Inhibitory subpopulations in preBötzinger Complex play distinct roles in modulating inspiratory rhythm and pattern

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Abstract

Inhibitory neurons embedded within mammalian neural circuits shape breathing, walking, chewing, and other rhythmic motor behaviors. At the core of the neural circuit controlling breathing is the preBötzinger Complex (preBötC), a nucleus in the ventrolateral medulla necessary for generation of inspiratory rhythm. In the preBötC, a recurrently connected network of glutamatergic Dbx1-derived (Dbx1$^+$) neurons generates rhythmic inspiratory drive to inspiratory premotoneurons and motoneurons. Functionally and anatomically intercalated among Dbx1$^+$ preBötC neurons are GABAergic and glycinergic neurons, whose functions have not been clearly determined. We first characterized the spatial distribution of molecularly-defined inhibitory preBötC subpopulations in double reporter mice expressing either the red fluorescent protein tdTomato or EGFP in GlyT2$^+$, GAD1$^+$, or GAD2$^+$ neurons. We found that, in postnatal mice, the majority of inhibitory preBötC neurons expressed a combination of GlyT2 and GAD2 while a much smaller subpopulation also expressed GAD1. To determine the functional role of these subpopulations, we used holographic photostimulation, a patterned illumination technique with high spatiotemporal resolution, to specifically excite small groups of GlyT2$^+$ or GAD1$^+$ preBötC subpopulations in rhythmically active medullary slices from Dbx1^{tdTomato};GlyT2^{EGFP} and Dbx1^{tdTomato};GAD1^{EGFP} double reporter mice. Stimulation of 4 or 8 GlyT2$^+$ preBötC neurons during endogenous rhythmic activity prolonged the interburst interval in a phase-dependent manner and increased the latency to burst initiation when bursts were evoked by stimulation of Dbx1$^+$ neurons. In contrast, stimulation of 4 or 8 GAD1$^+$ preBötC neurons did not affect interburst interval or latency to burst initiation, but did prolong both endogenous and evoked burst duration when stimulation occurred during the burst. We conclude that the majority of inhibitory preBötC neurons express both GlyT2 and GAD2 and affect breathing rhythm by delaying burst initiation while a smaller GAD1$^+$ subpopulation affects inspiratory patterning by prolonging burst duration.

Keywords: inhibition, breathing, central pattern generator, motor systems

Introduction

Breathing, walking, and other rhythmic motor behaviors are controlled by networks of excitatory and inhibitory neurons that coordinate and adjust muscle activity to meet physiological demand. At the core of the neural circuit controlling breathing is the preBötzinger Complex (preBötC), a compact nucleus in the ventrolateral medulla that generates breathing rhythm and is hypothesized to organize other rhythmic orofacial movements (Ashhad et al., 2022; Moore et al., 2014; Smith et al., 1991). In the preBötC, a recurrently connected network of glutamatergic Dbx1-derived (Dbx1$^+$) neurons in the preBötC generates rhythmic inspiratory drive to inspiratory premotoneurons and motoneurons (Ashhad et al., 2022; Bouvier et al., 2010; Gray et al., 2010; Koizumi et al., 2008). Also within the preBötC are neurons that are glycinergic (defined by expression of the vesicular glycine transporter GlyT2; GlyT2$^+$) (Koizumi et al., 2013; Morgado-Valle et al., 2010; Winter et al., 2009), and GABAergic (defined by expression of either or both of the two isoforms of glutamic acid decarboxylase, GAD1/GAD67 and GAD2/ GAD65 (Erlander et al., 1991); GAD1/2$^+$; Fig. 2A) (Koizumi et al., 2013; Kuwana et al., 2006). GlyT2$^+$ and GAD1/2$^+$ neurons are functionally and anatomically intercalated among Dbx1$^+$ neurons, with many, but not all, showing inspiratory-modulated activity (Koizumi et al., 2013; Kuwana et al., 2006; Morgado-Valle et al., 2010; Winter et al., 2009). Co-expression of GlyT2 with either GAD2 or GAD1 in preBötC and thus co-release of glycine and GABA is observed postnatally (Hirrlinger et al., 2019; Koizumi et al., 2013; Oke et al., 2023; Rahman et al., 2013). However, a developmental switch leads to many neurons expressing either GlyT2 or GAD2 in adult animals (Hirrlinger et al., 2019; Oke et al., 2023).

Inhibition regulates many aspects of breathing (Baertsch et al., 2018; Dutschmann and Dick, 2012; Ezure and Tanaka, 2004; Ezure et al., 2003; Janczewski et al., 2013; Kubin et al., 2006; Shao and Feldman, 1997; Sherman et al., 2015; Smith et al., 2007) and plays a role in the etiology of Rett syndrome, a neurodevelopmental disorder that affects breathing (Abdala et al., 2010; Chao et al., 2010). However, there are conflicting interpretations over the role of preBötC inhibitory neurons in breathing (Janczewski et al., 2013; Marchenko et al., 2016; Smith et al., 2007). Pharmacological block of GlyT2$^+$ transmission *in vitro* or *in vivo* has minimal effects on respiratory rhythm generation (Janczewski et al., 2013; Shao and Feldman, 1997). However, ~40% of preBötC GlyT2$^+$ neurons show endogenous respiratory-related activity (Koizumi et al., 2013; Morgado-Valle et al., 2010; Winter et al., 2009). Optogenetic stimulation of medullary GlyT2$^+$ neurons *in vivo* delays inspiration, modulates tidal volume, and causes apneas (Ausborn et al., 2018; Fortuna et al., 2019; Hulsmann et al., 2021; Sherman et al., 2015), but has also been shown to increase inspiratory frequency by reducing excitability during inspiration, shortening post-I refractoriness, and post-inhibitory rebound (Baertsch et al., 2018). Optogenetic photoinhibition of these neurons increases tidal volume and shortens expiratory duration (Sherman et al., 2015; Vafadari et al., 2023). preBötC GAD1$^+$ neurons project ipsilaterally to downstream XII premotoneurons and motoneurons at the same coronal level in the medullary slice (Koizumi et al., 2013), indicative of a role in modulating inspiratory burst pattern as activity is transmitted from the preBötC to XII nerve. Disinhibition of the BötC and preBötC in *in situ* preparations that generate a motor rhythm with distinct inspiratory, post-I, and expiratory phases causes inspiratory and post-I phases to fuse (Dutschmann and Paton, 2002), even producing tonic activity on respiratory motor nerves that have been interpreted as

indicating that inhibition is necessary for rhythmogenesis (Marchenko et al., 2016). Proposed roles for preBötC inhibitory neurons thus include contributing to preBötC excitation-inhibition balance (Ashhad and Feldman, 2020; Ramirez and Baertsch, 2018), ensuring the alternating silence of antagonist inspiratory and expiratory muscles (Ezure et al., 2003; Janczewski et al., 2013), modulating motor nerve burst pattern (Janczewski et al., 2013; Shao and Feldman, 1997), inducing apneas for regulatory and protective reflexes and volitional behaviors (Ezure and Tanaka, 2004; Janczewski et al., 2013; Kubin et al., 2006), and, more controversially, generating rhythm (Janczewski et al., 2013; Sherman et al., 2015; Smith et al., 2007).

These observations fall short of providing a clear picture for the role of preBötC inhibitory neurons in breathing (Abdala et al., 2015; Ashhad et al., 2022; Dick et al., 2018; Feldman and Kam, 2015; Ramirez and Baertsch, 2018). Pharmacological and optogenetic manipulations are not restricted to preBötC neurons, instead affecting unspecified numbers and types of inhibitory neurons throughout the medulla, and the functional effects of specifically activating or inhibiting preBötC GAD1/2$^+$ neurons have not been determined. Thus, the question remains what specific role preBötC GlyT2$^+$ and GAD1/2$^+$ neurons play in breathing.

We hypothesized that unrecognized heterogeneity among inhibitory preBötC neurons might explain the myriad roles proposed for these neurons. To characterize the molecular heterogeneity of preBötC inhibitory neurons, we used double reporter mice where red and green fluorescent proteins were expressed in GlyT2$^+$, GAD1$^+$, or GAD2$^+$ neurons and found that the majority of preBötC inhibitory neurons in the postnatal mouse express both GlyT2 and GAD2 while a much smaller subpopulation also expresses GAD1. To determine whether

molecularly-defined preBötC inhibitory neurons showed any functional differences, we used holographic photostimulation, which can dynamically and selectively excite or inhibit sets of neurons *within* a population with exceptional spatiotemporal resolution (Kam et al., 2013; Lutz et al., 2008; Zahid et al., 2010), in rhythmic medullary slices from double reporter mice. Holographic photostimulation of GlyT2$^+$ neurons delays burst initiation in the preinspiratory period, consistent with a role for preBötC GlyT2$^+$ neurons in regulating the timing of the start of inspiration, whereas application of the same stimulation protocol to GAD1$^+$ neurons during the inspiratory burst increases burst duration. We therefore reveal functional differences among embedded preBötC inhibitory microcircuits in the clinically important motor program for breathing.

Results

GAD1 defines a distinct preBötC inhibitory subpopulation.

