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Neurochemistry of the Kölliker-Fuse nucleus from a respiratory perspective

Adrienn G. Varga^{1,2}, Sebastian N. Maletz¹, Jordan T. Bateman^{1,2}, Brandon T. Reid¹, Erica S. Levitt^{1,2}

¹Department of Pharmacology and Therapeutics, University of Florida, Gainesville, FL 32610

²Center for Respiratory Research and Rehabilitation, Department of Physical Therapy, University of Florida, Gainesville, FL 32610

Abstract

The Kölliker-Fuse nucleus (KF) is a functionally distinct component of the parabrachial complex, located in the dorsolateral pons of mammals. The KF has a major role in respiration and upper airway control. A comprehensive understanding of the KF and its contributions to respiratory function and dysfunction requires an appreciation for its neurochemical characteristics. The goal of this review is to summarize the diverse neurochemical composition of the KF, focusing on the neurotransmitters, neuromodulators and neuropeptides present. We also include a description of the receptors expressed on KF neurons and transporters involved in each system, as well as their putative roles in respiratory physiology. Finally, we provide a short section reviewing the literature regarding neurochemical changes in the KF in the context of respiratory dysfunction observed in SIDS and Rett syndrome. By overviewing the current literature on the neurochemical composition of the KF, this review will serve to aid a wide range of topics in the future research into the neural control of respiration in health and disease.

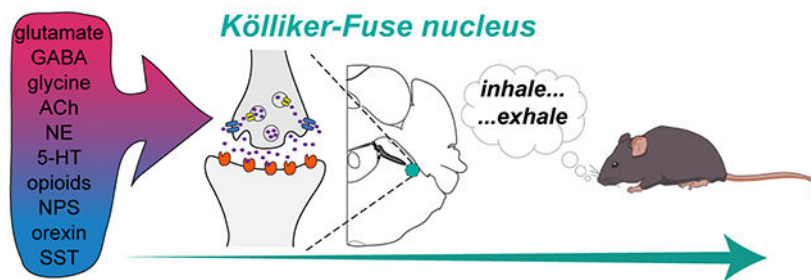
Graphical Abstract

The Kölliker-Fuse nucleus (KF) plays an important role in respiration and upper airway control. The purpose of this review is to summarize the neurochemical composition of the KF, discussing the neurotransmitters, neuromodulators and neuropeptides present. We also describe the receptors and transporters involved in each system, and provide a summary of their roles in the control of breathing. In addition, we briefly discuss the neurochemical changes in the KF in SIDS and Rett syndrome. This review will serve to support a wide range of topics in the future research into the neural control of breathing in health and disease.

Correspondence to: Adrienn G. Varga, University of Florida, 1200 Newell Drive, Gainesville, FL 32610. adrienn.varga@ufl.edu.

Conflict of Interest

The authors declare no competing financial interests.



Keywords

respiration; SIDS; Rett syndrome; GABA; glutamate; opioids

Introduction

The parabrachial complex is a collection of multiple nuclei in the dorsolateral pons surrounding the superior cerebellar peduncle. The three major subdivisions of the parabrachial complex, the lateral and medial parabrachial nuclei and the Kölliker-Fuse nucleus (KF; Figure 1), can be found in animals (Dutschmann and Dick 2012) and humans (Lavezzi et al. 2004b). Each of the pontine nuclei have a different neurochemical composition, as well as anatomical connections, in accordance with distinct functional roles. Due to its important role in respiratory physiology, we highlight the KF and its neurochemical identity in this review.

Respiration is generated and controlled by the coordinated efforts of respiratory nuclei in the pons and medulla. Rhythm generators in the ventrolateral medulla, consisting of the pre-Bötzinger complex, the post-inspiratory complex, and the lateral parafacial nucleus, are thought to generate inspiration, post-inspiration, and expiration respectively (Smith et al. 1991; Anderson et al. 2016; Huckstepp et al. 2015). The Bötzing complex is a collection of mostly inhibitory neurons that fire during expiration, providing a major source of inhibition for the network (Ezure et al. 2003a; Schreihofer et al. 1999). Neural output from rhythm generators is relayed to premotor neurons in the ventral respiratory group. Medullary respiratory neurons receive modulatory input from pontine structures, including the KF, which is required for normal breathing (Fung and St. John 1995; Dutschmann and Herbert 2006; Dutschmann and Dick 2012; Smith et al. 2007). Together, these nuclei (with others not mentioned here) form a highly interconnected network that produces a three-phase respiratory pattern (inspiration, post-inspiration, and expiration) that constitute a normal breath (see more information on respiratory rhythm in the following reviews: Smith et al., 2013; Del Negro et al., 2018; Ramirez & Baertsch, 2018a, 2018b). The precise control of breathing phases relies on the continuous balance of excitatory and inhibitory neurotransmission in respiratory neural populations. Thus, the neurochemical identity of respiratory nuclei provides valuable information about their functional roles in respiration and respiratory related behaviors, such as swallowing and cough.

Lumsden (1923) described the KF as the “inspiratory off-switch,” important for the transition of inspiration to expiration. Since then, it has been shown that the KF is required

for normal breathing and upper airway patency (Fung and St. John 1995; Dutschmann and Herbert 2006; Dutschmann and Dick 2012; Smith et al. 2007). The KF is composed of heterogeneous populations of neurons, most of which have a respiratory function (Cohen and Wang 1959; Dick et al. 1994; Dutschmann and Herbert 2006; Ezure and Tanaka 2006). Excitatory KF neurons project to the majority of the core respiratory nuclei in the brainstem, including the pre-Bötzinger complex, lateral parafacial nucleus, Bötzing complex, and rostral ventral respiratory group (Geerling et al. 2017; Song et al. 2012). Through these projections, the KF directly participates in the control of respiration, by modulating respiratory rate and pattern (Lumsden 1923; St-John et al. 1971; Cohen 1971; Cohen and Shaw 2004; Fung and St. John 1995; Jodkowski et al. 1994; Gautier and Bertrand 1975; Smith et al. 2007). As demonstrated by Fung and St John (1995), chemical lesion or pharmacological inhibition of the KF changes rate and pattern, by eliminating the post-inspiratory phase and prolonging inspiration. Conversely, pharmacological excitation of the KF prolongs the duration of the post-inspiratory phase (Dutschmann and Herbert 2006). These studies, in conjunction with others, support the critical role of the KF in the neural control of breathing.

KF neurons also project to a range of cranial nerves, for instance the vagus and hypoglossal nerves (Browaldh et al. 2016; Geerling et al. 2017). These projections are important in the coordinated control of the upper airways with breathing during orofacial behaviors involving similar anatomical pathways, such as swallowing, cough and vocalizations (Browaldh et al. 2016; Bautista et al. 2014; Jakus et al. 2008). Additionally, through connections with regions mediating the arousal component of hypoxic/hypercapnic chemoreflexes, the KF may also play a role in the state-dependent adjustments of respiratory rate and pattern (Kaur and Saper 2019). For instance, the KF receives chemosensory information from the lateral hypothalamus, retrotrapezoid nucleus (RTN), medullary raphe, the nucleus of the solitary tract (NTS) and the locus coeruleus (LC) (Guyenet and Bayliss 2015; Herbert et al. 1990; Hancock and Fougousse 1976; Kaur and Saper 2019; Peyron et al. 1998; Robertson et al. 2013; Yokota et al. 2016).

With this in mind, the purpose of this review is to illustrate the unique neurochemical makeup of the KF, mainly in the context of respiration. We will begin by emphasizing the various neurotransmitters, neuromodulators, neuropeptides, receptors, and transporters expressed in the KF. To gain an understanding of the neurochemical composition of the KF, we drew primarily from autoradiography, immunohistochemistry, *in situ* hybridization and anatomical tracing studies. In some of these studies the authors were not focused on KF, but included the KF in images and analysis as part of the parabrachial complex more generally. For these studies, we report findings about the parabrachial complex and extract, when possible, information pertaining to KF. Additionally, the described approaches have their pitfalls (e.g. antibody and probe selectivity, background or artifactual staining). Therefore, we also reference physiological studies to functionally confirm or dispute the conclusions made by histological methods. In the later portions of this review, we address neurochemical changes in the KF that lead to breathing disorders. Importantly, we do not intend for this to be an exhaustive review of the KF's function, since many reviews exist on the physiological role of the pons in breathing (Browaldh et al. 2016; Dutschmann and Dick 2012; Smith et al. 2007; Smith et al. 2013; Mörschel and Dutschmann 2009).

We hope that this review will be a valuable resource in understanding the complex neurochemical makeup of the KF, and by identifying gaps in our knowledge, it will aid future research on the neural control of breathing in health and disease.

