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# The Expression of Galanin in the Parafacial Respiratory Group and its Effects on Respiration in Neonatal Rats

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**Abstract—The inhibitory peptide galanin is expressed within the retrotrapezoidal nucleus (RTN) – a key central chemoreceptor site that also contains the active expiratory oscillator. It was previously reported that microinjection of galanin into pre-Bötzinger complex – containing the inspiratory oscillator – exerts inhibitory effects on inspiratory motor output and respiratory rhythm. In neonatal rats, the present study aimed to investigate: (1) expression of galanin within the parafacial respiratory group (pFRG), which overlaps anatomically and functionally with the adult RTN, and; (2) effects of galanin on respiratory rhythm using the *in vitro* brainstem-spinal cord preparation. We showed that 14 ± 2% of Phox2b-immunoreactive (ir) neurons in the parafacial region were also galanin-ir. Galanin peptide expression was confirmed within 3/9 $CO_2$-sensitive, Phox2b-ir Pre-Inspiratory neurons (Pre-I) recorded in parafacial region. Bath application of galanin (0.1–0.2 μM): (1) decreased the duration of membrane depolarization in both Pre-I and inspiratory pFRG neurons, and; (2) decreased the number of C4 bursts that were associated with each burst in Pre-I neurons within the pFRG. In preparations showing episodic breathing at baseline, the respiratory patterning reverted to the 'normal' pattern of single, uniformly rhythmic C4 bursts ($n$ = 10). In preparations with normal respiratory patterning at baseline, slowing of C4 rhythm ($n$ = 7) resulted although rhythmic bursting in recorded Pre-I neurons remained unperturbed ($n$ = 6). This study therefore demonstrates that galanin is expressed within the pFRG of neonatal rats, including neurons that are intrinsically chemosensitive. Overall the peptide has an inhibitory effect on inspiratory motor output, as previously shown in adults.** 

**Key words: respiratory central pattern generator, neuropeptide, episodic breathing, parafacial respiratory group, retrotrapezoid nucleus, Pre-Inspiratory.**

## INTRODUCTION

Galanin is an inhibitory, 29 amino-acid peptide expressed in cardio-respiratory related and other areas of the brainstem (Melander et al., 1986; Krukoff et al., 1992; Spirovski et al., 2012). It acts through G protein-(or trimeric G protein)-coupled receptors: the activation of which primarily results in the hyperpolarization of neurons. Galanin is notably present within a subset of neurons within the retrotrapezoid nucleus (RTN) in the parafacial region in adult rodents (Stornetta et al., 2009; Bochorishvili et al., 2012; Spirovski et al., 2012; Shi et al., 2017), but is also expressed in other nuclei such as the rostral ventral respiratory group, A1 noradrenergic

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*Abbreviations:* ACSF, artificial cerebrospinal fluid; C4, C4 nerve rootlet; Insp, inspiratory; NTS, nucleus of the solitary tract; pFRG, parafacial respiratory group; Phox2b, transcription factor Phox2b; Post-I, Post-Inspiratory; Pre-I, Pre-Inspiratory; RTN, retrotrapezoidal nucleus; TTX, tetrodotoxin; -ir, immunoreactive; Te, expiratory period; Ti, inspiratory period; Ttotal, whole respiratory cycle period; VGlut2, vesicular glutamate transporter type 2.

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cell group and nucleus of the solitary tract (NTS) (Spirovski et al., 2012).

Galaninergic RTN neurons project toward nuclei in all major subgroups of the pontomedullary respiratory central pattern generator, including the pre-Bötzinger complex, the caudolateral subnucleus of the solitary tract and the Kölliker-Fuse nucleus (Bochorishvili et al., 2012). Galanin-synthesizing RTN neurons are activated following prolonged hypercapnia but not hypoxia (Spirovski et al., 2012). The specific physiological conditions under which galanin is released from such neurons is currently unclear, particularly since galanin is often co-localised with markers of excitatory neurotransmission (e.g., vesicular glutamate transporter 2 VGlut2; Bochorishvili et al., 2012). Nevertheless, targeted microinjections of galanin peptide into sub-compartments of the respiratory central pattern generator in adult rats elicit marked changes in respiratory rhythm and patterning that are consistent with the peptide's inhibitory nature (Abbott et al., 2009). For example, microinjection of galanin into the pre-Bötzinger complex (which contains the inspiratory oscillator) evokes persistent apnea and/or severe respiratory dysrhythmia with a reduction of inspiratory duration.