We first examined the spatial distribution of neurons expressing the transcription factor Dbx1, which defines a necessary excitatory preBötC subpopulation (Bouvier et al., 2010; Gray et al., 2010), and the glycinergic inhibitory marker GlyT2 (Zeilhofer et al., 2005), in P0-P4 double reporter mice expressing the red fluorescent protein tdTomato in Dbx1$^+$ neurons and enhanced green fluorescent protein (EGFP) in GlyT2$^+$ neurons. Double reporter mice were generating using a cre-lox strategy crossing Dbx1^{cre} (Bielle et al., 2005) with Rosa26$^{LoxP\text{-}Stop\text{-}LoxP\text{-}tdTomato}$ (Madisen et al., 2010) in combination with GlyT2^{EGFP} BAC transgenic reporter mice (Zeilhofer et al., 2005). We counted neurons expressing tdTomato, EGFP, or both fluorescent proteins within a 150 µm radius hemi-cylinder ventral to nucleus ambiguus that extended rostrocaudally from

360 to 480 µm caudal to the caudal pole of VII, a region corresponding anatomically to preBötC (Ruangkittisakul et al., 2014). Within this region, there were similar numbers of Dbx1^{+} and GlyT2^{+} neurons (Dbx1^{+}: 662.8 ± 189.9; GlyT2^{+}: 636.3 ± 132.8; n=4; Figure 1A-C). Coexpression of Dbx1 and GlyT2 in this region was observed (175.3 ± 62.5; n=4; Figure 1A-C), constituting 26.4% of Dbx1^{+} neurons and 27.5% of GlyT2^{+} neurons. No tdTomato labeling was observed in preBötC from cre-negative Dbx1$^{+/+}$;Rosa26$^{\text{LoxP-Stop-LoxP-tdTomato}}$ animals (data not shown).

To determine the molecular phenotype of preBötC inhibitory neurons, we characterized the pairwise spatial distribution and levels of co-expression of inhibitory markers in the preBötC of P0-P4 transgenic double reporter mice expressing either the red fluorescent protein tdTomato and enhanced green fluorescent protein (EGFP) in GlyT2^{+}, GAD1^{+}, or GAD2^{+} neurons. Double reporter mice were generated using a cre-lox strategy, crossing GlyT2^{cre} (Foster et al., 2015) or GAD2^{cre} (Taniguchi et al., 2011) with Rosa26$^{\text{LoxP-Stop-LoxP-tdTomato}}$ in combination with GlyT2^{EGFP} and GAD1^{EGFP} BAC transgenic reporter mice (Chattopadhyaya et al., 2004; Zeilhofer et al., 2005).

In GAD2^{tdTomato};GlyT2^{EGFP} mice (GAD2^{cre}:Rosa26$^{\text{LoxP-Stop-LoxP-tdTomato}}$:GlyT2^{EGFP}), GlyT2^{+} and GAD2^{+} neurons were found in approximately equal numbers with GlyT2^{+} slightly outnumbering GAD2^{+} (GlyT2^{+}: 722 ± 106.7; GAD2^{+}: 658.3 ± 107.1; n=3; Figure 1D-F). Co-expression of GlyT2 and GAD2 was high (GlyT2^{+}/GAD2^{+}: 431 ± 66; n=3) with 59.7% of GlyT2^{+} expressing GAD2 and 65.5% of GAD2^{+} expressing GlyT2 (Figure 1D-F). In contrast to the high density of GAD2^{+} and GlyT2^{+} preBötC neurons, significantly fewer GAD1^{+} neurons were observed in preBötC. In GAD2^{tdTomato};GAD1^{EGFP} mice (GAD2^{cre}:Rosa26$^{\text{LoxP-Stop-LoxP-tdTomato}}$:GAD1^{EGFP}) mice, GAD1^{+} neurons were much lower than GAD2^{+} neurons in preBötC (GAD2^{+}: 792 ± 125.4; GAD1^{+}: 41 ± 13.9; n=3;

Figure 1G-I). Most GAD1^{+} neurons expressed GAD2 (GAD2^{+}/GAD1^{+}: 26.3 ± 10.4; n=3) with 3.3% of GAD2^{+} neurons expressing GAD1 and 64.3% of GAD1^{+} neurons expressing GAD2 (Figure 1G-I). A similar distribution was observed in GlyT2^{tdTomato};GAD1^{EGFP} mice (GlyT2^{cre}:Rosa26$^{LoxP\text{-}Stop\text{-}LoxP\text{-}tdTomato}$:GAD1^{EGFP}). In these mice, GAD1^{+} neurons were significantly lower than GlyT2^{+} neurons (GlyT2^{+}: 662 ± 81.1; GAD1^{+}: 60.3 ± 24; n=3; Figure 1J-L). Most GAD1^{+} neurons expressed GlyT2 (GlyT2^{+}/GAD1^{+}: 43.3 ± 18.5; n=3) with 6.5% of GlyT2^{+} neurons expressing GAD1 and 71.8% of GAD1^{+} neurons expressing GlyT2 (Figure 1J-L). Thus, we found that inhibitory neuron were heterogeneous in their expression of these molecular markers and suggest that two major subpopulations of inhibitory neurons in the preBötC are GAD2^{+}/GlyT2^{+} coexpressing neurons and GAD1^{+} neurons that likely also express GAD2 and/or GlyT2.

Threshold preBötC GlyT2^{+} stimulation delays burst initiation

We sought to examine whether these inhibitory preBötC subpopulations had distinct functional roles in inspiratory rhythm and pattern generation. A major issue with functional studies involving preBötC inhibitory neurons *in vitro* and *in vivo* is the difficulty in restricting perturbations to preBötC neurons with the high density of inhibitory neurons in nearby BötC and inhibitory afferents from other medullary sites. To selectively target preBötC GlyT2^{+} neurons and assess their functional role, we used holographic stimulation in rhythmically active medullary slices obtained from neonatal from Dbx1^{tdTomato};GlyT2^{EGFP} (Dbx1^{cre} x Rosa26$^{LoxP\text{-}Stop\text{-}LoxP\text{-}tdTomato}$ x GlyT2^{EGFP}) double reporter mice, which allowed us to localize our neurons to preBötC, selectively activate specific subpopulations, and rigorously determine their role in inspiratory rhythmogenesis (Kam et al., 2013; Lutz et al., 2008; Zahid et al., 2010). Glutamate

uncaging of a 10 µm soma-centered spot over a single preBötC GlyT2$^+$ neuron adjacent to these Dbx1$^+$ neurons resulted in a burst of action potentials (Figure 2A), similar to the responses of Dbx1$^+$ neurons to photostimulation (Kam et al., 2013; Sun et al., 2019).

We first confirmed that targeted neurons were in preBötC. preBötC Dbx1$^+$ neurons are necessary for inspiratory rhythmogenesis and minimally colocalize with GlyT2$^+$ (Bouvier et al., 2010; Gray et al., 2010). Consistent with the Dbx1$^+$ neurons being in preBötC, we observed no colocalization of Dbx1$^+$ and GlyT2$^+$ in our stimulation field (Figure 2B). To determine whether the neurons were in preBötC, we photostimulated Dbx1$^+$ neurons and calculated a probability of stimulating burst (P_{stim}) by dividing the number of successfully evoked bursts by the number of laser stimuli. Holographic photostimulation of 8 Dbx1$^+$ neurons at intervals slightly shorter than the endogenous rhythm resulted in generation of inspiratory bursts in a median [IQR] of 94% [76%,95%] of stimuli (n=9; Figure 2C), consistent with these neurons playing a role in inspiratory rhythmogenesis.

In the same field, we selected 4 or 8 GlyT2$^+$ neurons for low frequency stimulation, which would allow sampling of all phases of endogenous rhythm. The interval between stimuli was greater than 2 times the average period to allow for unstimulated cycles in between stimulated cycles. The phase of stimulation was defined as the time of stimulation after a burst divided by the average interburst interval (T_E) of unstimulated cycles (control T_E). We categorized the phase of stimulation into the postinspiratory period (post-I; <20% of control T_E immediately following a burst), mid-expiration (mid-E; 20-90% of control T_E after post-I), preinspiratory period (pre-I;

90-110% of control T_E following mid-E if stimulation did not occur during a burst), and inspiration (stimulation occurring during a burst) and measured the effect on T_E.

Photostimulation of 4 preBötC GlyT2$^+$ neurons adjacent to the photostimulated Dbx1$^+$ neurons during post-I and mid-E did not affect T_E, but pre-I stimulation resulted in a 10% increase in T_E compared to control that was statistically significant (Friedman test, $\chi^2(3)=10.2$, p=0.02, post-hoc Wilcoxon signed rank test with Bonferroni-Holm correction: ctrl vs. post-I: p=0.6, ctrl vs. mid-E: p=1, ctrl vs. pre-I: p=0.004, n=9; Figure 2D, E). Amplitude and duration (T_I) of the burst subsequent to photostimulation of the 4 GlyT2$^+$ neurons was not significantly different from control bursts (Friedman test, amp.: $\chi^2(3)=3.3$, p=0.35, T_I: $\chi^2(3)=0.3$, p=0.95, n=9; Figure 2D, E). While stimulation of 8 GlyT2$^+$ neurons during post-I and mid-expiration also did not affect the T_E, stimulation during the preinspiratory period produced a significant increase in T_E (Friedman test, $\chi^2(3)=14.1$, p=0.003, post-hoc Wilcoxon signed rank test with Bonferroni-Holm correction: ctrl vs. post-I: p=1, ctrl vs. mid-E: p=0.7, ctrl vs. pre-I: p=0.004, n=9; Figure 2F, G). Amplitude and T_I of the subsequent burst following stimulation of 8 GlyT2$^+$ neurons at any phase were not significantly different from control (Friedman test, amp.: $\chi^2(3)=1.3$, p=0.7, T_I: $\chi^2(3)=0.3$, p=0.6, n=9; Figure 2F, G). When 4 or 8 GlyT2$^+$ neurons were activated during an inspiratory burst, we observed no effects on inspiratory burst amplitude or duration (Wilcoxon signed rank test, 4 neurons, amplitude, p=0.1, T_I, p=0.2, 8 neurons, amplitude, p=0.2, T_I, p=0.07, n=9; Figure 2D-G).

We hypothesized that the specific effect of preinspiratory stimulation of 8 GlyT2$^+$ neurons on T_E was due to preinspiration being a particularly sensitive period of increasing synchronization, critical for rhythmogenesis. A similar process may be initiated by photostimulation of 4-9

preBötC neurons (Kam et al., 2013). In particular, the latency observed when triggering synchronous activity in a small group of Dbx1$^+$ neurons represents the same assembly mechanism occurring during the preinspiratory period (Kam et al., 2013; Sun et al., 2019). If true, stimulation of 8 GlyT2$^+$ neurons following stimulation of 8 Dbx1$^+$ neurons should produce a prolonged latency to burst generation, analogous to the increased period observed when 8 GlyT2$^+$ neurons were photostimulated during the preinspiratory period during endogenous rhythm. We therefore took advantage of the ability of the holographic system to display multiple patterns to stimulate Dbx1$^+$ neurons and GlyT2$^+$ neurons sequentially and examine this process rigorously.

