-BötC inspiratory activity, as both: 1) substance P modulation of TRPC3/7 channels (Ben-Mabrouk & Tryba, 2010); and, 2) mGluR5 modulation of TRPC3/7 channels enhances inspiratory rhythm regularity. Taken together, our data suggest there is convergence of multiple synaptic mechanisms that can activate ICAN-carrying TRP channels to boost neuronal excitability during burst generation. The synaptic activation of TRP-channels appears to amplify excitatory synaptic drive, providing a mechanism to ensure triggering inspiratory bursts and thereby reduce the cycle-by-cycle variability of inspiratory rhythms. As such, modulation of TRP channels may be a novel therapeutic target to reduce irregular breathing patterns, such as those clinically observed in disorders of central respiratory control.

While blocking mGluR5 significantly reduces inspiratory population burst area and duration, it should be noted that because multiple synaptically-mediated pathways (e.g. mGluR, SubP) can activate TRP channels, we would not necessarily expect that blocking mGluR5 alone would have a dramatic effect on inspiratory population burst area and/or duration. However, blocking mGluR5 resulted in a dramatic increase in the irregularity of the respiratory rhythm, suggesting that endogenous activation of mGluR5 can function to ensure reliable triggering of inspiratory activity at regular intervals.

Activation of mGluR5 enhances the regularity of pre-BötC inspiratory activity

In mammals, the pre-BötC initiates inspiratory efforts at regular intervals that are essential to sustain terrestrial life. Several studies suggest that the pre-BötC neural network is essential for eupnea (Ramirez *et al.*, 1998; Gray *et al.*, 2001; Wenninger *et al.*, 2004; McKay *et al.*, 2005; McKay & Feldman, 2008; Tan *et al.*, 2008). Within the pre-BötC, glutamate mediated AMPA-ergic synaptic transmission is critical for inspiratory rhythmogenesis, as blockade of AMPAR eliminates pre-BötC inspiratory activity (Funk *et al.*, 1993; Koshiya & Smith, 1999; Thoby-Brisson & Ramirez, 2001; Ireland *et al.*, 2008). However, metabotropic glutamate receptor (mGluR) activation is also thought to play an essential functional role in inspiratory rhythmogenesis within the parafacial respiratory group and/or retrotrapezoid

nucleus (pFRG/RTN) and pre-BötC, wherein distinct mGluR subtypes differentially modulate excitability of respiratory motoneurons and interneurons (Nattie & Li, 1995a; Nattie & Li, 1995b; Hay *et al.*, 1999; Mironov & Richter, 2000; Pace *et al.*, 2007a; Mironov, 2008). In particular, the activation of mGluR has been hypothesized to strongly influence pre-BötC network activity patterns. For example, prior studies found that activation of mGluR group III (subtype GluR 8) in the pre-BötC is critical to the generation of fictive sigh (sigh-like) activity (Lieske & Ramirez, 2006). Further studies proposed that group I mGluR (mGluR1/5) mediated signaling triggers activation of ICAN, the latter of which significantly contributes to inspiratory drive potentials in pre-BötC neurons (Pace *et al.*, 2007a; Mironov, 2008), as well as intrinsic bursting activity (Pena *et al.*, 2004; Ben-Mabrouk & Tryba, 2010). Here, we show mGluR5 activation, rather than mGluR1, plays a major role in modulating cycle-to-cycle variability of the inspiratory rhythm generated by the pre-BötC. We further clarify the roles of mGluR1 versus mGluR5 in modulating the inspiratory rhythm generated by the pre-BötC. These data suggest that activation of mGluR5 among pre-BötC neurons enhances the regularity of pre-BötC inspiratory activity.

Differential group I (mGluR1/5) modulation of the regularity of fictive eupnea

Mironov (2008) observed an increase in inspiratory drive potentials/currents in pre-BötC inspiratory neurons and an enhancement of fictive eupneic hypoglossal (XII) burst frequency following application of DHPG, a group I (mGluR1/5) agonist. Further, selectively blocking mGluR1 (with LY-367385) decreases inspiratory drive currents and decreases fictive eupneic inspiratory burst frequency (Mironov, 2008). We extend these findings to indicate that although inspiratory drive currents in pre-BötC neurons are enhanced by mGluR1 activation and reduced following blockade of endogenous mGluR1 activity (Mironov, 2008), these changes did not translate into significant alterations in fictive eupneic ($\int$ VRG) burst area, the burst duration or burst irregularity.