Glutamate

Glutamate receptor expression in the KF

Glutamate is the key excitatory neurotransmitter in most brainstem respiratory neurons (reviewed in (Bonham 1995)). Two major classes of glutamate receptors are expressed in the mammalian brain: ionotropic and metabotropic. Ionotropic AMPA, NMDA and kainite receptors are ligand-gated ion-channels, whereas metabotropic glutamate receptors (mGluR₁₋₈) are G protein-coupled receptors. Expression of these glutamate receptors in the respiratory network changes during development (reviewed in Wong-Riley & Liu, 2005).

Glutamate injections into the KF elicit a characteristic post-inspiratory apnea and/or tachypnea, which is a useful step in functionally localizing the KF (Chamberlin and Saper 1994; Dutschmann and Herbert 2006; Saunders and Levitt 2020; Barnett et al. 2018). Multiple studies provide functional evidence for the presence of glutamate receptors in the KF (Dutschmann and Herbert 1998; Lara et al. 2002; Bautista and Dutschmann 2014a; Kuna and Remmers 1999; Dutschmann and Herbert 2006).

AMPA receptors are expressed in a wide range of variants due to multiple forms and combinations of subunits (GluA1-4). Most AMPA receptors contain the subunit GluA2, which makes them impermeable to Ca²⁺ (Brusa et al. 1995; Jonas and Spruston 1994; Sommer et al. 1991). Detected by immunohistochemistry, the KF of rats contains a moderate amount of GluA1 and GluA2/3, and a low amount of GluA4 (Chamberlin and Saper 1995). In addition to the variety in AMPA subunits, flip or flop splice forms of those subunits also have a substantial impact on the channel's kinetic and gating properties (Monyer et al. 1991; Lee et al. 1998). More than half of the analyzed KF neurons showed strong mRNA expressions of the GluA2 (formerly GluRB) flip and GluA3 (formerly GluRC) flip subunits. Similarly, the majority of KF neurons expressed GluA1 (formerly GluRA) flop and GluA3 (formerly GluRC) flop mRNAs (Guthmann and Herbert 1999a). Such subunit assemblies likely result in AMPA receptors that have increased glutamate sensitivity, longer desensitization times and higher levels of steady state currents (Mosbacher et al. 1994; Guthmann and Herbert 1999a).

NMDA receptors are heterogeneous due to the diversity of the splice forms and subunits that can form various assemblies and result in distinct NMDA receptors (Feldmeyer and Cull-Candy 1996). This heterogeneity in structure leads to differences in channel properties. The structural differences can affect, for instance, the receptor's affinity to glutamate, the strength of the Mg²⁺ block, or deactivation kinetics, leading to more global physiological consequences. The KF expresses an abundance of NR1, NR2B and NR2D subunits confirmed by immunohistochemistry against the proteins, as well as in situ hybridization for mRNA detection in rat brains (Guthmann and Herbert 1999b; Dutschmann et al. 1998; Kron et al. 2008). During development, NR2B expression decreases and NR2D expression increases in the KF (Kron et al. 2008). The NR2D isoform has longer deactivation times,

lower affinity for glutamate and a weaker Mg^{2+} block (Feldmeyer and Cull-Candy 1996). Consistent with a switch from NR2B to NR2D expression, NMDA-mediated synaptic currents in the KF were smaller and decayed slower in brain slices from rats at P14-21 than P1-5 (Kron et al. 2008).

Because noxious nasal stimulation increases the expression of immediate early gene cFos in NR1-labeled KF neurons, these neurons have been described as potential relay sites for the nasotrigeminal reflex circuit, at least in rats (Dutschmann et al. 1998). Furthermore, NR1-labeled KF neurons project to the Bötzing complex and are implicated in control of post-inspiration (Song et al. 2015).

Currently eight different mGluRs have been identified, termed mGluR1-8 (Hollmann 1994). Based on their composition and pharmacology, the eight receptor subtypes have been classified into three groups. Group I contains mGluR1 and mGluR5 and couples to Gq proteins. Group II contains mGluR2 and mGluR3, and couples to Gi/o proteins. Group III contains mGluR4 and mGluR6-8, and couples to Gi/o proteins. Immunohistochemistry against mGluR1 and mGluR5 (group I receptors) and mGluR2/3 (group II) in rats resulted in strong to moderate staining for all tested receptor subtypes in the KF (Guthmann and Herbert 1999c). The strongest immunoreactivity was observed against mGluR1, while staining for mGluR5 and mGluR2/3 was also present, but it was less prominent. This study also revealed that group I receptors are postsynaptic, with mGluR1 located on KF neuron dendrites and mGluR5 located on the soma. Group II receptors on the other hand, are presumably on presynaptic terminals or reside on glial cells. Studies into the different contributions of mGluRs to KF function and respiratory network function are very important, yet lacking.

Glutamate transporters in the KF

Glutamate transporters can be divided into two main classes, excitatory amino acid transporters (EAATs) and vesicular glutamate transporters (VGLUTs). Currently there are five known EAATs (EAAT1-5). EAAT1-4 are expressed in the brain, however EAAT 1 and 2 are mostly present in glia and EAAT5 has only been found in the retina (for review see (Zhou and Danbolt 2013). The presence of EAAT2 (also known as GLT-1) in the KF has not been specifically investigated. However, low levels of immunocytochemical labeling for different variants of GLT-1 can be detected in the KF and rest of the parabrachial complex in mouse brains (images of KF coronal sections obtained from the open access online microscopic image repository associated with the data set described by (Holmseth et al. 2009); <http://www.rbwb.org/>). EAAT3 can be found in neurons throughout the entire brain, and is solely localized to the soma and dendrites (Shashidharan et al. 1997). EAAT4 is most highly expressed in cerebellar Purkinje cells in mouse, rat and human brains, but is also found throughout the rodent forebrain (Massie et al. 2008). Neither EAAT3 nor EAAT4 can be detected in the brainstem of adult rodents (Furuta et al. 1997; Nieoullon et al. 2006).

The second family contains three known types of VGLUTs: VGLUT1-3. VGLUTs have a much lower affinity for glutamate than EAATs (Shigeri et al. 2004). VGLUT1 is predominantly expressed in the cortex, cerebellum, and hippocampus, while VGLUT2 is more restricted to the thalamus, deep cerebellar nuclei and the brainstem (Freneau et al.

2001). Most neurons located in the rostral parts of the KF are glutamatergic and express VGLUT2, however VGLUT2 expressing KF neurons are more sparse in the caudal regions of the nucleus, as shown in mice (Geerling et al. 2017; Kaur et al. 2013; Niu et al. 2010). VGLUT3 can be detected in the parabrachial complex, however its presence in the KF has not been examined yet (Guo et al. 2005).

Sources of glutamate in the KF

The majority of KF (and parabrachial complex) neurons are glutamatergic, however the density of glutamatergic cells decreases progressively towards the caudal portions of the KF (Geerling et al. 2017). These glutamatergic KF neurons send extensive axonal projections to caudal targets in the rodent brain, such as the NTS, medullary reticular formation, Bötzing complex, pre-Bötzing complex, and rostral ventral respiratory group (Ezure and Tanaka 2006; Yokota et al. 2007; Herbert et al. 1990; Geerling et al. 2017). A large proportion of parabrachial complex neurons express the transcription factor, Forkhead box protein 2 (FoxP2), however KF neurons only show minor expression for this marker. Most FoxP2 expressing neurons are glutamatergic and express VGLUT2 mRNA in the entire parabrachial complex, including the sparse FoxP2+ KF neurons (Geerling et al. 2011; Geerling et al. 2017). The developmental origin of the densely packed, rostral KF glutamatergic neurons is unknown.

KF neurons also receive glutamatergic modulation from a wide range of brainstem respiratory nuclei (Figure 2). For instance, excitatory, somatostatin (SST)-expressing pre-Bötzing complex neurons project to the KF (Yang and Feldman 2018). These excitatory projections between the pre-Bötzing complex and KF are hypothesized to be important in the control of respiratory phase-transitions in the context of breathing, as well as behaviors strictly coordinated with breathing (sniffing, whisking, swallowing, vocalizations, etc.).

Glutamatergic projections to the KF also originate from the marginal layer of the ventral surface of the medulla (the area corresponding to the retrotrapezoid nucleus [RTN]), detected by a combination of retrograde labeling and in situ hybridization for VGLUT2 mRNA in rats (Weston et al. 2004). While this study did not specifically look at projections to the KF, the example injection sites and summary figures indicate the inclusion of the KF. In a more recent study, a majority of retrograde labeled varicosities from the RTN to the KF were positive for VGLUT2 confirming glutamatergic input from RTN (Silva et al. 2016). Additionally, arousal inducing chemosensory information may arrive indirectly to the KF from the carotid bodies, through the nucleus of the solitary tract (NTS; Song et al., 2011; Kaur et al., 2013). Labeling and antidromic activation experiments performed in rats indicate that hypoxia-activated neurons in the NTS send excitatory projections to the KF that may be glutamatergic (Gozal et al. 1999; Song et al. 2011).