Holographic photostimulation of 8 Dbx1$^+$ neurons at intervals just shorter than the endogenous interval resulted in entrainment of the rhythm (P_{stim}: 94.1% [89%,94%] n=9; Figure 3A, E). An important parameter in generating these evoked burst is the latency between laser stimulation and burst initiation, which we hypothesize represents a synchronization process essential for rhythmogenesis. For stimulation of 8 Dbx1$^+$ neurons, the latency between laser stimulation and burst initiation was 147 ms [132 ms,161 ms] (n=9; Figure 3A, E).

Having established a baseline P_{stim} and latency for burst generation with photostimulation of 8 Dbx1$^+$ neurons, we examined the effects of stimulating 4 or 8 GlyT2$^+$ neurons at 50 or 100 ms following Dbx1$^+$ stimulation (Figure 3B, C). While sequential stimulation of 4 or 8 GlyT2$^+$ neurons 50 ms after stimulation of 8 Dbx1$^+$ neurons did not significantly alter the latency preceding the burst (Figure 3B), increasing the interval between 8 Dbx1$^+$ and 4 or 8 GlyT2$^+$ stimulation to 100 ms significantly increased the latency by 39% [35%,44.7%] for 4 GlyT2$^+$ neurons and by

47.6% [40.6%,64.8%] for 8 GlyT2$^+$ neurons (Friedman test, $\chi^2(4)=26.8$, $p=2x10^{-5}$, post-hoc Wilcoxon signed rank test with Bonferroni-Holm correction: 50 ms: ctrl vs. 4 GlyT2$^+$: p=0.9, ctrl vs. 8 GlyT2$^+$: p=0.3, 100 ms: ctrl vs. 4 GlyT2$^+$, p=0.004, ctrl vs 8 GlyT2$^+$, p=0.004; n=9, Figure 3B,C, E). The probability of stimulation and amplitude and duration of evoked bursts were not significantly affected by 4 or 8 GlyT2$^+$ stimulation at 50 or 100 ms intervals (Friedman test, P_{stim}: $\chi^2(4)=0.6$, p=1, amp.: $\chi^2(4)=1.51$, p=0.8, T_I: $\chi^2(4)=5.8$, p=0.2, n=9, Figure 3B, C, E). We increased the delay between 8 Dbx1$^+$ stimulation and either 4 or 8 GlyT2$^+$ stimulation to activate inhibitory neurons during the evoked burst (Figure 3D). GlyT2$^+$ stimulation during an evoked burst did not significantly alter burst amplitude or duration (Wilcoxon signed rank test, amp.: 4 GlyT2$^+$, p=0.4, 8 GlyT2$^+$, p=0.1; T_I: 4 GlyT2$^+$, p=0.3, 8 GlyT2$^+$, p=0.9, n=9, Figure 3D, E). Thus, stimulating 4 or more preBötC GlyT2$^+$ neurons specifically delays burst initiation.

Threshold preBötC GAD1$^+$ stimulation prolongs burst duration

The ability to target specific numbers of neurons allowed us to directly compare whether the smaller triple-expressing preBötC GAD1$^+$ neurons differed functionally from preBötC GlyT2$^+$ neurons that did not express GAD1. To assess the functional role of the GAD1$^+$ preBötC subpopulation, we used holographic stimulation in rhythmic medullary slices from Dbx1^{tdTomato};GAD1^{EGFP} (Dbx1^{cre} x Rosa26$^{LoxP\text{-}Stop\text{-}LoxP\text{-}tdTomato}$ x GAD1^{EGFP}) double reporter mice to specifically target these neurons. Glutamate uncaging of a 10 µm soma-centered spot over a single preBötC GAD1$^+$ neuron adjacent to Dbx1$^+$ neurons resulted in a burst of action potentials (Figure 4A), similar to the responses of Dbx1$^+$ neurons to photostimulation (Kam et al., 2013; Sun et al., 2019). We again confirmed the location of the field in preBötC by examining

colocalization of Dbx1$^+$ and GAD1$^+$ and determining whether photostimulating 8 Dbx1$^+$ neurons entrained inspiratory rhythm. We did not observe co-expression of Dbx1 and GAD1 in our target fields (Figure 4B). Photostimulation of 8 Dbx1$^+$ neurons produced robust entrainment of the rhythm (P_{stim}: 89.5% [80%,97.2%]; n=7; Figure 4C), consistent with the field being in preBötC.

Following the protocol for preBötC GlyT2$^+$ functional studies, we photoactivated 4 or 8 GAD1$^+$ neurons during endogenous rhythm. In contrast to GlyT2$^+$ stimulation, there were no statistically significant effects of the number of GAD1$^+$ neurons stimulated or the phase on T_E (Friedman test, 4 neurons.: $\chi^2(3)=6.4$, p=0.09, 8 neurons: $\chi^2(3)=2.3$, p=0.5, n=7; Figure 4D-G). The amplitude and duration of the burst following stimulation of 4 or 8 GAD1$^+$ neurons at any phase was also not significantly affected (Friedman test, 4 neurons: amp.: $\chi^2(3)=0.8$, p=0.9, T_I: $\chi^2(3)=1.5$, p=0.7, 8 neurons amp.: $\chi^2(3)=3.5$, p=0.3, T_I: $\chi^2(3)=3.5$, p=0.3, n=7; Figure 4D-G). In contrast to GlyT2$^+$ stimulation, photostimulation of 4 or 8 GAD1$^+$ neurons during inspiratory bursts significantly affected burst duration, but not amplitude (Wilcoxon signed rank test, amplitude: 4 GAD1$^+$ vs ctrl, p=0.2, 8 GAD1$^+$ vs ctrl, p=0.7, T_I, 4 GAD1$^+$ vs ctrl, p=0.04, 8 GAD1$^+$ vs ctrl, p=0.02; n=8; Figure 4D-G)

We next determined whether GAD1$^+$ stimulation could affect rhythmogenic processes using sequential stimulation. Photostimulation of 8 Dbx1$^+$ neurons recapitulated the effects on P_{stim} and latency observed with Dbx1^{tdTomato};GlyT2^{EGFP} reporter mice (Figure 5A, E). When Dbx1$^+$ stimulation was followed by stimulation of 4 or 8 GAD1$^+$ neurons with a 50 or 100 ms delay, no significant differences in P_{stim} or latency were observed (Friedman test, P_{stim}: $\chi^2(4)=4.3$, p=0.4,

latency: $\chi^2(4)=6.9$, p=0.1, amp.: $\chi^2(4)=12.7$, p=0.01, adjusted p>0.05 all pairs, T_I: $\chi^2(4)=2$, p=0.7, n=6; Figure 5B, C, E). To determine the effect of GAD1$^+$ stimulation during the evoked burst, we increased the delay following 8 Dbx1$^+$ stimulation. 4 GAD1$^+$ stimulation during bursts evoked by 8 Dbx1$^+$ prolonged burst duration by 59% and 93%, respectively (ctrl: 0.24 ms [0.21 ms,0.32 ms], 4 GAD1$^+$: 0.39 ms [0.30 ms,0.50 ms], 8 GAD1$^+$: 0.47 ms [0.26 ms, 0.59 ms]; Wilcoxon signed rank test, 4 GlyT2$^+$, p=0.03, 8 GlyT2$^+$, p=0.03, n=9, Figure 5D, E). While 4 GAD1$^+$ stimulation during evoked burst did not affect burst amplitude, 8 GAD1$^+$ stimulation during evoked bursts caused a small, but statistically significant decrease in amplitude (ctrl: 6.9 a.u. [3.6 a.u. ,9.4 a.u.], 8 GAD1$^+$: 4.2 a.u. [1.7 a.u., 7.9 a.u.], Wilcoxon signed rank test,4 GAD1$^+$, p=0.4, 8 GAD1$^+$, p=0.03, n=9; Figure 5D, E). We therefore demonstrate that stimulation of >4 preBötC GAD1$^+$ neurons during endogenous and evoked bursts increases their duration and stimulation of 8 preBötC GAD1$^+$ neurons during evoked bursts decreases burst amplitude, revealing distinct functional roles for preBötC GlyT2$^+$ and GAD1$^+$ neurons.

Discussion

Inhibition appears to play several roles in modulation of breathing, but how these functions are generated by inhibitory neurons in the preBötC has not been clearly determined (Abdala et al., 2015; Ashhad et al., 2022; Dick et al., 2018; Feldman and Kam, 2015; Ramirez and Baertsch, 2018). We rigorously characterized the distribution and co-expression of the major inhibitory neuron markers in preBötC and determined that preBötC contains two major molecularly-defined inhibitory subpopulations in neonates, delineated by the presence or absence of GAD1. We then used holographic photostimulation to both ensure that the neurons were in preBötC

and determine their functional role in endogenous rhythm and evoked burst generating processes. We found that GlyT2^{+} stimulation in the preinspiratory phase resulted in prolongation of the period and analogously that photostimulation following threshold Dbx1^{+} stimulation resulted in increases in latency to burst initiation. In contrast to GlyT2^{+} stimulation, photostimulation of the same number of GAD1^{+} neurons did not delay endogenous or evoked inspiratory burst generation, but did prolong burst duration when activated during endogenous or evoked inspiratory bursts. These results point to distinct functional roles for preBötC GAD1^{+} and GAD1^{-} neurons in the breathing central pattern generator.