Like mGluR1 antagonists, blockade of mGluR5 activity (with MPEP) also reduces inspiratory drive currents in pre-BötC neurons and reduces fictive eupneic frequency (Mironov, 2008). However, unlike blocking mGluR1, blockade of mGluR5 additionally increases fictive eupneic ($\int$ VRG) burst irregularity. As discussed below, we propose that differential group I (mGluR1/5) modulation of fictive eupnea can be explained by the distinct mechanisms by which mGluR1 versus mGluR5 blockade decreases inspiratory drive potentials/currents in pre-BötC inspiratory neurons.

As suggested by Pace *et al.* (Pace *et al.*, 2007a), insight into mechanism(s) by which mGluR1 activation underlies inspiratory bursting can be gleaned by studying other central pattern generating (CPG) networks, such as lamprey locomotion as well as respiratory activity. For example, activation of mGluR1 contributes to rhythm-related burst generation in lamprey spinal neurons (Kettunen *et al.*, 2002; Kettunen *et al.*, 2003) as well as inspiratory neonatal mice XII motoneuron activity, by blocking K^+ currents (Sharifullina *et al.*, 2004). In pre-BötC inspiratory neurons, mGluR1 activation also enhances inspiratory drive potentials, *via* an IP_3R -independent signaling pathway, by blocking K^+ currents (Pace *et al.*, 2007a). Thus, blocking endogenous mGluR1 activation (with LY-367385) would be expected to enhance K^+ currents and reduce inspiratory burst frequency and, indeed we observed a decrease in burst frequency.

In contrast to mGluR1 blockade, reducing mGluR5 activation with MPEP causes a more pronounced reduction in inspiratory drive potentials *via* an IP_3R -dependent signaling pathway (Pace *et al.*, 2007a) that activates TRP-channels (Crowder *et al.*, 2007; Mironov, 2008). In particular, mGluR5-signaling triggers TRPC3/7 channel activation (Berg *et al.*, 2007) that are expressed in the pre-BötC and whose activation entails IP_3R -dependent signaling pathways that we proposed underlies ICAN-dependent inspiratory pacemaker

activity and additionally improves the regularity of fictive eupnea (Ben-Mabrouk & Tryba, 2010). Here, we also propose that, unlike mGluR1, mGluR5-mediated signaling improves inspiratory rhythm regularity, by: 1) augmenting the intrinsic membrane component of inspiratory drive potentials *via* TRPC3/7 activation; and, 2) triggering TRPC3/7-dependent intrinsic bursting. Thus, we suggest that blocking mGluR5 results in irregular inspiratory rhythmogenesis within the pre-BötC, likely by a combined reduction in: 1) inspiratory drive potentials during inspiratory non-pacemaker neuronal bursting; and, 2) TRPC3/7-dependent intrinsic bursting activity. These data suggest that mGluR5 activates TRPC3/7 channels to recruit inspiratory neuron bursting, which enhances the regularity of the rhythm.

One might expect that activation of mGluR5 and mGluR1 would increase the $\int$ VRG inspiratory burst area and/or duration. However, the mGluR group I (mGluR1 and 5) agonist, DHPG, significantly decreased the $\int$ VRG inspiratory burst area and duration (Fig. 2A3). We speculate that this decrease is due to a DHPG-mediated depolarization of both inspiratory and non-inspiratory neurons, leading to enhanced network excitability. In contrast, activation of mGluR5 would be expected to trigger TRPC3/7 channels particularly during ongoing inspiratory bursts. The net effect, we speculate, results in an increase in network excitability both during the burst and intra-burst periods. The resultant shift in baseline excitability between and during bursts, may account for the overall reduction in inspiratory network burst area and duration measured after DHPG application. After DHPG application, subsequent application of SKF-96365 would be expected to decrease excitability during synaptically-driven bursts, yet blocking TRPC channels with SKF-96365 does not appear to be sufficient to reverse the effects of DHPG on burst area and duration, suggesting that additional mechanisms may be involved (Fig. 2B2).