Functional role of glutamate in the KF

Representing the most prevalent type of neurotransmitter in the KF, glutamatergic modulation of KF neurons has widespread functional implications. One of the most important and most heavily studied functions of the KF is in the control of respiratory rate and pattern. NMDA receptor activation in the KF/parabrachial complex stimulates

respiratory rate in normal, as well as hypoxic conditions, and influences sleep-wake states (Boon and Milsom 2008; Bonis et al. 2010a; Chamberlin and Saper 1994). Sequential microinjections of both AMPA- and NMDA-antagonists into the KF and parabrachial complex of decerebrate rabbits results in substantially decreased respiratory rate (Navarrete-Opazo et al. 2020). In rats, blockade of NMDA receptors in the KF abolishes the post-inspiratory phase of breathing in baseline and post-hypoxia conditions (Song et al. 2015), and reduces nasotrigeminal reflex induced respiratory depression and bradycardia (Dutschmann and Herbert 1998). Conditional deletion of Vglut2 from specific portions of the parabrachial complex, including the KF, results in delayed or lack of arousal responses to increased CO₂, indicating a potential role in the chemosensory control of respiration in mice (Kaur et al. 2013).

In the arterially perfused working heart-brainstem preparation of the rat, glutamate microinjections into the KF prolong the post-inspiratory phase of respiration (which has direct effects on respiratory rate by prolonging expiration potentially through modulation of the rhythm generator), and causes glottis closure in the upper airways (Dutschmann and Herbert 2006). Direct application of glutamate receptor agonists or antagonists into the KF has been shown to modulate the activity of multiple cranial nerves, many of them with important roles in upper airway control, including the recurrent laryngeal, trigeminal and hypoglossal nerves (for a review see: (Browaldh et al. 2016)). For instance, unilateral kainic acid, NMDA or AMPA microinjections in the KF lead to the tonic excitation of the hypoglossal nerve (Kuna and Remmers 1999). These experiments provide further support for the KF's pivotal role in upper airway patency.

GABA

GABA receptor expression in the KF

The main inhibitory neurotransmitter in the nervous system, gamma-aminobutyric acid (GABA), binds to two types of receptors. The first group, GABA-A receptors are ionotropic, composed of five homologous or heterologous subunits, forming 19 different isoforms of a ligand-gated Cl⁻ channel that is the functional receptor (Olsen and Sieghart 2008). The second group, GABA-B receptors are metabotropic G-protein coupled receptors encoded by three GABA-B receptor genes (Kaupmann et al. 1998).

A detailed study on GABA-A receptor expression in the parabrachial complex, and specifically addressing the KF, was conducted by Guthmann et al. (1998). In this study, immunolabeling of GABA-A receptor subunits revealed the presence of γ 2-subunit on the cell bodies and dendritic profiles of KF neurons in rats. The α 1 and β 2/3 subunits were mainly present on KF neuron dendrites, while they were also detectable on parabrachial neuron somas. Moderate staining was present on KF somas for α 2 and α 3 subunits, however, these subunits were not evident on dendritic processes in the KF.

Such detailed specific description of the expression of GABA-B receptor variants in the KF is not available. Nevertheless, strong immunohistological labeling of GABA-B-R1 variant was shown in general in the 'pontine nuclei' (Margeta-Mitrovic et al. 1999), while a different study found that the GABA-B-R1b variant (the more prevalent version of GABA-

B-R1) was seldom expressed in the pons (Fritschy 1999). While the exact variant of GABA-B receptors in the KF is currently not known, acute KF slice experiments conducted in our laboratory provides functional evidence for the presence of GABA-B receptors on KF neurons. Application of the GABA-B agonist baclofen leads to the activation of G-protein coupled inwardly rectifying K⁺ (GIRK) conductance in a large population (~60%) of KF neurons in both rats and mice (Levitt et al. 2015; Levitt and Williams 2018; Varga et al. 2020). Furthermore, the GABA-B receptor positive neurons are also sensitive to opioids (Varga et al. 2020; Levitt and Williams 2018).

GABA transporters in the KF

Out of the currently known GABA transporter genes, the ones prevalently present in the central nervous system are GAT1 and GAT3 (for a review on GABA transporters see (Zhou and Danbolt 2013)). In the rat KF, an in situ hybridization study detected the presence of both GAT1 and GAT3 mRNA (Durkin et al. 1995). Meanwhile, also performed in rats, immunohistochemical characterization of the KF region revealed contrasting results, with prominent GAT3 immunoreactivity, but no GAT1 labeling at all (Guthmann et al. 1998). Immunolabeling for GAT3 was exclusive to glia and neuronal processes, but not cell bodies, which is in agreement with previous reports on GAT3 expression (Ribak et al. 1996; Minelli et al. 1996; Minelli et al. 1995; Guthmann et al. 1998). Arguably, the presence of GAT1 mRNA detectable by in situ hybridization does not necessitate the expression of the GAT1 protein (detectable by immunohistochemistry), nevertheless further supportive evidence is required to reconcile this discrepancy. Additionally, the GABA vesicular transporter (Vgat) has been shown to co-localize with FoxP2 in the caudal portions of the KF (Geerling et al. 2017).

Sources of GABA in the KF

In the mammalian brain, GABA is synthesized via decarboxylation of glutamate into GABA and CO₂, by the catalytic enzyme glutamic acid decarboxylase (GAD). GAD is encoded by two genes in two isoforms, GAD65 and GAD67 (Erlander et al. 1991). While both isoforms are present throughout the neuron, GAD65 is more prominent in axon terminals (Kaufman et al. 1991). GAD immunolabeling has been shown in KF somas as well as axon terminals (Ford et al. 1995; Guthmann et al. 1998). The regional distribution of GAD65 and GAD67 mRNA and/or protein is densely clustered and overlaps in the caudal portions of the KF and the lateral parabrachial nucleus. Both classes of GAD show overlapping, but scattered expression patterns in the rest of the parabrachial complex in rodent brains (Guthmann et al. 1998; Geerling et al. 2017). Thus far two subpopulations of GAD67-expressing neurons have been identified in the caudal KF, with one group co-expressing the transcription factor FoxP2, while the other group lacking FoxP2 (Gray 2008; Miller et al. 2012; Geerling et al. 2011; Geerling et al. 2017). The KF also receives GABAergic inhibitory input from the RTN, NTS, and the Bötzing complex (Figure 2; Ezure et al., 2003; Silva et al., 2016).

Functional role of GABA in the KF

Although the majority of KF neurons are glutamatergic, the more caudal KF contains GABAergic clusters of neurons that also have extensive projections throughout the brainstem (Geerling et al. 2017). The most prominent GABAergic projections originating

from the KF target the sensory trigeminal nuclei, showing dense labeling at the level of pars interopolaris and extending with sparse projections all the way to the medullary reticular formation (Geerling et al. 2017). Most of these projections contact ipsilateral target sites. Geerling and colleagues (2017) also found GABAergic projections from KF to the hypoglossal and facial motor nuclei, the NTS and the ventrolateral medulla (including the Böttinger and pre-Böttinger complexes). Rostral GABAergic projections from the KF extend to the periaqueductal gray, midbrain reticular formation and a subset of hypothalamic nuclei (Geerling et al. 2017).

A range of pharmacological studies have provided clues about the role of GABAergic signaling in the KF (to review, see (Browaldh et al. 2016)). Direct administration of GABA-A receptor agonists into the KF has a strong effect on respiratory related functions. For instance, locally applied muscimol decreases respiratory rate, increases the length of inspiration and expiration, increases resting diaphragm EMG amplitude, decreases diaphragm EMG frequency, and decreases genioglossus EMG amplitude in anesthetized, vagotomized rats (Silva et al. 2016). In a different study, using the in situ preparation of the rat, GABA-A agonist isoguvacine abolished post-inspiration and eupneic hypoglossal activity, while also disrupting the breathing pattern (Bautista and Dutschmann 2014a). Isoguvacine injected into the KF also leads to apneusis, abolishes laryngeal adductor activity (Dutschmann and Herbert 2006), and causes drastic changes in the baseline respiratory pattern that have been implicated in the coordination of swallows (Bautista and Dutschmann 2014b; Bautista et al. 2014).

Disinhibition of the KF by bicuculline, a GABA-A receptor antagonist, disrupts the swallowing reflex and leads to delayed swallowing in the in situ preparation of the rat (Bautista and Dutschmann 2014b). Such failure to properly coordinate swallowing and breathing could lead to aspiration. Injection of bicuculline into the KF exacerbates respiratory depression and bradycardia evoked by ethmoidal nerve (sensory branch of the trigeminal nerve) stimulation (Dutschmann and Herbert 1998). These data combined with the anatomical data showing dense KF projections to the trigeminal nuclei, support the KF's potential role in coupling respiratory and trigeminal inputs, perhaps to coordinate orofacial/whisking behaviors with breathing, or to initiate the diving reflex (Dutschmann and Herbert 1996; Geerling et al. 2017; Dutschmann and Herbert 1998).