The molecular heterogeneity of inhibitory preBötC neurons is consistent with previous studies examining numbers of molecularly-defined inhibitory neurons in preBötC and extend our understanding of the spatial distribution and molecular expression of these neurons. Using double reporter Dbx1^{tdTomato};GlyT2^{EGFP} mice to visualize Dbx1^{+} and GlyT2^{+} populations in the same tissue, we found approximately equal numbers of Dbx1^{+} and GlyT2^{+} neurons. In previous studies using GlyT2^{EGFP} mice, GlyT2^{+} neurons were found to constitute approximately half of inspiratory-modulated neurons in preBötC, with some having intrinsic bursting properties (Morgado-Valle et al., 2010; Winter et al., 2009). We found more overlap between Dbx1^{+} and GlyT2^{+} neurons in our histological analysis than previously reported (Bouvier et al., 2010; Gray et al., 2010; Morgado-Valle et al., 2010; Winter et al., 2009); although, we did not see significant overlap in the acute slices. GlyT2, GAD1, and GAD2 transcripts were detected in Dbx1^{+} preBötC neurons (Hayes et al., 2017), so our analysis may have included neurons outside the ventrolateral medulla and preBötC as defined in previous studies and/or there may be some non-specific cre-expression in the Dbx1^{cre} mouse. We found similar numbers of GlyT2^{+}

and GAD2$^+$ neurons in preBötC, perhaps due to their substantial coexpression, consistent with previous studies (Hirrlinger et al., 2019; Oke et al., 2023). While GlyT2$^+$ and GAD2$^+$ neurons constituted a large number of preBötC neurons, there were significantly fewer GAD1$^+$ neurons in preBötC.

Quantitative differences in our results and those reported in other studies may be due to different boundaries for preBötC and, in some cases, a focus on inspiratory-modulated neurons. preBötC, like many functionally-defined structures in the reticular formation, does not have clearly delineated borders, despite the position of these structures being highly stereotyped (Feldman and Kam, 2015). As neither Dbx1, nor any of the inhibitory markers, delineate preBötC boundaries, we opted to use a hemicylindrical volume that hews roughly to a functionally defined rostrocaudal extend defined relative to caudal pole of facial and a transverse area corresponding roughly to the lateral part of the paragigantocellular reticular nucleus or the medial part of the lateral reticular nucleus (Ruangkittisakul et al., 2014).

Molecular coexpression of GlyT2, GAD2, and GAD1 further highlights the heterogeneity of preBötC inhibitory neurons. In our study, GlyT2 and GAD2 were frequently co-expressed in preBötC neurons. GAD1, where expressed, also overlapped frequently with both GlyT2 and GAD2, and the high degree of overlap between GlyT2 and GAD2 points to a significant proportion of triple-expressing GAD1$^+$/GAD2$^+$/GlyT2$^+$ preBötC inhibitory neurons. Using a split-Cre system to track expression of GlyT2 and GAD2, a significant number of preBötC neurons were found to express both GlyT2 and GAD2 in neonatal mice, but that this coexpression was developmentally reduced, with many preBötC inhibitory neurons expressing only GlyT2 or

GAD2 in adults (Hirrlinger et al., 2019; Oke et al., 2023). GlyT2 and GAD1 coexpression was demonstrated with single cell multiplex RT-PCR of samples from GAD1^{+} neurons and GlyT2 immunostaining in GAD1^{EGFP} mice (Koizumi et al., 2013). While GAD2 and GAD1 are frequently coexpressed in cortical GABAergic neurons (Erlander et al., 1991; Soghomonian and Martin, 1998), we find that GAD1/GAD2 coexpression is more limited in neonatal preBötC. In other neurons, GAD2 has been found to localize to synaptic terminals while GAD1 appears concentrated in the somatic compartment (Erlander et al., 1991; Soghomonian and Martin, 1998). Functionally, GAD2 may therefore serve to replenish the neurotransmitter pool of GABA while GAD1 may play a metabolic role (Erlander et al., 1991; Soghomonian and Martin, 1998). Our results are also consistent with most neonatal preBötC inhibitory neurons co-releasing GABA and glycine (Rahman et al., 2013). Coexpression across these inhibitory markers was not complete, so singly expressing neurons or neurons expressing other combinations, such as GAD1^{+}/GAD2^{+} or GAD1^{+}/GlyT2^{+} double expressing neurons could constitute other functionally distinct subpopulations. Whether GlyT2 or GAD2 is developmentally downregulated in GAD1^{+} neurons or GAD1 is also downregulated during maturation remains to be determined. In neonatal mice, however, we conclude that there are two major inhibitory subpopulations: neurons coexpressing GAD2 and GlyT2, and those expressing GAD2, GAD1, and GlyT2.

We aimed to determine whether the GAD1^{+} and GAD1^{-} preBötC populations play functionally distinct roles in inspiratory rhythm and pattern generation *in vitro*. Pharmacological blockade of inhibitory transmission, local microinjection, and opsin expression induced in whole animal transgenic mice or viral infection often affect nearby BötC neurons, and, in the case of whole animal opsin expression, inhibitory inputs from afferents throughout the brainstem (Ausborn et

al., 2018; Baertsch et al., 2018; Fortuna et al., 2019; Huff et al., 2022; Hulsmann et al., 2021; Sherman et al., 2015; Vafadari et al., 2023). To overcome this technical obstacle, we used holographic photostimulation to restrict activation to a small number of neurons and to establish that the targeted inhibitory neurons were in preBötC by showing that activating adjacent Dbx1$^+$ neurons entrained inspiratory rhythm.

We found that photostimulating 4 or 8 GlyT2$^+$ preBötC neurons during the preinspiratory period produced a phase delay. These data are consistent with brief optogenetic activation of GlyT2$^+$ neurons causing phase-dependent delays in inspiratory activity *in vivo* with phase delays being strongest when stimulation occurred in the preinspiratory period (Sherman et al., 2015). Prolonged stimulation prolongs the expiratory period and can produce apnea (Fortuna et al., 2019; Sherman et al., 2015). Repeated optogenetic stimulation may also terminate inspiration or increase breathing frequency by eliciting rebound excitation (Baertsch et al., 2018; Fortuna et al., 2019; Hulsmann et al., 2021). Whether these other effects are due to activation of a greater number of preBötC GlyT2$^+$ neurons, stimulation of GlyT2$^+$ neurons in both preBötC and BötC, or indiscriminate activation of all inhibitory subtypes due to the use of the vesicular GABA transporter (VGAT) promoter, so inhibitory neurons and afferents, remains to be tested.

We also found that activation of 4 or 8 GAD1$^+$ preBötC neurons unexpectedly increased burst duration when stimulation occurred during the inspiratory burst. Previous studies pointed to a role for inhibitory neurons in decreasing burst amplitude and duration. Pharmacological blockade of $GABA_A$ receptors increased amplitude and modestly increased duration (Baertsch et al., 2018; Janczewski et al., 2013; Shao and Feldman, 1997). Optogenetic GlyT2$^+$ and VGAT$^+$

neuron stimulation *in vivo* decreased inspiratory duration or terminated inspiration (Baertsch et al., 2018; Hulsmann et al., 2021; Sherman et al., 2015). However, these studies did not specifically activate GAD1$^+$ preBötC neurons. Our data are thus the first to show how targeted activation of GAD1$^+$ preBötC neurons affects inspiration.

While these threshold effects were statistically significant, the effect size was relatively small. Further molecular and functional heterogeneity within these subpopulations may explain the small effects sizes (Koizumi et al., 2013; Zheng et al., 2019). Intrinsic properties of inhibitory neurons expressing GAD1, GlyT2, or both do not appear to differ significantly across these subpopulations, but do show some differences with excitatory inspiratory neurons (Burgraff et al., 2022; Koizumi et al., 2013; Picardo et al., 2013; Picardo et al., 2019; Reising et al., 2022; Zheng et al., 2019). Other sources of variability that may dilute the effects of photostimulation include differences in slice preparations and in single neuron responses to photoactivation. Some GlyT2$^+$ neurons could also be GAD1$^+$ neurons, and there is a high probability that GAD1$^+$ neurons expressed GlyT2$^+$. Nonetheless, the probability of the GlyT2$^+$ neurons also being GAD1$^+$ is quite small and the differential effects of stimulating GlyT2$^+$ vs. GAD1$^+$ support distinct functionality between these two subpopulations in spite of the small number of neurons activated and numerous potential sources of variability.

The effects of single or low neurons stimulation have revealed fundamental properties of neural circuits in mammals (Doron and Brecht, 2015) and provide unique constraints for computational models of preBötC (Feldman and Kam, 2015). Single primary motor cortical neuron stimulation can initiate whisking, demonstrating how modulation of one or a few

neurons can alter a rhythmic motor behavior (Brecht et al., 2004). Additionally, these threshold effects, particularly threshold numbers of neurons, are critical network parameters that affect neural circuit dynamics (Feldman and Kam, 2015). The 4-9 inspiratory preBötC neuron threshold for triggering an evoked burst *in vitro* has served as a useful constraint for computational models that point to experimentally testable network parameters and mechanisms (Ashhad et al., 2023; Guerrier et al., 2015; Phillips and Rubin, 2022; Song et al., 2015). These data may therefore constrain computational models that include inhibitory preBötC neurons and point to novel mechanisms of inhibitory regulation of breathing.