Effects of group I mGluR (mGluR1/5) modulation of L-type calcium channels on fictive eupnea

Group I mGluRs have been linked to activation of L-type calcium (Ca^{2+}) channels. For example, in the cerebellum, activation of mGluR1 and/or mGluR5 typically leads to Ca^{2+} entry through TRP-channels followed by Ca^{2+} entry through L-type calcium channels (Collin *et al.*, 2009). Group I mGluRs are also linked to activation of L-type Ca^{2+} channels in pre-BötC neurons (Mironov & Richter, 1998). Thus, it is possible that blocking group I mGluR alters the inspiratory rhythm regularity by reducing L-type Ca^{2+} channel activation. However, it should be noted that blocking L-type Ca^{2+} channels (with nifedipine) in pre-BötC neurons has no significant effect on inspiratory drive potentials or XII motor output during fictive eupnea (Pace *et al.*, 2007a). Further, blocking L-type Ca^{2+} channels also has no significant effect on fictive eupneic burst frequency, amplitude or irregularity (Lieske & Ramirez, 2006). Taken together, these data suggest that mGluR5 activation of TRPC3/7 channels, rather than L-type Ca^{2+} channels, can play a major role in modulating the regularity of fictive eupneic inspiratory activity generated by the pre-BötC.

Differential group I mGluR regulation of pre-BötC and hypoglossal inspiratory activation

In the pre-BötC, inspiratory bursts require intact AMPA-R transmission, are synchronized (in part) by electrical coupling and continues in the absence of inhibitory synaptic transmission (Pace & Del Negro, 2008). Both group I mGluRs (mGluR1 and mGluR5) clearly play a role in modulating fictive eupneic bursting generated by the pre-BötC. However, unlike hypoglossal (XII) motoneurons (Sharifullina *et al.*, 2005), rhythmic inspiratory drive potentials in the pre-BötC are more heavily dependent on mGluR5 activation (rather than mGluR1) (Pace *et al.*, 2007a), whose downstream signaling appears to play a major role in rhythm regularity.

Like pre-BötC neurons, inspiratory bursts generated by motoneurons within the hypoglossal nucleus (XII) also require intact AMPA-R transmission, are synchronized by electrical coupling and continue to generate inspiratory activity in the absence of inhibitory synaptic transmission (Sharifullina *et al.*, 2005). However, unlike pre-BötC neurons, XII inspiratory bursts are mediated more by mGluR1 activation (rather than mGluR5) which “paces bursting by cyclic activation of ATP-sensitive K⁺ channels” (Sharifullina *et al.*, 2005). These data suggest that mGluR5 activation (more than mGluR1) plays a stronger role in inspiratory rhythmogenesis within the pre-BötC, whereas mGluR1 activation (rather than mGluR5) more strongly contributes to inspiratory motor outflow.

Important limitations to the current study

Given the differential role of group I mGluR in modulating pre-BötC neuronal activity versus XII motoneuron activity, bath-application of agonists and/or antagonists may have additional effects on cells throughout the respiratory brain-slice preparation. To minimize this issue, our study monitored group I mGluR effects on fictive eupneic activity generated by the inspiratory pre-BötC network (within the VRG), rather than monitoring XII inspiratory activity, as done in some studies. As pre-BötC network activity triggers subsequent inspiratory XII motor patterns (Chevalier *et al.*, 2008), our experiments suggest that mGluR5-mediated enhancement of inspiratory rhythmogenesis within the pre-BötC should improve inspiratory motor activity, but the effects of altering availability of mGluR5 activity within the pre-BötC on breathing activity in a more intact respiratory network remains to be tested.

Using agonists and antagonists, prior studies indicate that both mGluR1 and mGluR5 contribute to inspiratory drive potentials in pre-BötC neurons (Pace & Del Negro, 2008). Here, we also show that both mGluR1 and mGluR5 are expressed in VRG-island preparations, containing the pre-BötC. However, to specifically determine the expression of mGluR1/mGluR5 in inspiratory neurons, a combination of labeling electrophysiologically identified neurons and mGluR immunohistochemistry would be needed to be done.