Based on the wide spectrum of results, it is clear that the KF is an important mediator of many respiratory related behaviors and GABAergic neurotransmission plays a crucial role in the coordination of the sequential steps involved in these behaviors. As discussed in the "Neurochemical alterations in the context of respiratory dysfunction" section, alterations in KF GABAergic neurotransmission have serious consequences in the pathophysiology of respiratory dysfunction.

Glycine

Glycine Receptor Expression

The amino acid glycine serves as one of the main inhibitory neurotransmitters in the brainstem and spinal cord. The glycine receptor (GlyR) is an ionotropic heteropentamer,

located primarily in the brainstem and spinal cord (Rajendra et al. 1997). GlyRs are ligand-gated chloride channels allowing for rapid postsynaptic inhibition. Glycinergic inhibition has been implicated in the modulation of respiratory frequency and pattern (Schmid et al. 1991; Janczewski et al. 2013). A functional GlyR consists of three α subunits and two β subunits, with each α subunit containing a glycine-binding site, while the β subunits are thought to anchor GlyR at synaptic sites (Herbert et al. 2000; Grudzinska et al. 2005).

The expression of mRNA for the $\alpha 1$, $\alpha 2$, and β subunits of the glycine receptor in the parabrachial complex and specifically in the KF has been confirmed by in situ hybridization (Fujita et al. 1991; Sato et al. 1991; Sato et al. 1992). GlyR $\alpha 1$ subunit expression in the KF has also been documented by immunohistochemistry, with differences in both staining intensity and pattern noted between the rostral and caudal portions. GlyR $\alpha 1$ immunoreactivity in the rostral KF is strong, granular and diffusely distributed throughout the fibers, whereas weaker staining of cell bodies and dendrites can be observed in the caudal KF of rats (Herbert et al. 2000).

Glycine Transporter Expression

Glycine transporters consist of 12 transmembrane domains and rely on the co-transport of Na^+/Cl^- into the cell with glycine (Zafra and Giménez 2008). There are two gene products for the glycine transporter, GlyT1 and GlyT2. GlyT1 is expressed mainly in glial cells, including in astrocytes, where it influences excitatory neurotransmission through the regulation of glycine levels at glutamatergic synapses containing the NMDA receptor, at which glycine acts as a co-agonist (Gabernet et al. 2005). Under this framework, reduced GlyT1 expression corresponds with a potentiation of NMDA receptor function. In the KF of rats, GlyT1 immunoreactivity is relatively weak compared to the surrounding parabrachial nuclei, despite strong expression of mRNA for the NR1 subunit of the NMDA receptor (Herbert et al. 2000).

The GlyT2 transporter is located on the axons and other presynaptic elements of glycinergic neurons, and mediates the uptake of glycine from the extracellular space before it is packaged into vesicles (Rousseau et al. 2008). The uptake of glycine by GlyT2 controls the intracellular concentrations of glycine and therefore determines the vesicular content of glycine (Rousseau et al. 2008). In the parabrachial complex, including the KF, GlyT2 expression is limited to areas that express either glycine or GlyR $\alpha 1$ immunoreactivity (Herbert et al. 2000).

Sources of Glycine in the KF

Glycine is made from serine using the enzymes serine hydroxyl-methyl-transferase and glycine decarboxylase (Hernandes and Troncone 2009). However, the concentrations of glycine available at terminals is significantly higher than can be synthesized locally, indicating that uptake by GlyT2 or other forms of local recapture are needed to maintain presynaptic glycine supplies (Rousseau et al. 2008). Glycine immunoreactivity in the KF takes the form of punctate labeling along axonal fibers and at the terminals of presumed afferent axons. Of these glycine-immunoreactive puncta, some are located adjacent to unlabeled cell bodies, suggesting that the KF contains axosomatic glycinergic contacts

(Herbert et al., 2000). Additionally, the presence of scattered cell bodies outlined by glycine immunoreactivity has also been consistently documented in the rat KF (Rampon et al., 1996; Herbert et al., 2000).

Using transgenic mice and Cre-dependent viral labeling, Yang & Feldman (2018) documented projections from GlyT2-positive glycinergic neurons in the pre-Bötzinger complex to many brainstem regions, including the KF (Figure 2). These glycinergic projections parallel projections from glutamatergic somatostatin-positive pre-Bötzinger neurons to many respiratory regions, but their function has not yet been identified.

While no other glycinergic projections to the KF have been directly confirmed, several regions that project to the KF are known to contain glycinergic neurons. Some pump cells of the NTS have been shown to express GlyT2 mRNA, and are thought to co-release GABA and glycine (Ezure and Tanaka 2004). NTS pump cells are known to project to the KF, and may therefore represent a source of glycinergic input (Ezure and Tanaka 2006). Afferent projections also extend to the KF from the inhibitory augmenting expiratory neurons of the Bötzinger complex (Ezure et al. 2003b), some of which co-release glycine and GABA (Schmid et al. 1996), have also been found.

Functional role of Glycine in the KF

While both GABAergic and glycinergic synaptic currents are present in KF neurons, the glycinergic component becomes more predominant later in development, at least in the rat brain (Kron et al. 2007b). Despite the role of the KF as a relay site in trigeminally-evoked bradycardia and respiratory suppression, it appears that transmission through glycine receptors in the KF is not essential for modulating nasotrigeminal reflex responses in rats (Dutschmann and Herbert 1998). While unilateral injections of the GABA-A antagonist bicuculline in the KF enhance trigeminally-evoked bradycardia and respiratory suppression, injections of the GlyR antagonist strychnine does not significantly alter these autonomic responses (Dutschmann and Herbert 1998).

Acetylcholine

Acetylcholine receptor expression in the KF

Acetylcholine (ACh) is a neurotransmitter that activates two flavors of receptor: the ionotropic nicotinic and metabotropic muscarinic acetylcholine receptor (nAChR and mAChR respectively). Both receptors are expressed throughout the central nervous system (Wamsley et al. 1984; Tribollet et al. 2004; Levey et al. 1991) and modulate the excitability and firing rate of neurons (Papke 2014; Felder 1995; Wess 1996). The nAChR is a ligand-gated pentameric cation channel that is expressed pre- and/or post-synaptically and formed by various compositions of α (α 2-10) and β (β 2-4) subunits. Activation by ACh or nicotine causes depolarization and increases neuronal excitability (Papke 2014). Conversely, the mAChR, activated by ACh or muscarine, is coupled to G-proteins and expressed in five different receptor subtypes (M1-M5). M1, M3, and M5 receptor subtypes are excitatory and couple to Gq, while M2 and M4 are inhibitory and couple to Gi/o (Wess 1996; Felder 1995).

Autoradiography studies performed in the lateral parabrachial nucleus identified the expression of $\alpha 7$ nAChR in the vicinity of the KF in rats (Tribollet et al. 2004). In human studies, an increase in $\alpha 7$ nAChR expression was observed in the KF of fetuses and early newborns with smoking mothers (Lavezzi 2018). $\alpha 2$ -containing nAChRs are also expressed in the parabrachial complex of rats and mice (Ishii et al. 2005).

There are few histological studies regarding the expression of mAChRs in the KF. Using a nonselective mAChR antagonist probe, mAChR expression was shown in the parabrachial complex by autoradiography (Wamsley et al. 1984), however, receptor subtype expression was not assessed. Examination of the Allen Brain Atlas suggests mAChR mRNA expression in the KF. Further support for mAChR expression specifically in the parabrachial complex and specifically in the KF comes from physiological studies detailed below in the “Functional Implications of Acetylcholine” section.

Sources of Acetylcholine in the KF

Acetylcholine is synthesized in cholinergic neurons by choline acetyltransferase (ChAT). ChAT is expressed in two pontine tegmental nuclei that are nearby the parabrachial complex: 1) the pedunclopontine tegmental nucleus, which is rostral to the parabrachial complex, and 2) the laterodorsal tegmental nucleus, which is medial to the parabrachial complex (Armstrong et al. 1983). Representing the main source of acetylcholine for most forebrain and hindbrain structures, local projections from the pontine tegmental nuclei likely contribute to KF cholinergic signaling (Figure 2; Woolf & Butcher, 1989). In addition, immunohistochemistry identified ChAT expression locally, in the parabrachial complex of cats (Jones and Beaudet 1987). Recently taking advantage of ChAT^{Cre} transgenic mice, lateral parabrachial and KF neurons have been shown to co-express cholinergic and glutamatergic markers transiently during development, which may be important for maturation of excitatory synapses (Nasirova et al. 2020).