While conceptually useful, are the threshold effects of stimulating GlyT2$^+$ and GAD1$^+$ preBötC neurons physiologically relevant? Entire classes of inhibitory neurons are often assumed to fire synchronously (Kvitsiani et al., 2013) even though activation of a few, or even single, neurons can significantly impact neural circuit dynamics and subserve functions other than blanket inhibition (Isaacson and Scanziani, 2011; Karnani et al., 2016a; Karnani et al., 2016b). Indeed, a major role for inhibition is in regulating excitation-inhibition balance where even minor alterations in the number or strength of inhibitory activity can alter neural circuit dynamics (Ashhad and Feldman, 2020; Isaacson and Scanziani, 2011; Ramirez and Baertsch, 2018). The overlapping connectivity and phasic activity of inhibitory and excitatory preBötC neurons supports such a role. Excitatory SST$^+$ and GlyT2$^+$ preBötC neurons overlap extensively in their efferent targets as well as in their inputs (Yang and Feldman, 2018; Yang et al., 2020). Morphological analysis of GAD1$^+$ and GlyT2$^+$ inspiratory preBötC neurons also showed overlap with glutamatergic preBötC neurons in their projections dorsomedially to XII premotoneuron and motoneuron areas as well as in their dendritic fields (Koizumi et al., 2013). GlyT2$^+$ and

VGAT$^+$ neurons have been found to be tonically active, expiratory-modulated, and inspiratory-modulated, some with intrinsic bursting properties (Baertsch et al., 2018; Burgraff et al., 2022; Ezure et al., 2003; Morgado-Valle et al., 2010; Oke et al., 2018; Winter et al., 2009). Similarly, the majority of GAD1$^+$ neurons also displayed inspiratory-modulated activity (Koizumi et al., 2013), suggesting that GlyT2$^+$ and GAD1$^+$ preBötC neurons co-terminate and are co-active with excitatory Dbx1$^+$ preBötC neurons.

These data support a model whereby GlyT2$^+$ and GAD1$^+$ preBötC neurons contribute to excitation-inhibition balance governing distinct respiratory-related processes: GlyT2$^+$ to burst initiation and inspiratory rhythmogenesis and GAD1$^+$ to burst duration and inspiratory patterning. When active during preinspiration, changes in GlyT2$^+$ preBötC neuronal activity may allow precise, rapid regulation of burst timing by advancing or delaying bursts when GlyT2$^+$ neuron activity is decreased or increased. GAD1$^+$ preBötC neuron activity during inspiration, on the other hand, modulates excitation-inhibition balance onto preBötC pattern generating neurons, inspiratory premotoneurons, and/or XII motoneurons to increase or decrease burst duration. Different excitation levels during the interburst interval and during inspiratory bursts and excitation-inhibition balance may explain how GlyT2$^+$ has an inhibitory effect on burst initiation while GAD1$^+$ appears to have an excitatory effect on burst duration. Activation of more GlyT2$^+$ or GAD1$^+$ preBötC neurons, as observed in optogenetic studies, shifts the excitation-inhibition balance towards inhibition and results in stronger inhibitory effects on both rhythm and pattern even when stimulation occurs outside of the preinspiratory or inspiratory period.

In summary, we discover functionally distinct inhibitory preBötC subpopulations defined by the presence or absence of GAD1. We find that threshold stimulation can precisely and rapidly regulate the initiation of inspiration through activation of GlyT2^{+} neurons and the duration of inspiration through activation of GAD1^{+} neurons. We suggest that these modest changes in GlyT2^{+} and GAD1^{+} preBötC activity alter excitation-inhibition balance in rhythm and pattern generating microcircuits, allowing for precise, dynamic control of breathing. We conclude that the activity of distinct subpopulations of inhibitory preBötC neurons constitute functional microcircuits that can rapidly shape breathing, expand dynamic range, and confer lability that allows breathing to respond to changes in metabolic demand and environmental conditions and to coordinate with other respiratory-related behaviors, such as swallowing and vocalization.

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Competing Interests statement

The authors declare no competing interests.

Materials and methods

Experimental procedures were carried out in accordance with the United States Public Health Service and Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals. All animals were handled according to approved institutional protocols at Rosalind Franklin University of Medicine and Science (#B14-16, #B18-10, #B21-03). All protocols were approved by the Rosalind Franklin University of Medicine and Science Institutional Animal Care and Use Committee (Animal Welfare Assurance #A3279-01). Animals were subject to regular veterinary inspection and only to the experimental procedures detailed here and maintained on a conventional 12hr light/12hr dark cycle with free access to food and water. Every effort was made to minimize pain and discomfort, as well as the number of animals.

Transgenic mice

Rosa26$^{\text{LoxP-Stop-LoxP-tdTomato}}$ (JAX Stock No. 7914) (Madisen et al., 2010) mice were crossed with either GlyT2^{EGFP} (Zeilhofer et al., 2005) or GAD1^{EGFP} (JAX Stock No. 7677) (Chattopadhyaya et al., 2004) BAC transgenic mice to generate Rosa26$^{\text{LoxP-Stop-LoxP-tdTomato}}$GlyT2^{EGFP} and Rosa26$^{\text{LoxP-Stop-LoxP-tdTomato}}$GAD1^{EGFP} mouse lines. These mice were then crossed with either Dbx1^{cre} (Bielle et al., 2005), GlyT2^{cre} (Foster et al., 2015), or GAD2^{cre} (JAX Stock No. 19022) (Taniguchi et al., 2011) mice to generate Dbx1^{tdTomato};GlyT2^{EGFP}, Dbx1^{tdTomato};GAD1^{EGFP}, GAD2^{tdTomato};GlyT2^{EGFP}, GAD2^{tdTomato};GAD1^{EGFP}, and GlyT2^{tdTomato};GAD1^{EGFP} double reporter mice. Animal genotypes were verified via real time polymerase chain reaction using primers specific for cre recombinase (Pierani et al., 2001; Rose et al., 2009; Weibel et al., 2013) or by direct visualization of fluorescent reporter.

Histology and imaging

Neonatal double reporter mice (P0-P4) were anesthetized by inhalation of isoflurane and transcardially perfused with 4% paraformaldehyde. A block of tissue from the pons to the rostral cervical spinal cord was isolated, fixed overnight in 4% paraformaldehyde, and cryoprotected in 30% sucrose. The fixed brainstem-spinal cord block was sliced coronally on a Leica CM3050S cryostat in 60 µm intervals from the obex to the superior vestibular nucleus.

Sections were cover-slipped in PVA-DABCO (Valnes and Brandtzaeg, 1985) and imaged on a confocal Leica SP8 microscope. Facial motor nucleus and Nucleus Ambiguus (NA) were used as landmarks to identify preBötC. For each section, the preBötC was centered within a 450 µm x 450 µm field and a z-stack (1.5 µm optical section) obtained using a 20x objective.

Z-stacks were imported into CellProfiler 4.2.1 (Carpenter et al., 2006) one channel at a time and run through a custom-designed image processing pipeline to automatically extract the total cell count and 3-dimensional coordinates of cell centroids. CellProfiler output and z-stacks were loaded into IgorPro for manual review and editing. Custom written code was utilized to overlay the coordinate information extracted from CellProfiler onto the raw images from each channel. A 3-dimensional distance threshold was then applied to determine whether neurons expressed both markers, i.e., were co-expressing. Cells within a 150 µm semicircle centered at the ventral pole of NA were counted.

Slice electrophysiology

Neonatal double reporter mice (P0- P4) of either sex were anesthetized with isoflurane and euthanized by thoracic transection. Brainstems were removed in cold artificial CSF (ACSF) containing the following: 124 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, and 30 mM dextrose, which we aerated with 95% O2 and 5% CO2. Brainstems were then pinned to an agar block with the rostral side up. We cut a single 550-600 µm thick transverse medullary slice with the preBötC at the rostral surface. To obtain slices with the preBötC at the surface, the rostral cut was made above the first set of XII nerve rootlets at the level of the dorsomedial cell column and principal lateral loop of the inferior olive, and the caudal cut captured the obex (Ruangkittisakul et al., 2014). Slices were placed rostral side up in a chamber and perfused at 2–3 ml/min with 28–30˚C recording ACSF. Baseline recording ACSF consisted of the cutting ACSF solution with K^+ raised to 9 mM and Ca^{2+} maintained at 1.5 mM for a robust burst rhythm (Kam et al., 2013). In all experiments, slices were allowed to equilibrate for 30 min to ensure that the frequency and magnitude of XII population activities reached steady-state. Respiratory activity reflecting suprathreshold action potential firing from populations of neurons was recorded from XII motor nucleus or nerve roots or as population activity directly from the preBötC using suction electrodes (tip size ~50 mm). For whole cell patch clamp recordings, $GlyT2^+$ or $GAD1^+$ preBötC neurons were selected visually using fluorescence microscopy and patched under IR-DIC video microscopy. Patch electrodes with a 2–3 M tip resistance were fabricated using a three-stage custom program on a Flaming-Brown micropipette puller (P-97, Sutter Instruments) and filled with solution containing the following (in mM): 135 KGluconate, 1.1 EGTA, 5 NaCl, 0.1 $CaCl_2$, 10 HEPES, 2 MgATP, and 0.3 Na_3GTP. In whole-cell current-clamp recordings, membrane potentials were brought to −55 mV using a

negative bias current. For whole-cell recordings, experiments in which variations in series and input resistance exceeded the initial value by 25% were discarded. Population and single cell recordings were amplified using a Multiclamp 700B (Molecular Devices), filtered at 2–4 kHz, integrated, and digitized at 10 kHz. Integration was performed on custom built Paynter filter with a 20– 100 ms time constant. Digitized data were analyzed off-line using custom procedures written for IgorPro (Wavemetrics)

Holography

Holographic photostimulation was performed on a Phasor SLM system (Intelligent Imaging Innovations, Inc) mounted around an epifluorescence upright microscope (Zeiss Axioscope). We used a 405 nm diode laser (CUBE 405–100; Coherent) to uncage MNI-glutamate (0.5 mM) and depolarize targeted neurons. An iterative Fourier transform algorithm, implemented in Slidebook 5 (Intelligent Imaging Innovations, Inc), computed the phase pattern on the SLM corresponding to the desired distribution of light intensity at the focal plane of the objective. A blocker was inserted in the intermediate Fourier plane to block the unmodulated light component (zero order spot) and replicate patterns (Golan et al., 2009). Neurons were targeted by centering 10 µm circular spots over the somata of the selected neurons. The laser intensity per spot was set at 1–3 mW, which was previously determined to elicit bursts of action potentials in the targeted neurons without the spread of glutamate to neighboring neurons (Kam et al., 2013). Each laser stimulation consisted of 5x0.8 ms pulses, delivered at 200 Hz. A threshold stimulus was determined as the minimum number of neurons and minimum laser power required to entrain preBötC bursts at slightly faster than the endogenous frequency with

a success rate >80%. The latency between laser stimulation and burst initiation was calculated as the time between the beginning of laser stimulation and the start of the next preBötC burst.