The parafacial respiratory group (pFRG) in neonatal animals is coextensive with the RTN in adults (Onimaru and Homma, 2003). Recent studies demonstrated that it is functionally akin to the lateral portion of the RTN (Huckstepp et al., 2015). Both the RTN and pFRG neurons are intrinsically $CO_2$ sensitive and exhibit similar neurochemical phenotypes (i.e., containing VGlut2 and the transcription factor Phox2b, and devoid of markers of cholinergic and noradrenergic transmission) (Mulkey et al., 2004; Stornetta et al., 2006, 2009; Onimaru et al., 2008; Onimaru et al., 2014). Strong evidence suggests that the pFRG is the dominant respiratory oscillator and is coupled with the inspiratory pre-Bötzinger oscillator in neonates (Onimaru and Homma, 2003, 2006). In adults, the oscillator within the lateral RTN appears to be conditional as it is profoundly inhibited in conditions of normal respiratory drive (Pagliardini et al., 2011; Huckstepp et al., 2015). Increases in chemical drive prompt its disinhibition and the expression of active expiration. Interestingly, during active expiration, the pFRG/lateral RTN oscillator again predominates since inspiratory rhythm is expressed in a quantal manner with respect to active expiratory activity in abdominal muscles (Janczewski and Feldman, 2006).

The aims of the present study were to investigate in neonatal Wistar rats (postnatal day P0–P2): (a) the expression of galanin peptide within the pFRG, and; (b) the effects of exogenous galanin application (0.1–0.2 μM) on the activity of the C4 nerve roots and Pre-I neurons of the pFRG. It was hypothesized that, similar to adult rats, galanin would be present within pFRG neurons and have inhibitory actions on respiratory rhythm. We show that galanin is indeed expressed within $CO_2$-sensitive, Phox2b-containing pFRG neurons. We demonstrate that exogenous galanin has inhibitory effects on the duration of both C4 (inspiratory) nerve and Pre-Inspiratory (Pre-I) neuron firing. Furthermore, galanin alters the expression of inspiratory C4 rhythm in relation to an underlying rhythm in Pre-I neuron firing.

## EXPERIMENTAL PROCEDURES

The experimental protocols were approved by the Animal Research Committee of Showa University, which operates in accordance with Law No. 105 for the care and use of laboratory animals of the Japanese Government.

### Immunohistochemistry for galanin peptide and Phox2B transcription factor in the pFRG region

Three neonatal rats (P1) were deeply anesthetized with atmospheric ether until loss of nociceptive reflexes before their brainstems were isolated. The brains were fixed in 4% paraformaldehyde for 2 h at 4 °C and then immersed in 18% sucrose overnight. The brainstems were transversely sectioned (50 μm) and mounted onto slides. Sections were incubated with blocking buffer containing primary antibodies against galanin (rabbit anti-galanin, 1:400, Peninsula Laboratories, LLC, San Carlos, CA, USA) and Phox2b (guinea pig anti-Phox2b, 1:400, Onimaru et al., 2008) overnight at 4 °C. Following washing, sections were incubated for 1 h with secondary antibodies (Alexa Fluor 488 conjugated to anti-rabbit IgG and Alexa Fluor 546 conjugated to anti-guinea pig IgG; both 1:1000, Molecular Probes/Invitrogen). Excess antibodies were then removed by washing prior to cover-slipping with Vectashield. The immunofluorescent sections were viewed and photographed under the 20×–40× objectives on a conventional epifluorescence microscope (Zeiss Axio Imager Z1).

The parafacial region was analyzed for numbers of neurons containing galanin and Phox2b. Although immunostaining for a cholinergic marker was not performed, the facial motor nucleus could be clearly visualised as the ventral medullary cluster of large, Phox2b-immunoreactive (ir) nuclei. Quantitation of galanin- and Phox2b-ir profiles was performed between the facial motor nucleus and the ventral medullary surface along the entire rostrocaudal extent of the facial motor nucleus. Every 50-μm section was processed and quantified. The number of sections per rat ranged from 13–15. Phox2b-ir profiles found caudal to the caudal end of the facial motor nucleus were excluded as these were likely C1 neurons. Furthermore, C1 cells were likely excluded since it has been previously documented that galanin does not colocalize with markers of noradrenaline synthesis that are present in C1 neurons (Melander et al., 1986; Stornetta et al., 2009). To prevent double-counting of galanin-ir neurons that are present in two separate sections, a correction factor of 0.91 was applied based on a section thickness of 50 μm, an approximate mean object thickness of 15 μm, and a minimum detectable "cap" thickness of 5 μm for a neuron that has been split: $C = 50/50 + 15 - (5 + 5)$ (Clarke, 1993; Spirovski et al., 2012). Since Phox2b antibodies only labeled nuclei, a cellular structure that is ~10-μm thick,



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