Acetylcholine transporters in the KF

Acetylcholine is packaged into synaptic vesicles by vesicular ACh transporter (VACHT; (Parsons et al. 1993). Coexpression of ChAT and VACHT in a neuron indicates functional cholinergic neurotransmission (Schäfer et al. 1994). *In situ* hybridization identified VACHT mRNA in the parabrachial complex of adult rats (Schäfer et al. 1994). Furthermore, this finding is observed in the Allen Brain Atlas, which shows VACHT expression in the KF and parabrachial nuclei. In the synaptic cleft, ACh is rapidly hydrolyzed to acetate and choline by acetylcholinesterase (AChE). Choline uptake and recycling is then performed by the choline transporter 1 (CHT1), which is distributed in low densities on fibers in the pontine nuclei (Misawa et al. 2001).

Functional Implications of Acetylcholine

Although few histological studies have confirmed the expression of mAChR in the KF, functional studies provide further support for local expression of this receptor subtype. Egan and North (1986) found that muscarine (mAChR agonist) hyperpolarized parabrachial complex neurons in rat intracellular recordings by acting through the M2 mAChR subtype. Additionally, microdialysis of atropine, a mAChR antagonist, into the KF of goats has been

shown to decrease pulmonary ventilation and breathing frequency at night (Bonis et al. 2010b). In a later study using the arterially perfused working heart-brainstem preparation, carbachol, a nonselective AChR agonist, was microinjected into the pons of rats (Brandes et al. 2011). While the study did not focus on the effects of AChR agonist on the KF, in a couple of animals carbachol unilaterally injected in the KF led to increases in respiratory rate, indicating the presence of mAChRs (Brandes et al. 2011). These functional studies, considered in conjunction with the above histological evidence, support mAChR expression in the parabrachial complex and more specifically the KF. Nevertheless, future research, with particular attention given to understanding the specific expression of mAChR subtypes (M1-M5) should provide important details regarding the cholinergic neurochemistry of the KF and its impact on breathing.

Norepinephrine

Norepinephrine receptor expression in the KF

Norepinephrine (NE), or noradrenaline, is a catecholamine synthesized by noradrenergic neurons in the brainstem. NE has differential effects on target neurons depending on the adrenergic receptor subtype(s) present. Adrenergic receptors are GPCRs that can be broadly classified as α 1, α 2, or β receptors, each of which has three subtypes (α 1A-C, α 2A-C, and β 1-3) (for review, see (Lefkowitz 1990; Strosberg 1993)). α 1 receptors are excitatory Gq-coupled, while α 2 receptors are inhibitory Gi/o-coupled. All three β receptor subtypes are Gs-coupled.

A wide range of histological methods, including autoradiography and *in situ* hybridization, indicate the presence of α 2 receptors in the pontine nuclei, including the parabrachial nuclei and the KF (Scheinin et al. 1994; Unnerstall et al. 1985; Herbert and Flügge 1995; Andrade et al. 2014; Davern 2014). Specifically, moderate to intense labeling for α 2A and α 2B-receptor subtypes has been documented in the KF of multiple species (Rosin et al. 1993; Wang et al. 1996; Tavares et al. 1996; Strazielle et al. 1999). To our knowledge, there are no reports directly addressing the presence of β adrenergic receptors in the KF. Since the pons and medulla have been shown to only express very low amounts of β -receptors, the KF likely only expresses a limited amount (if any) of this adrenergic receptor subtype (Palacios and Kuhar 1982).

Norepinephrine transporter in the KF

The norepinephrine transporter (NET) plays a key role in regulating noradrenergic signaling in the brain by maintaining intracellular NE stores and via NE reuptake from the synapse into the presynaptic terminals (Torres et al. 2003). NET mRNA labeling has been described in locations consistent with the presence of catecholaminergic cell groups A4, A5, A6 (or locus coeruleus), A7, A1/C1 and A2/C2 (Lorang et al. 1994). Additionally, the presence of NET correlates with the level of noradrenergic innervation, with autoradiography demonstrating moderate levels of the transporter in the KF region of the mouse brain (Strazielle et al. 1999).

Sources of norepinephrine in the KF

The primary source of NE throughout the brain is the locus coeruleus (LC), however other noradrenergic areas also contribute (Robertson et al. 2013; Swanson and Hartman 1975; Schwarz and Luo 2015; Jones and Moore 1977). Indeed, noradrenergic neurons form an almost continuous column of NE producing nuclei in the hindbrain (Robertson et al. 2013). The cellular makeup and developmental origin of the nuclei is somewhat heterogeneous, with each neuron subtype derived from one of six distinct rhombomere-based genetic lineages (r1-r6, (Robertson et al. 2013)). The parabrachial complex, including the KF, receives axonal projections from r1-derived neurons that make up the majority of the LC, as well as neurons of r3, r4 and r5 origin, which are present in the subcoeruleus area, A7, A5, and the adrenergic/noradrenergic C1/A1 and C2/A2 areas in the mouse medulla (Figure 2; Robertson et al., 2013). Projections from the LC and subcoeruleus to KF have also been demonstrated by retrograde tracing studies in cats and monkeys (Hancock and Fougousse 1976). Additionally, a range of anatomical studies performed on cat, rat and mouse brains, indicate the presence of noradrenergic cells in the KF, many of which refer to the region as KF/A7, thus combining the KF with the noradrenergic A7 nucleus (Hermanson and Blomqvist 1995; Stevens et al. 1982; Reddy et al. 1991; Strazielle et al. 1999). Some of these studies imply that the noradrenergic neurons located in the KF functionally belong to the A7 nucleus, but cannot be properly distinguished due to lack of anatomical borders. However, a subset of experiments (done on the same species) indicate that the KF and A7 are independent pontine nuclei, both of which contain NE producing neurons that differentially express a range of other neuropeptides (Figure 2; Holstege & Kuypers, 1982; Clark & Proudfit, 1991; Reddy et al., 1991).

Functional role of norepinephrine in the KF

Most results on the role of noradrenergic KF neurons come from anatomical tracing studies and provide consistent results from a range of species. However, direct functional and physiological evidence is still lacking. The majority of NE producing KF neurons project to the superficial laminae of the dorsal horn and the thoracic sympathetic cell column, and these projections have an inhibitory effect on spinal motoneurons (Reddy et al. 1991; Clark and Proudfit 1991; Holstege and Kuypers 1982; Stevens et al. 1982). The specific role or roles of noradrenergic KF neurons in modulating spinal control of behavior remains unknown.

Serotonin

Serotonin receptor expression in the KF

There are seven general classes of serotonin receptors that include 14 known receptor subtypes (Hoyer et al. 2002; Barnes and Sharp 1999). Serotonin receptors are GPCRs with the exception of 5-HT₃ receptors that are ligand-gated ion channels (Bockaert et al. 2006; Maricq et al. 1991; Van-Hoof and Yakel 2003). Of all serotonin receptor types, the 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT₄, and 5-HT₇ receptors have been implicated in the involvement of the mammalian respiratory network and therefore speculated to be present in the KF (Barrett et al. 2012; Erickson et al. 2007; Yamauchi et al. 2008; Richter et al. 2003; Dhingra et al. 2016). Current studies have explicitly shown the expression of G_{i/o}-coupled 5-

HT_{1A}, and G_q-coupled 5-HT_{2A} and 5-HT_{2B} receptors in the rodent KF (Dhingra et al. 2016; Niebert et al. 2011).

Sources of serotonin in the KF

In the central nervous system, serotonin is primarily produced in the raphe nuclei found in the midbrain and brainstem (Törk 1990). Among these, the caudal raphe projects extensively to the pontine respiratory group, which includes the KF (Figure 2; Steinbusch, 1981; Steinbusch et al., 1981; Hilaire et al., 2010). Afferent projections from the KF to the nucleus raphe magnus of the medulla have also been shown, thus completing reciprocal connections between the raphe and the KF (Gang et al. 1991; Gang et al. 1990; Gang et al. 1993).

Functional role of serotonin in the KF

Although the activation of 5-HT receptors has been implicated in a wide variety of neurological processes, its effects on respiration provide the most insight into the role of serotonin in the KF (for a review on 5-HT's role in respiration see Hodges & Richerson, 2008).

Intracerebroventricular (ICV) administration of a 5-HT_{1A} receptor antagonist, WAY100635, increases respiratory variability and silences upper airway muscle respiratory activity in rats (Besnard et al. 2012). In contrast, ICV administration of a 5-HT_{1A} receptor agonist, 8-OH-DPAT, increases upper airway muscle activity (Besnard et al. 2012). Given the importance of the KF in coordination of the upper airways, these effects could be mediated in part by the KF. 5-HT_{1A} receptor activation leads to a substantial increase in the immediate early gene Fos expression in many respiratory nuclei, including the KF (Besnard et al. 2012). Administration of 8-OH-DPAT, a 5-HT_{1A} receptor agonist, directly into the KF improves respiratory regularity in a mouse model of Rett syndrome, providing further evidence for functional 5-HT_{1A} receptors in the KF (Abdala et al. 2010). The importance of KF 5-HT_{1A} receptors on variability was also confirmed in a later study where microinjection of the antagonist WAY100635 into the KF led to a significant increase in respiratory pattern variability, characterized by spontaneous central apneas in mice (Dhingra et al. 2016). Together these data provide evidence for the KF's involvement in the serotonergic modulation of the upper airways and respiratory pattern control.