Data analysis and statistics

Semiautomated event detection of respiratory-related activity recorded in XII output or preBötC population recordings was performed using custom procedures written in IgorPro. Multiple criteria, including slope and amplitude thresholds, were used to select events automatically, which were then confirmed visually.

Data are expressed as mean±standard deviation or median [interquartile range (IQR)]. Box plots show median, IQR, and 10% and 90% thresholds. In figures displaying average data, data points from individual experiments are displayed in gray. To determine sample sizes, *a priori* power analysis was performed using G*power 3.1.9.2 (Faul et al., 2009). In this program, the appropriate statistical test along with a desired power of 95% at a 5% significance level was selected for each type of experiment with effects sizes based on published or preliminary data. These parameters yielded minimum total sample sizes of 6 for all tests. Sample sizes represent the number of biological replicates for each group. Statistical analysis was performed using IgorPro. As the data were not normally distributed (Shapiro Wilk test), non-parametric tests were used. Multiple comparisons were evaluated using non-parametric Kruskal-Wallis or Friedman test for unpaired or repeated measures designs, respectively. Post-hoc Nemenyi or Wilcoxon signed rank tests with Bonferroni-Holm corrections were then used to determine significance for within group comparisons of unpaired or repeated measures designs. Statistical significance was set at $p < 0.05$.

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Figure legends

Figure 1. Spatial distribution of preBötC inhibitory subpopulations

Confocal z-projections of 60 um sections showing tdTomato, EGFP, and merged channels; rostrocaudal distribution of counts of tdTomato, EGFP, and co-expressing neurons; and Venn diagram representing the average total counts across three sections from preBötC of (A-C) $Dbx1^{tdTomato}GlyT2^{EGFP}$, (D-F) $GlyT2^{EGFP}GAD2^{tdTomato}$, (G-I) $GAD1^{EGFP}GAD2^{tdTomato}$, and (J-L) $GAD1^{EGFP}GlyT2^{tdTomato}$ mice. Dotted semicircles represents area where neurons are counted. Counts are the sum of neurons from left and right preBötC for the specified sections. Scale bar, 100 µm.

Figure 2. Stimulation of preBötC $GlyT2^+$ neurons during endogenous rhythm increases T_E.

(A) DIC and fluorescence images of a $Dbx1^{tdTomato}GlyT2^{EGFP}$ mouse. Whole cell current clamp response of a $GlyT2^+$ neuron (white arrow) to photostimulation (green arrow). Scale bar, 10 mV, 100 ms. **(B)** Fluorescent images of preBötC from $Dbx1^{tdTomato}GlyT2^{EGFP}$ mouse. Scale bar, 100 µm. **(C)** Fluorescent tdTomato image of preBötC. White arrows mark targeted neurons. Integrated XII (∫XII) trace shows response to photostimulation of 8 $Dbx1^+$ neurons. Scale bar, 200 ms. **(D)** *left,* Fluorescent image showing 4 preBötC $GlyT2^+$ neurons targeted for photostimulation (white arrows). *right,* Overlay of cycles of ∫XII activity under control conditions with no stimulation, and when stimulation occurred during post-inspiratory (post-I), expiratory (exp), and preinspiratory (pre-I) phases. Green arrows mark time of stimulation. Average control inspiratory burst shape and burst shape when stimulation occurred during the inspiratory burst (bottom left). Green arrow marks time of stimulation. **(E)** Grouped amplitude

(amp.), burst duration (T_I), and interburst interval (T_E) for cycles with no stimulation (ctrl), and when stimulation of 4 GlyT2$^+$ preBötC neurons occurred during post-I, exp., and pre-I. T_I and amp are also shown when stimulation occurred during bursts (insp.). *, $p<0.05$, Friedman test, post-hoc Wilcoxon signed rank test with Bonferroni-Holm correction, n=9. (**F)** *left,* Fluorescent image showing 8 preBötC GlyT2$^+$ neurons targeted for photostimulation (white arrows). *right,* Overlay of cycles of ∫XII activity under control conditions with no stimulation, and when stimulation occurred during post-inspiratory (post-I), expiratory (exp), and preinspiratory (pre-I) phases. Green arrows mark time of stimulation. Average control inspiratory burst shape and burst shape when stimulation occurred during the inspiratory burst (bottom left). Green arrow marks time of stimulation. **(G)** Grouped amplitude (amp.), burst duration (T_I), and interburst interval (T_E) for cycles with no stimulation (ctrl), and when stimulation of 8 GlyT2$^+$ preBötC neurons occurred during post-I, exp., and pre-I. T_I and amp are also shown when stimulation occurred during bursts (insp.). *, $p<0.05$, Friedman test, post-hoc Wilcoxon signed rank test with Bonferroni-Holm correction, n=9.

Figure 3. Stimulation of preBötC GlyT2$^+$ neurons increases the latency to burst initiation for evoked bursts.

(A-D) *left,* Fluorescent images of preBötC from a Dbx1^{tdTomato}GlyT2^{EGFP} mouse and, *right,* overlaid traces from the ∫XII recording of evoked bursts triggered by **(A)** 8 Dbx1$^+$ neurons, **(B)** 8 Dbx1$^+$ neurons followed by 4 or 8 GlyT2$^+$ neurons after a 50 ms delay, **(C)** 8 Dbx1$^+$ neurons followed by 4 or 8 GlyT2$^+$ neurons after a 100 ms delay, and **(D)** 8 Dbx1$^+$ neurons followed by 4 or 8 GlyT2$^+$ neurons after a >100 ms delay (during the evoked burst). White arrows indicate

targeted neurons, Red arrows indicate time of 8 Dbx1$^+$ stimulation, Green arrows indicate time of 4 or 8 GlyT2$^+$ stimulation 50 ms, 100, ms, and > 100 ms after Dbx1$^+$ stimulation. Dotted lines indicate average beginning and end of burst evoked by 8 Dbx1$^+$ neurons. **(E)** Grouped data showing amplitude, burst duration (T_I), probability of stimulation (P_{stim}), and latency to burst initiation following stimulation of Dbx1$^+$ and GlyT2$^+$ neurons. *, $p<0.05$, Friedman test, post-hoc Wilcoxon signed rank test with Bonferroni-Holm correction, n=9.

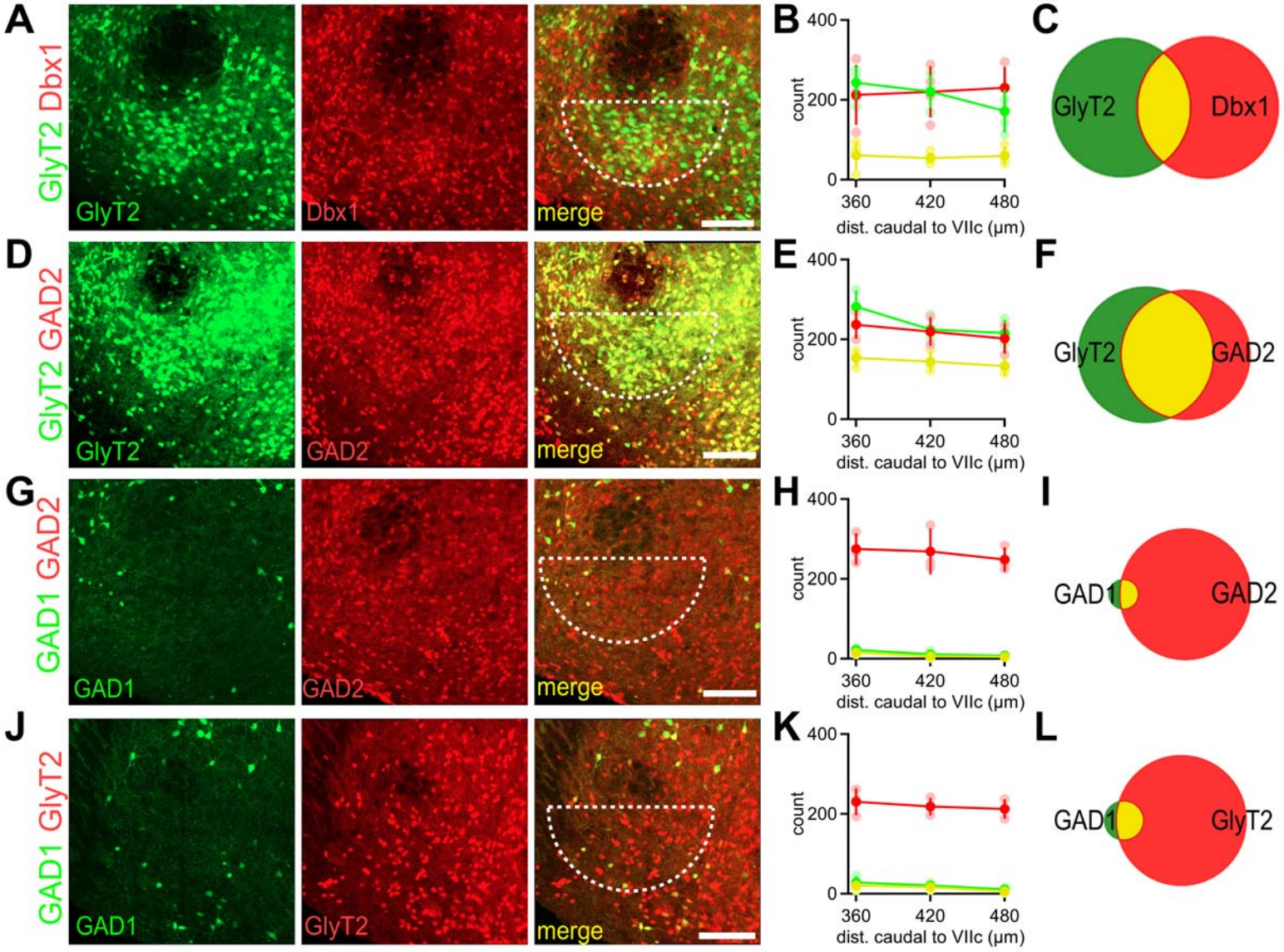
Figure 4. Stimulation of preBötC GAD1$^+$ neurons during endogenous bursts increases T_I.