Our *in vitro* data also suggest that group I mGluR activates ICAN-carrying TRPC channels to improve the regularity of the $\int$ VRG inspiratory rhythm. However, while ICAN has been measured in inspiratory neurons following mGluR1/5 activation elsewhere (Mironov, 2008), a limitation of our current study is that we did not directly measure ICAN in VRG inspiratory neurons during activation of mGluR1/5.

Finally, because endogenous activation of mGluR5 appears to have a more profound effect on the inspiratory rhythm, than mGluR1 signaling, we used superfusion techniques to limit the effects of MPEP on respiratory-related neurons situated outside the pre-BötC and test the role of mGluR5. These data also suggested that mGluR5 activation within the pre-BötC plays an important role in generating a reliable inspiratory rhythm, by reducing irregular inspiratory rhythms. In summary, our data suggest that mGluR1/5 are not essential for inspiratory rhythmogenesis, but mGluR5 plays a major role in activating ICAN, which ensures that rhythmic bursting occurs at regular intervals – as blocking ICAN results in irregular respiratory activity (see also (Ben-Mabrouk & Tryba, 2010)).

Acknowledgments

Authors thank Dr. Catherine Kaczorowski and Sandra Chuppa for editing the manuscript and Sandra Chuppa for technical support. Supported by NIH R01HL079294 and NIH R01HL079294-04S1 (to AKT) from the National Heart, Lung, and Blood Institute.

Abbreviations

pre-BötC	pre-Bötzinger Complex
VRG	Ventral Respiratory Group
mGluR	metabotropic glutamate receptor
TRPC	Transient receptor potential canonical

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Fig. 1. Mouse medullary slice preparations containing the neural network for inspiratory rhythm generation, the pre-Botzinger Complex

A) Transverse brainstem slice preparation with the ventral respiratory group (VRG). **B)** Raw VRG population activity (VRG) is recorded with an extracellular electrode, placed on the surface of the slice (bottom trace) and integrated VRG activity ($\int$ VRG) (top trace). Integrated $\int$ VRG population inspiratory bursting is shown on a longer time scale. **C1)** The rhythmic VRG-island preparation containing the pre-BötC was isolated from the respiratory brainstem slice preparation. Rhythmic $\int$ VRG population activity was recorded from the VRG-island after its isolation from the rest of the brainstem slice (bottom trace). **C2)** Western blots verified the expression of mGluR5 and mGluR1 in the VRG-island, containing the pre-BötC. Cortex and cerebellum tissues served as positive controls for group 1 mGluR expression and GADPH was used as a loading control.

Fig. 2. The group I mGluR (1/5) agonist, DHPG improves the regularity of fictive eupnea and this effect can be reversed by TRPC antagonist, SKF-96365

Integrated VRG activity ($\int$ VRG) with fictive inspiratory bursting, **A1**) during fictive eupneic activity under control conditions, in artificial cerebral spinal fluid (ACSF); **A2**) after bath-application of the group I mGluR (1/5) agonist, DHPG; and **B1**) subsequent co-application of the TRPC channel antagonist, SKF-96365. **A3**) Following bath-application of DHPG, fictive eupneic inspiratory rhythmic bursts become more regular (less irregular), shorter in duration and more frequent (* $P < 0.05$; ** $P < 0.01$, paired t-test). **B2**) Subsequent co-application of the TRPC channel antagonist SKF-96365 reverses the DHPG improvement in rhythm regularity, without significantly changing the burst frequency. Statistical tests were made on raw data. C) After blocking TRPC channels with SKF-96365, subsequent additional blockade of INaP eliminated the inspiratory rhythm.