Opioids

Opioid receptor expression in the KF

Opioid receptors are G_{i/o} protein-coupled receptors that are widely distributed throughout the mammalian brain and have been implicated in a multitude of physiological processes depending on their location, ranging from analgesia to reward and addiction, stress resilience and homeostatic control (Toubia and Khalife 2019; Adams et al. 1986). The three major types of opioid receptors are delta (DOR), kappa (KOR) and mu opioid receptors (MOR; for reviews on opioid receptors see Waldhoer et al., 2004; Williams et al., 2013; Manglik, 2019). MORs are abundantly expressed in the KF based on immunohistochemistry and *in situ* hybridization in rats and analysis of genetic knock-in mice with mCherry-tagged MOR (Chamberlin et al. 1999; Ding et al. 1996; George et al. 1994; Erbs et al. 2014). Moderate

intensity of DOR expression was documented by both immunohistochemistry and *in situ* hybridization in the parabrachial complex (Cahill et al. 2001). Similarly, DOR positive fibers and varicosities, as well as DOR immunoreactive cell bodies were detected in the parabrachial complex and A7 area (Arvidsson et al. 1995). KOR expression has not been documented in the KF.

Physiological studies in rodents also support the expression of MORs in the KF (Levitt et al. 2015; Varga et al. 2020) and nearby parabrachial nuclei (Prkic et al. 2012; Miller et al. 2017). In single-cell recording of KF neurons in brain slice, about 60% of KF neurons express somatodendritic MORs (Levitt et al. 2015). There is no functional evidence of somatodendritic DORs, since the effects of the MOR/DOR agonist [Met⁵]-enkephalin are blocked by the selective MOR antagonist CTAP (Levitt et al. 2015) and are absent in neurons with MORs deleted (Varga et al. 2020).

Sources of endogenous opioids in the KF

Endogenous opioid peptides have important roles in a wide range of physiological functions, including analgesia, stress relief, feeding behaviors, and importantly, cardiorespiratory control (Adams et al. 1986; Toubia and Khalife 2019). Endogenous opioids are produced via enzymatic splicing of pre-pro-peptides and the functional products of splicing are categorized as: β -endorphin that binds to MOR, enkephalins that bind to DOR and MOR, and dynorphins that bind to KOR and MOR (Corder et al. 2018; Waldhoer et al. 2004). β -endorphin is cleaved from pro-opiomelanocortin in the hypothalamus/pituitary and NTS (Figure 2; Lee & Wardlaw, 2007; Cerritelli et al., 2016). Similarly, endogenous enkephalins are cleaved from the precursor protein pre-pro-enkephalin. Functional enkephalin neuropeptides, Met- and Leu-enkephalin, have high affinity for DOR and also bind to MOR (Udenfriend and Kilpatrick 1983; Corder et al. 2018; Adams et al. 1986). Endogenous opioids are released in response to pain, stress and/or inflammation, and even in response to the administration of exogenous opioids (Schlen and Bentley 1980; Holaday et al. 1977; Adams et al. 1986).

Enkephalin is produced locally in the KF (Figure 2; Blomqvist et al., 1994; Hermanson & Blomqvist, 1995). *In situ* hybridization in rats revealed a large population of parabrachial complex neurons, including KF neurons, that express pre-pro-enkephalin mRNA (Hermanson and Blomqvist 1997; Engström et al. 2001). KF neurons containing pre-pro-enkephalin send projections to the paragigantocellular nucleus of the ventral medulla, as well as the spinal cord, and participate in analgesia (Blomqvist et al. 1994). Similarly, β -endorphin can be detected with immunolabeling in moderate to high levels in the parabrachial complex of rats, however this study did not specifically investigate peptide expression in the KF (Palkovits and Eskay 1987). On the other hand, endomorphin-1 and endomorphin-2 immunoreactive fibers are distributed differentially in the KF, with endomorphin-2 showing dense labeling and endomorphin-1 only present on a few fibers in rats and guinea pigs (Martin-Schild et al. 1999). Endomorphin-2 labeling is more intense in the KF of mice compared to rats (Martin-Schild et al. 1999).

Functional role of opioids in the KF

While not much is known about the exact function of endogenous opioids in the KF, there are multiple studies focusing on the effects of exogenous opioids. Exogenous MOR agonists have been shown to play a substantial role in opioid-induced respiratory rate depression and breathing pattern degradation in multiple species (Prkic et al. 2012; Miller et al. 2017; Lalley et al. 2014; Levitt et al. 2015; Saunders and Levitt 2020; Varga et al. 2020). Opioid agonists directly hyperpolarize a population of KF neurons by activating GIRK conductance (Levitt et al. 2015). Further details on the KF's role in opioid-induced respiratory depression are out of the scope of this review.

Neuropeptide S

Neuropeptide S receptor expression in the KF

Neuropeptide S (NPS) is a regulatory neuropeptide expressed in the brainstem. The NPS system has been associated with modulation of many different central functions, including stress/anxiety, arousal, feeding and digestion, addiction, memory, and pain (for review see Guerrini et al., 2010). Expression of mRNA for the NPS precursor peptide in rodents appears to be limited to a few pontine areas, including the parabrachial complex, and specifically the KF. However, expression of the NPS receptor, NPSR, is more widespread, with mRNA distributed throughout areas associated with sleep, anxiety, and sensory processing, including parts of the hippocampus, hypothalamus, and the amygdaloid cortex, as well as in the piriform cortex (Xu et al. 2007). NPSR is a G_q and G_s-coupled receptor, producing increases in intracellular Ca²⁺ and cAMP after NPS administration (Reinscheid et al. 2005; Xu et al. 2004). Compared to cortical and thalamic areas, low expression of NPSR mRNA has been found in the brainstem (Reinscheid and Xu 2005). In the lateral parabrachial nucleus and KF of mice, only a few scattered NPSR-expressing fibers were found with immunohistochemistry, while in situ hybridization for NPSR mRNA revealed moderate expression in the lateral parabrachial nucleus (Clark et al. 2011).

Sources of Neuropeptide S in the KF

Using in situ hybridization for NPS precursor mRNA, Clark et al. (2011) demonstrated that in the mouse brain, NPS-producing neurons were isolated to the KF and the pericoerulear area (Figure 2). In contrast, NPS-producing neurons in the rat were limited to the area of the principle sensory trigeminal nuclei, the pericoerulear area (LC and nearby catecholaminergic clusters), and the lateral parabrachial nucleus, with a few scattered fibers in the dorsomedial hypothalamus and amygdala. In rats, the number of NPS-producing cells is greater in the LC cluster than the lateral parabrachial cluster, while in mice, the number of NPS-producing cells in the parabrachial cluster was greater than in the LC cluster (Liu et al. 2011). Additionally, the lateral parabrachial cluster is considered to align with the KF in mice, but not in rats. However, the parabrachial cluster in both species was found to co-express corticotropin-releasing factor, suggesting they may be functionally similar, despite slight differences in spatial location. In humans, expression of NPS precursor mRNA has been documented in the parabrachial nuclei and KF (Adori et al. 2015).

Functional Role of Neuropeptide S in the KF

In other brain regions, such as the pericoerulear cluster, NPS neurons have been shown to be glutamatergic, suggesting a close association between NPS and excitatory signaling. In the rat parabrachial complex, NPS-expressing neurons have been shown to co-express corticotropin-releasing factor, implicating NPS signaling in arousal and anxiety (Xu et al. 2007). Short term stress, induced via a 10-minute swim test or a 20-minute prolonged restraint test, caused expression of cFos protein in NPS-producing neurons, though co-localization was lesser than in the LC cluster of NPS-producing neurons (Liu et al. 2011). This implies that both the KF and LC clusters of NPS-producing neurons are activated by stress, suggesting they may play a role in anxiety, arousal and other stress-mediated behaviors. Additionally, it is thought that the NPS-producing neurons of the KF region may receive synaptic inputs from Orexin A/hypocretin-positive cells, suggesting a potential role for the KF-NPS system in the arousal-mediated modulation of respiration in mice (Liu et al. 2011). Arousal signaling in the parabrachial complex is particularly important in respiratory control, with the KF and parabrachial nuclei shown to play a role in state-dependent, high CO₂-induced arousal (or hypercapnic arousal) - a staple of sleep apnea (Kaur et al. 2013; Kaur and Saper 2019; Kaur et al. 2017).