(A) DIC and fluorescence images of a Dbx1^{tdTomato}GAD1^{EGFP} mouse. Whole cell current clamp response of a GAD1$^+$ neuron (white arrow) to photostimulation (green arrow). Scale bar, 10 mV, 100 ms. **(B)** Fluorescent images of preBötC from Dbx1^{tdTomato}GAD1^{EGFP} mouse. Scale bar, 100 µm. **(C)** Fluorescent tdTomato image of preBötC. White arrows mark targeted neurons. Integrated XII (∫XII) trace shows response to photostimulation of 8 Dbx1$^+$ neurons. Scale bar, 200 ms. **(D)** *left,* Fluorescent image showing 4 preBötC GAD1$^+$ neurons targeted for photostimulation (white arrows). *right,* Overlay of cycles of ∫XII activity under control conditions with no stimulation, and when stimulation occurred during post-inspiratory (post-I), expiratory (exp), and preinspiratory (pre-I) phases. Green arrows mark time of stimulation. Average control inspiratory burst shape and burst shape when stimulation occurred during the inspiratory burst (bottom left). Green arrow marks time of stimulation. **(E)** Grouped amplitude (amp.), burst duration (T_I), and interburst interval (T_E) for cycles with no stimulation (ctrl), and when stimulation of 4 GAD1$^+$ preBötC neurons occurred during post-I, exp., and pre-I. T_I and amp are also shown when stimulation occurred during bursts (insp.). *, $p<0.05$, Friedman test,

post-hoc Wilcoxon signed rank test with Bonferroni-Holm correction, n=7 for post-I, exp., and pre-I, n=8 for insp. (**F)** *left,* Fluorescent image showing 8 preBötC GlyT2$^+$ neurons targeted for photostimulation (white arrows). *right,* Overlay of cycles of ∫XII activity under control conditions with no stimulation, and when stimulation occurred during post-inspiratory (post-I), expiratory (exp), and preinspiratory (pre-I) phases. Green arrows mark time of stimulation. Average control inspiratory burst shape and burst shape when stimulation occurred during the inspiratory burst (bottom left). Green arrow marks time of stimulation. **(G)** Grouped amplitude (amp.), burst duration (T_I), and interburst interval (T_E) for cycles with no stimulation (ctrl), and when stimulation of 8 GlyT2$^+$ preBötC neurons occurred during post-I, exp., and pre-I. T_I and amp are also shown when stimulation occurred during bursts (insp.). *, $p<0.05$, Friedman test, post-hoc Wilcoxon signed rank test with Bonferroni-Holm correction, n=7 for post-I, exp., and pre-I, n=8 for insp.

Figure 5. Stimulation of preBötC GAD1$^+$ neurons during evoked bursts alters burst pattern. **(A-D)** *left,* Fluorescent images of preBötC from a Dbx1^{tdTomato}GAD1^{GFP} mouse and, *right,* overlaid traces from the ∫XII recording of evoked bursts triggered by **(A)** 8 Dbx1$^+$ neurons, **(B)** 8 Dbx1$^+$ neurons followed by 4 or 8 GAD1$^+$ neurons after a 50 ms delay, **(C)** 8 Dbx1$^+$ neurons followed by 4 or 8 GAD1$^+$ neurons after a 100 ms delay, and **(D)** 8 Dbx1$^+$ neurons followed by 4 or 8 GAD1$^+$ neurons after a >100 ms delay (during the evoked burst). White arrows indicate targeted neurons, Red arrows indicate time of 8 Dbx1$^+$ stimulation, Green arrows indicate time of 4 or 8 GAD1$^+$ stimulation 50 ms, 100, ms, and > 100 ms after Dbx1$^+$ stimulation. Dotted lines indicate average beginning and end of burst evoked by 8 Dbx1$^+$ neurons. **(E)** Grouped data showing