Fig. 3. Blocking group I mGluR (1 and 5) does not eliminate the inspiratory rhythm, but increases irregular rhythmic bursting and decreases the burst frequency
 Integrated VRG activity ($\int$ VRG) with fictive inspiratory bursting, **A1**) during fictive eupneic activity under control conditions, in artificial cerebral spinal fluid (ACSF), **A2**) after blockade of mGluR5 activation with MPEP and after **A3**) additionally blocking mGluR1 with LY367385. **A4**) In the presence of MPEP and LY36785, the inspiratory rhythm continues, but becomes more irregular, and lower in frequency, relative to that in control (ACSF) conditions ($P^* < 0.05$, paired t-test). **B**) After blocking mGluR1 and mGluR5, subsequent bath application of the persistent sodium current blocker, riluzole eliminates the rhythm. Statistical tests were made on raw data.

Fig. 4. Blocking endogenous activation of mGluR 5 with MPEP results in shorter, less frequent and more irregular fictive eupneic inspiratory bursting

Integrated VRG activity ($\int$ VRG) with fictive inspiratory bursting, **A1**) during fictive eupneic activity, under control conditions, in artificial cerebral spinal fluid (ACSF); and, **A2**) after blocking endogenous mGluR5 with MPEP. **A3**) In the presence of MPEP, the inspiratory rhythm becomes more irregular and the inspiratory burst area, burst duration and frequency are reduced relative to that recorded in control (ACSF) conditions (* $P < 0.05$; *** $P < 0.001$, paired t-test). **A4**) After blocking mGluR5 with MPEP, subsequent bath-application of the persistent sodium current (INaP) antagonist, riluzole eliminated the inspiratory rhythm. Statistical tests were made on raw data.

Fig. 5. Superfusion of MPEP into the pre-BötC region increases inspiratory burst irregularity, decreases burst area, duration and frequency

A) Schematic illustration of unilateral superfusion of MPEP or ACSF (vehicle control) into the pre-BötC, while recording integrated VRG activity ($\int$ VRG) with fictive inspiratory bursting. **A1)** Fictive eupneic activity under control conditions, in artificial cerebral spinal fluid (ACSF); and, **A2)** after blocking endogenous mGluR5 with MPEP. **A3)** In the presence of MPEP, the inspiratory rhythm becomes more irregular and the inspiratory burst area, burst duration and frequency are reduced relative to that recorded in control (ACSF) conditions (* $P < 0.05$, paired t-test). Integrated VRG activity ($\int$ VRG) with fictive inspiratory bursting is shown, **B1)** during fictive eupneic activity under control conditions in artificial cerebral spinal fluid (ACSF); and, **B2)** after superfusion of ACSF (vehicle control). **B3)** In contrast to MPEP, vehicle control injections of ACSF did not significantly alter inspiratory burst regularity, burst area, duration or frequency. Statistical tests were made on raw data.

Fig. 6. Blocking endogenous activation of mGluR 1 with LY367385 results a reduction in the frequency of fictive eupneic inspiratory bursting

Integrated VRG activity ($\int \text{VRG}$) with inspiratory bursting, **A1**) during fictive eupneic activity, under control conditions, in artificial cerebral spinal fluid (ACSF); and, **A2**) after blocking endogenous mGluR1 with LY367385. **B**) In the presence of LY367385, the inspiratory rhythm frequency is reduced relative to that recorded in control (ACSF) conditions (** $P < 0.01$, paired t-test). Statistical tests were made on raw data.

Fig. 7. Blocking mGluR5 with MPEP increases inspiratory neuron burst irregularity and decreases neuron burst frequency while additionally blocking mGluR1 with LY367385 does not further alter these parameters

Fictive eupneic bursts (∫VRG) and inspiratory rhythmic neuron activity recorded in **A1**) control conditions (ACSF) and, **A2**) after blocking mGluR5 with MPEP. **A3**) Blocking mGluR5 with 20μM MPEP increases the inspiratory bursting irregularity, decreases inspiratory burst area and burst frequency without altering the inspiratory bursting duration. **A4**) Subsequent blockade of mGluR1 with LY367385 does not further alter **A5**) the bursting regularity, burst area, burst duration or burst frequency (* $P < 0.05$; ** $P < 0.01$, paired t-test). **B**) The subsequent blockade of the persistent sodium current (INaP) with riluzole abolished the respiratory rhythm activity. Statistical tests were made on raw data.