The NPS peptide is predominantly produced in areas implicated in the control of respiration (Xu et al. 2007). The NPS/NPSR system has also been demonstrated to modify respiratory parameters *in vivo*. ICV administration of NPS in wild-type and NPSR1-deficient mice resulted in receptor-dependent increases in respiratory frequency and decreases in tidal volume, demonstrating that administration of NPS can directly alter respiratory function through actions initiated in the central nervous system (Zhu et al. 2011).

Orexin

Orexin receptor expression in the KF

Orexins (ORX) or hypocretins are excitatory neuropeptides expressed in two splice variants (ORX-A and ORX-B) in the central nervous system (reviewed in Wang et al., 2018). Orexin receptors (OXR) are GPCRs with two identified isoforms, OX1R and OX2R (Sakurai et al. 1998). ORX-A binds to both OX1R and OX2R with equal affinity, while ORX-B binds mainly to OX2R (Langmead et al. 2004). Marcus and colleagues (2001) have found that OX1R and OX2R mRNA are differentially expressed in the rat brain, as identified by *in situ* hybridization, with both OX1R and OX2R mRNA only weakly expressed in the KF and rest of the parabrachial complex (Marcus et al. 2001). In contrast, using immunohistochemistry, Yokota et al. (2016) identified a substantial amount of OX2R-expressing neurons in the rat KF. Tracing methods indicate that the projection targets of these OX2R-positive KF neurons are mainly the rostral ventral respiratory group, the phrenic nucleus in the spinal cord, and the hypoglossal nucleus (Yokota et al. 2016). Further supportive data for the existence of OX2Rs in the KF and the projection targets of these neurons came from physiological experiments also conducted in rats (Dutschmann et al. 2007). In these experiments ORX-B microinjected into the KF lead to an increase in both hypoglossal and phrenic nerve output in the *in situ* working heart-brainstem preparation, likely resulting from ORX-B binding to the OX2R on KF neurons.

Sources of orexin in the KF

Orexins are exclusively produced by hypothalamic orexinergic neurons, located primarily in the tuberal part of the hypothalamus (Peyron et al. 1998; Nambu et al. 1999; Modirrousta et al. 2005; De Lecea et al. 1998; Sakurai et al. 1998). Among many other roles, it has been hypothesized that orexin is important in modulating respiration via multiple respiratory projection targets (Williams and Burdakov 2008; Deng et al. 2007; Zhang et al. 2005). Indeed, anatomical tracing studies showed that orexinergic neurons send axonal projections to the KF and other parabrachial nuclei (Figure 2; Peyron et al., 1998). Yokota and colleagues (2016) demonstrated through retrograde cholera toxin B subunit injections in the KF that up to ~23% of labeled neurons originating in the hypothalamus are immunoreactive for orexin. These neurons are predominantly distributed in the medial and perifornical regions of the hypothalamus, and additional labeling can be detected in the lateral region of the hypothalamic orexin region (Figure 2). The orexin-neuron axon terminals make asymmetrical synapses with somata and dendrites of KF neurons (Yokota et al. 2016).

Functional role of orexin in the KF

ORX serves an important role in regulating energy homeostasis and food intake (Sakurai 2002; Gregor Sutcliffe and De Lecea 2000; Willie et al. 2001), reward and addiction (Harris and Aston-Jones 2006), autonomic function (Kuwaki 2008; Williams and Burdakov 2008; Willie et al. 2001) and sleep-wake states (Sakurai 2007). Most relevant to this review, it has been shown that lack of orexin may lead to sleep disorders, such as narcolepsy (Chemelli et al. 1999; Willie et al. 2001) and sleep apnea (Nakamura et al. 2007). The wide range of functions served by the orexin system are controlled by two subpopulations of ORX neurons in the hypothalamus, with neurons located in the lateral hypothalamus being involved in food intake, reward and addiction, and neurons in the medial and perifornical hypothalamic regions modulating wakefulness and arousal states (Harris and Aston-Jones 2006). Based on the results of Yokota et al. 2016, the majority of hypothalamic neurons projecting to the KF originate in the medial and perifornical areas, hinting at a potential contribution of ORX-modulated KF neurons to the control of sleep-wake state and arousal. This hypothesis is in line with studies demonstrating increased activity of parabrachial complex, including KF area neurons during wakefulness (Sieck and Harper 1980; Lydic and Orem 1979). Additionally, Dutschmann et al. (2007) found that ORX-B microinjected into the KF of rats leads to transient increases in respiratory rate. This increase in breathing also coincides with an increase in hypoglossal motor output, linking orexinergic modulation of KF neural output to increased upper airway patency. This function has clear implications not only in the state-dependent control of respiration, but also in the reduced upper airway resistance, characteristic of obstructive sleep apnea (Dutschmann et al. 2007).

Somatostatin

Somatostatin receptor expression in the KF

Somatostatin (SST; also called growth hormone release-inhibiting hormone and somatotropin release-inhibiting factor) is a neuropeptide that is expressed in three prosomatostatin-derived peptide isoforms (SST-14, SST-28 and SST-28(1-12)) throughout the mammalian central nervous system (Bruno et al. 1992; O'Carroll et al. 1992; Günther et

al. 2018). SST achieves its inhibitory effects via $G_{i/o}$ protein-coupled SST receptors. Six different receptor subtypes have been described for SST to date, SST1-5, with SST2 receptors present in two forms, SST2a and SST2b (Vanetti et al. 1992; Vanetti et al. 1994; Yasuda et al. 1992; Bruno et al. 1992; O'Carroll et al. 1992). In human brains high levels of SST receptor expression has been identified by autoradiographic studies in the parabrachial complex that is highest during the early stages of fetal development and goes through an abrupt decrease during the perinatal period (Carpentier et al. 1996; Carpentier et al. 1997). Importantly, the location of binding sites generally overlaps with SST-positive fibers in infants (Chigr et al. 1989; Carpentier et al. 1997).

Sources of somatostatin in the KF

SST is initially secreted as pre-pro-somatostatin, an inactive precursor protein, which is then cleaved into pro-somatostatin. Pro-somatostatin is further processed into the above described active peptide isoforms (Kumar and Grant 2010). The active peptide SST is widely produced throughout the body, including in the gastrointestinal mucosa, the pancreas, and importantly throughout the central nervous system (Arimura et al. 1975; Polak et al. 1975; Patel and Reichlin 1978; Finley et al. 1981; Johansson et al. 1984). The main source of SST in the brain is the hypothalamus, however other sites, such as areas in the cortex, brainstem and spinal cord, also contribute to the distribution of the neuropeptide (Barnett 2003; Johansson et al. 1984; Finley et al. 1981). For instance, in the mouse brain, SST-expressing excitatory pre-Bötzing complex neurons project to the KF (Figure 2; Yang & Feldman, 2018). Immunocytochemistry on cat brains has revealed a dense distribution of SST-28 (1-12) immunoreactive fibers as well as cell bodies in the KF area, however the pons in general had less immunoreactive structures compared to the medulla (De León et al. 1992). In addition, the parabrachial complex has been shown to have moderate to high densities of immunoreactive neurons for SST labeling (Figure 2; Johansson et al., 1984). To our knowledge, there is no direct evidence for the KF's involvement in SST production. However, the results of retrograde tracing experiments combined with pre-pro-somatostatin mRNA labeling indicate that the KF represents one of the major sources of SSTergic projections to the rostral ventrolateral medulla and thus may be directly involved in the distribution of SST throughout the adult rat brain (Bou Farah et al. 2016).

Functional role of somatostatin in the KF

Taking into consideration the wide range of projections from the KF to most brainstem respiratory nuclei and the high density of SST receptor expression in most of these regions, KF SSTergic neurons likely play a key role in cardiorespiratory control (Bou Farah et al. 2016; Barnett 2003; Günther et al. 2018; Dutschmann and Dick 2012). However, the direct effect of SST on KF neurons and respiratory and/or cardiovascular control remains unknown.

Neurochemical alterations in the context of respiratory dysfunction

Sudden Infant Death Syndrome (SIDS)

SIDS is defined as the sudden unexpected death of a seemingly healthy infant during sleep, the cause of which remains unknown even after a complete autopsy (Krous et al. 2004). The

underlying cause of SIDS is not clear, however the most compelling hypothesis (“triple risk hypothesis”) is supported by both anatomical and functional evidence (Filiano and Kinney 1994). According to this hypothesis, the main culprit in SIDS is the abnormal development of autonomic areas of the brain, which leaves the infants vulnerable to external stressors, such as hypercapnia or hypoxia.