amplitude, burst duration (T_I), probability of stimulation (P_{stim}), and latency to burst initiation following stimulation of Dbx1$^+$ and GAD1$^+$ neurons. *, $p<0.05$, Friedman test, post-hoc Wilcoxon signed rank test with Bonferroni-Holm correction, n=6 for 50 ms and 100 ms delays, n=9 for >100 ms delays.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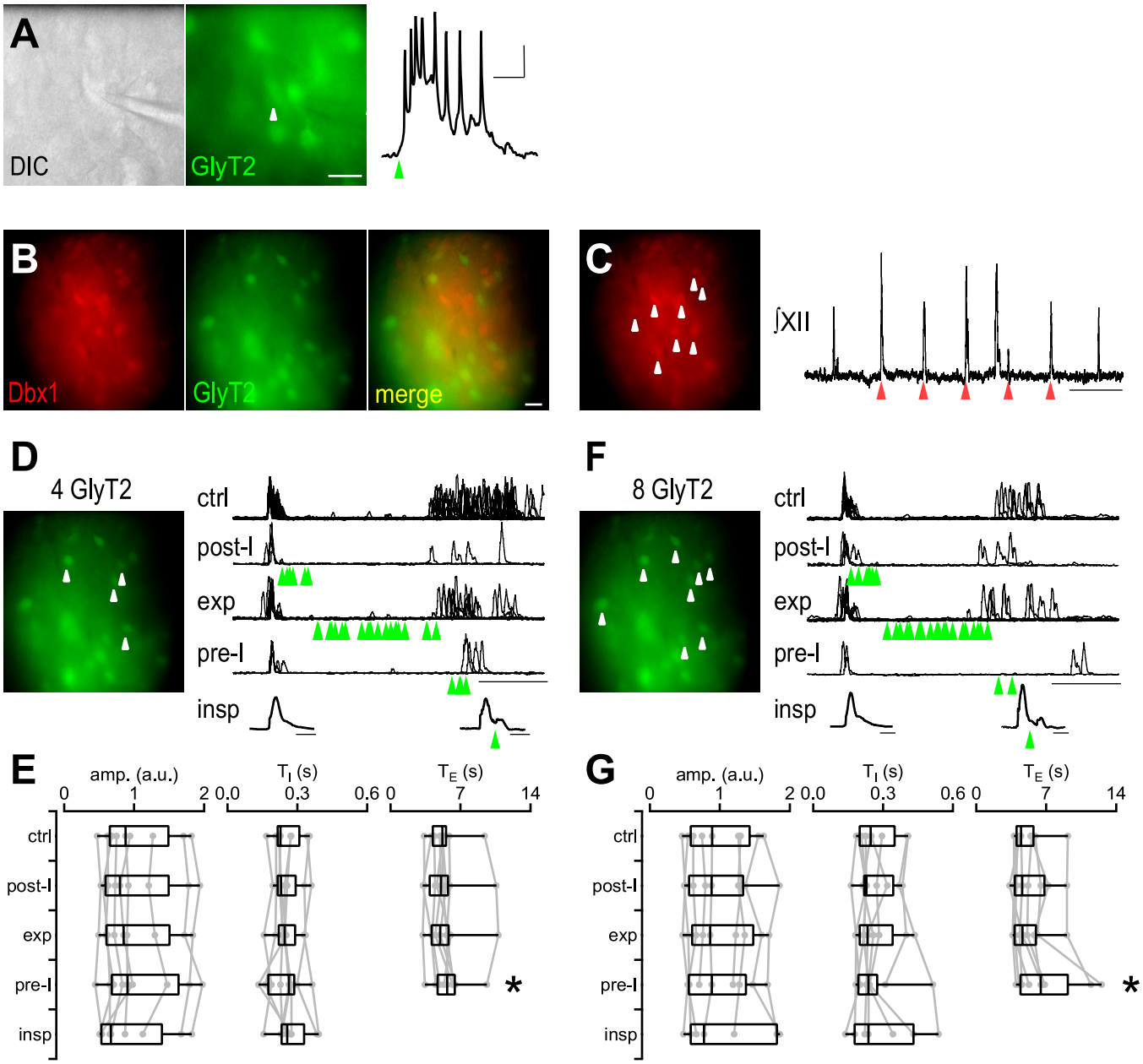
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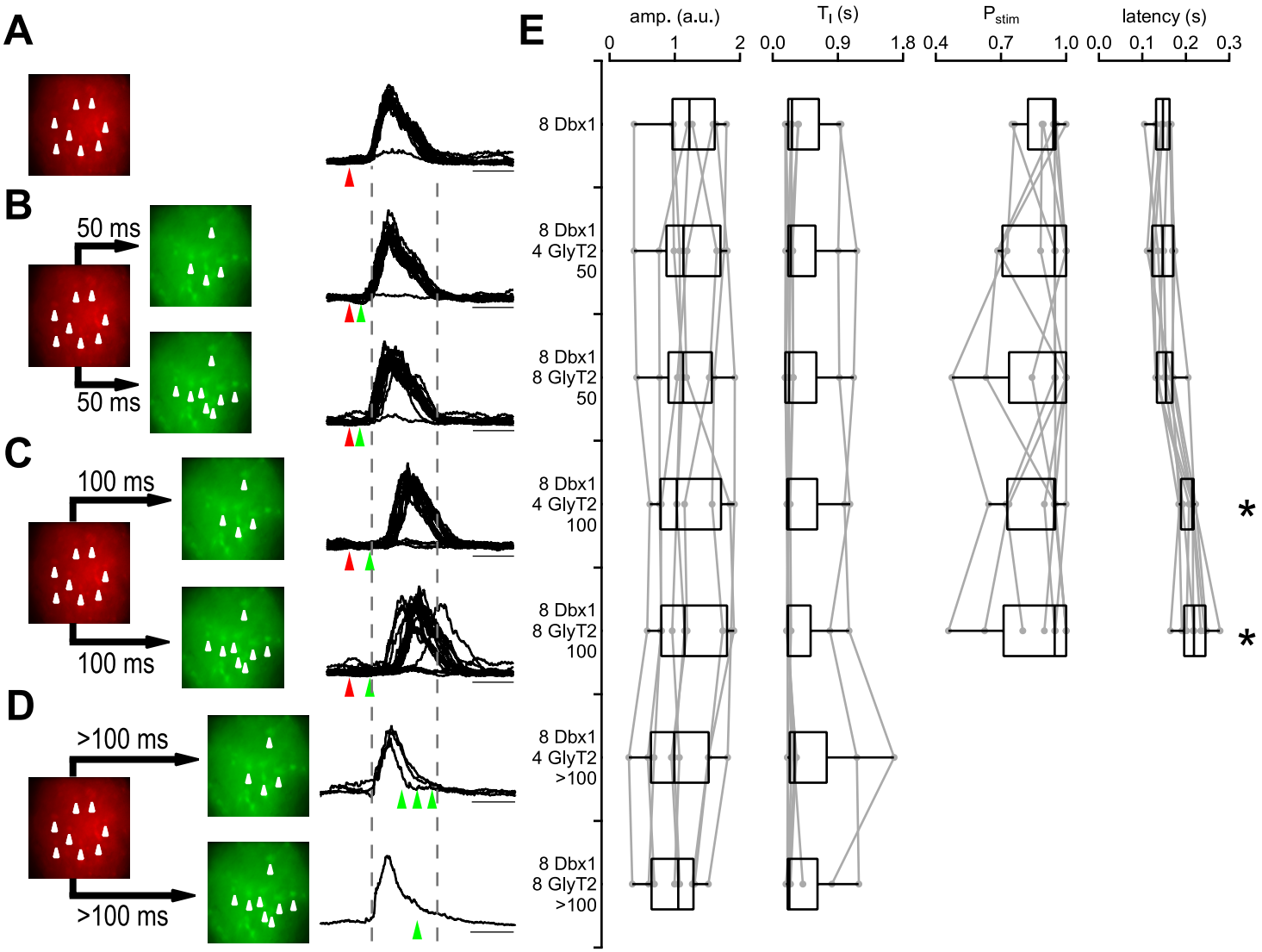
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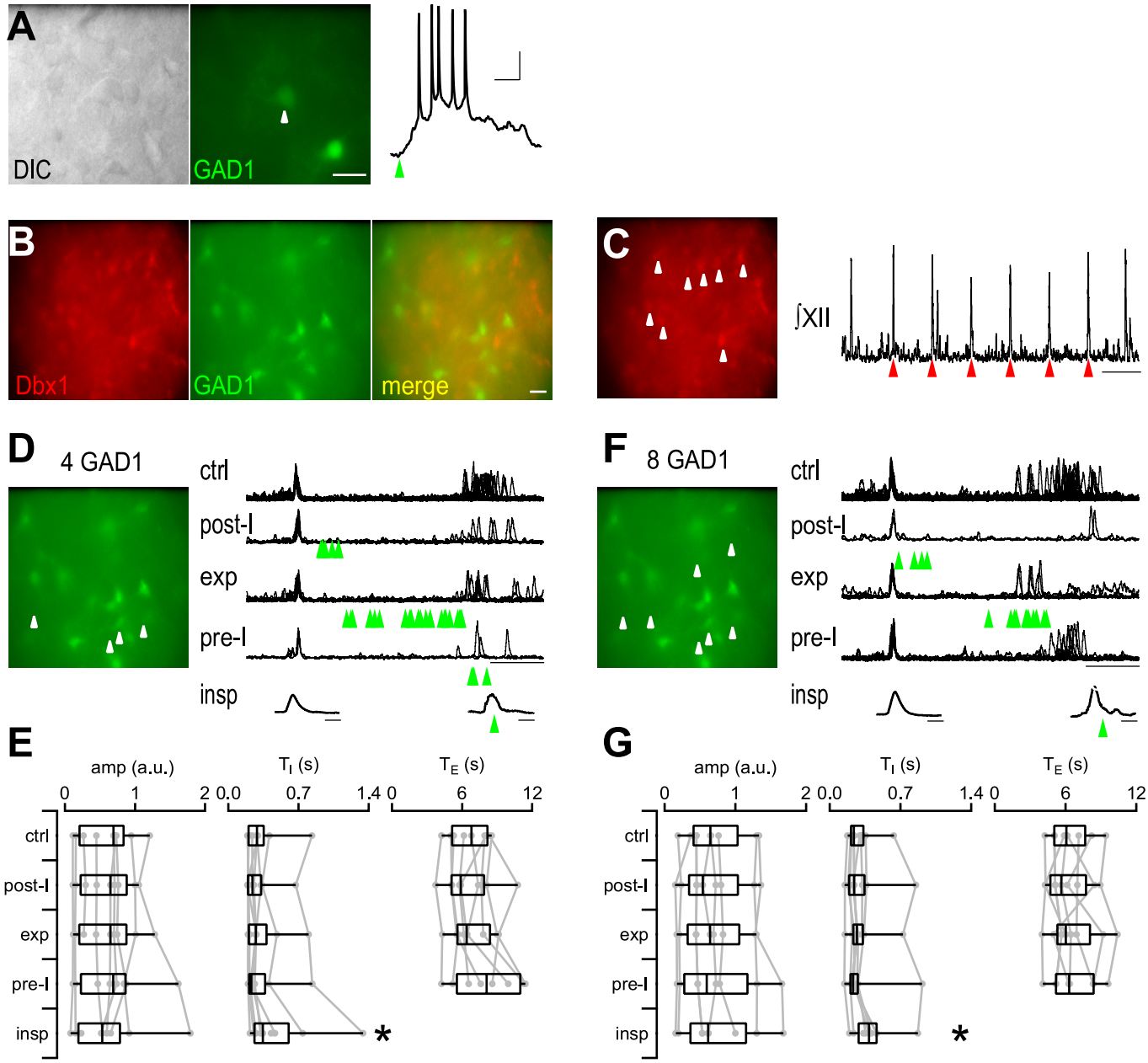
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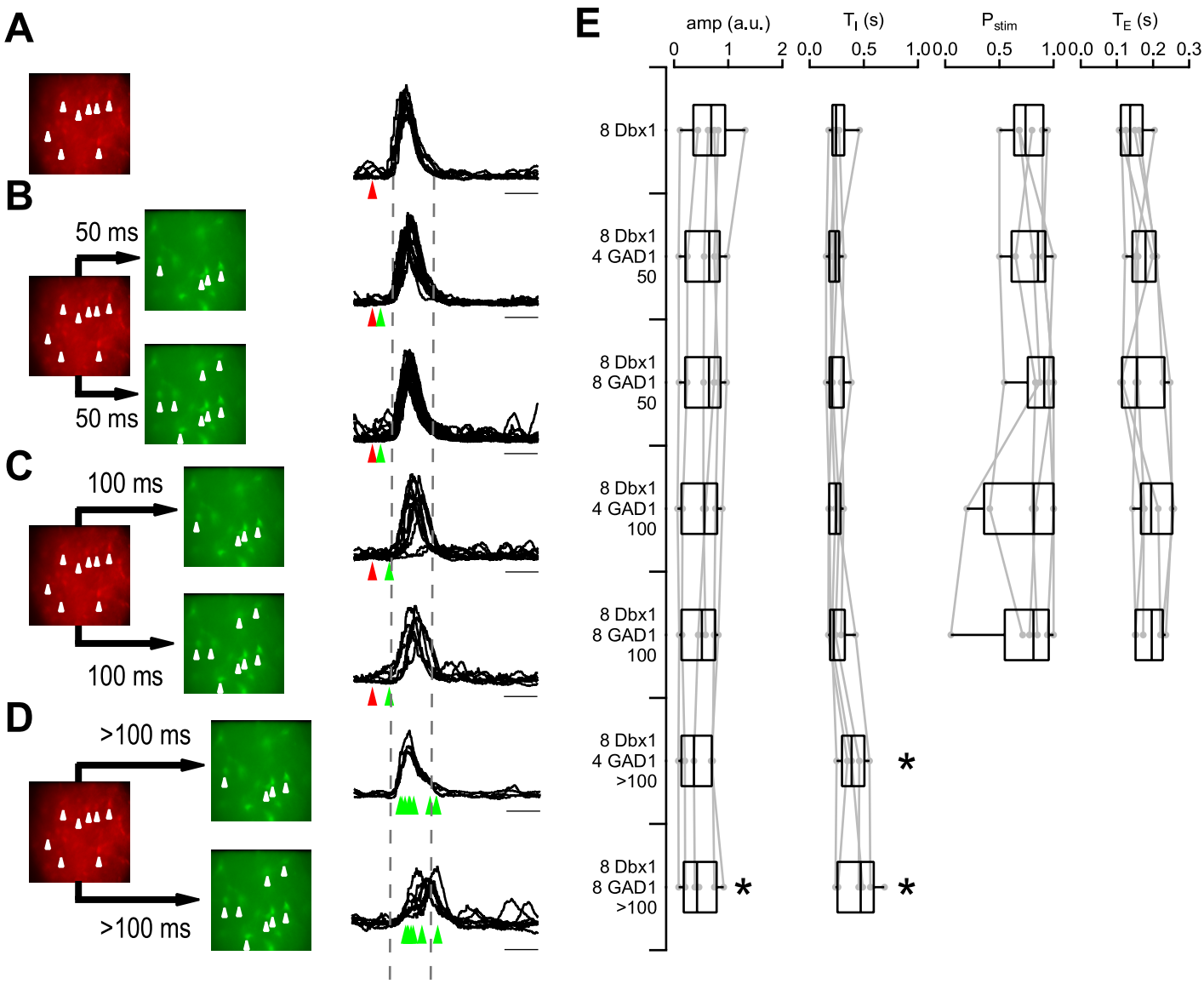
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