The KF’s crucial role in coordinating breathing in not only normoxia, but also high CO₂ and/or low O₂ conditions, makes it a prime target for elucidating the underlying abnormalities in SIDS (Damasceno et al. 2014; Lavezzi 2015). Indeed, evidence provided by human histological studies suggests an array of KF abnormalities in fetal, newborn and infant brains (Lavezzi et al. 2004a; Lavezzi et al. 2014; Lavezzi 2015; Lavezzi et al. 2016). A major contributor to late fetal (but not newborn or infant) unexplained deaths is an underdeveloped KF (Lavezzi et al. 2004a; Maturri and Lavezzi 2011). Beyond morphology, neurochemical alterations of the KF are also linked to SIDS. This includes differences in orexinergic innervation and receptor expression in the KF of control and SIDS subjects (Lavezzi et al. 2016). Specifically, in control brains, KF neurons receive extensive orexinergic projections measured by immunohistochemical labeling of OX1R, while such varicosities are minimal or absent in SIDS brains. OX2R labeling is weak or absent in control and SIDS brains, suggesting that there may not be a functional abnormality linked to OX2R in SIDS.

Another important characteristic of KF neurons in SIDS and sudden intrauterine unexplained death syndrome (SIUDS) is the decreased amount of brain-derived neurotrophic factor (BDNF) expression associated with morphological immaturity (Lavezzi et al. 2014; Lavezzi 2015). BDNF is required for the healthy development of brainstem respiratory nuclei and the projections connecting them (Caravagna et al. 2013; Lu 2003; Katz et al. 2009; Balkowiec and Katz 1998). Specifically, BDNF plays an important role in the maturation of GABAergic and glutamatergic fast synaptic transmission in the KF, as supported by data from rats (Kron et al. 2007b; Kron et al. 2007a; Kron et al. 2008). Thus, alterations in BDNF expression pathways may be the causative agents of KF morphological hypoplasia and potentially alterations in KF GABAergic and glutamatergic dysfunction in SIDS and SIUDS (Lavezzi et al. 2014; Lavezzi 2015).

One of the major risk factors of SIDS is smoking, so not surprisingly the majority of the specimens examined in the above described studies were exposed to harmful chemicals, including nicotine, present in cigarettes during pregnancy and/or following birth (Lavezzi et al. 2014; Lavezzi et al. 2016). Interestingly, deficiencies in both OX1R and BDNF expression highly correlate with maternal smoking (Lavezzi et al. 2016; Lavezzi et al. 2014). Future studies should address the potential causal role of nicotine or other harmful cigarette chemicals in abnormal orexin receptor and BDNF expression in the KF.

Rett syndrome (RTT)

Alterations in KF BDNF expression levels and consequent GABAergic dysfunction are also relevant in Rett syndrome (RTT; Vermehren-Schmaedick et al., 2012; Lavezzi et al., 2014, 2016; Lavezzi, 2015; Abdala et al., 2016). RTT results from mutation in a X-linked gene affecting the expression of the nuclear protein methyl-CpG-binding protein 2 (MeCP2),

where developmental and functional abnormalities accumulate across the brain and involving multiple neurotransmitter systems (Amir et al., 1999). Among other symptoms, RTT is characterized by irregular respiratory patterns, such as periodic breathing and frequent central and/or obstructive apneas, which indicate the heavy involvement of brainstem expiratory/post-inspiratory respiratory nuclei (Bissonnette et al. 2014; Bissonnette and Knopp 2008; Abdala et al. 2010; Carotenuto et al. 2013; Lugaesi et al. 1985; Stettner et al. 2007). Indeed, anatomical investigation of the KF in a mouse model of RTT showed a substantial decrease in the number of local GABAergic processes and perisomatic bouton-like puncta compared to control mice (Abdala et al. 2016). Additionally, GABA receptor antagonists microinjected into the KF of healthy rats induces RTT-like respiratory patterns (Abdala et al. 2016). Finally, local application of GABA reuptake blockers in the KF rescue the respiratory phenotype in RTT and restores breathing patterns in RTT mice, indicating a major role for the KF in RTT respiratory symptomology (Abdala et al. 2010).

The serotonergic system also has potential in correcting breathing irregularity in RTT. Sarizotan, a mixed serotonin 5-HT_{1A} receptor agonist and dopamine D₂ receptor partial agonist, and F15599, a selective serotonin 5-HT_{1A} agonist, are both effective at improving respiratory dysfunction in mouse models of RTT (Abdala et al. 2014b; Levitt et al. 2013). The KF may be a site of action because administration of the serotonin 5-HT_{1A} receptor agonist 8-OH-DPAT into the KF of MeCP2 deficient mice improved breathing irregularity (Abdala et al. 2010; Abdala et al. 2014a).

Summary

We summarized the neurochemical composition of the KF, including neurotransmitters, neuromodulators, and hormones present. We also addressed the receptors and transporters involved in each system and discussed their potential physiological function. Finally, we provide a brief summary of literature regarding neurochemical changes in the KF in the context of respiratory dysfunction. We predict that future research into the neurophysiology of respiration will benefit from this review.

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Abbreviations:

5-HT	5-hydroxytryptamine, serotonin
ACh	acetylcholine
AChE	acetylcholinesterase
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BDNF	brain-derived neurotrophic factor

cAMP	cyclic adenosine-monophosphate
ChAT	choline acetyl transferase
CHT1	choline transporter 1
CSF	cerebrospinal fluid
DOR	delta opioid receptor
EAAT	excitatory amino acid transporter
EMG	electromyography
FoxP2	Forkhead box protein 2
GABA	γ -aminobutyric acid
GAD	glutamic acid decarboxylase
GAT	GABA transporter
GIRK	G protein-coupled inwardly-rectifying potassium channel
GluA	subunits of the glutamate AMPA receptor
GluN	subunits of the glutamate NMDA receptor
GlyR	glycine receptor
GlyT	glycine transporter
GPCR	G-protein-coupled receptor
ICV	intracerebroventricular
KF	Kölliker-Fuse nucleus
KOR	kappa opioid receptor
LC	locus coeruleus
mAChR	muscarinic acetylcholine receptor
MeCP2	methyl-CpG-binding protein 2
mGluR	metabotropic glutamate receptor
MOR	mu opioid receptor
nAChR	nicotinic acetylcholine receptor
NE	norepinephrine
NET	norepinephrine transporter
NMDA	N-methyl-d-aspartate

NPS	neuropeptide S
NPSR	neuropeptide S receptor
NTS	nucleus of the solitari tract
ORX	orexin
RTT	Rett syndrome
SST	somatostatin
SIDS	sudden infant death syndrome
SIUDS	sudden intrauterine unexplained death syndrome
VAcHt	vesicular acetylcholine transporter
VGLUT	vesicular glutamate transporter
VGAT	vesicular GABA transporter

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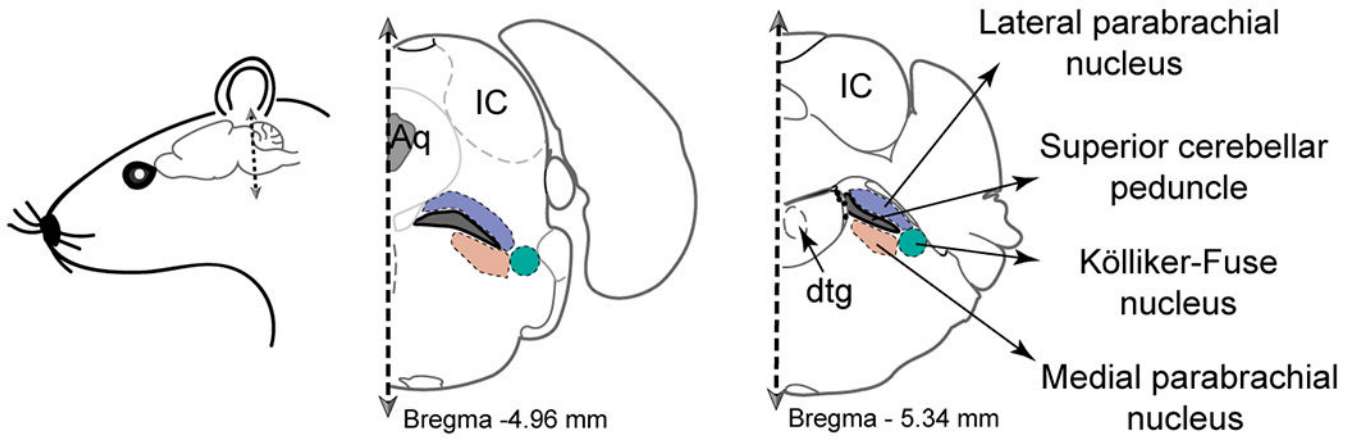


Figure 1.

Location of the KF in the adult mouse brain. In the schematics of coronal hemi-sections, the green shaded area indicates the location of the KF in the proximity of the lateral parabrachial nucleus (in purple) and the medial parabrachial nucleus (in orange) surrounding the superior cerebellar peduncle (in gray). Other landmarks included in the diagram: Aq – Aqueduct; IC – inferior colliculus; dtg – dorsal tegmental nucleus.

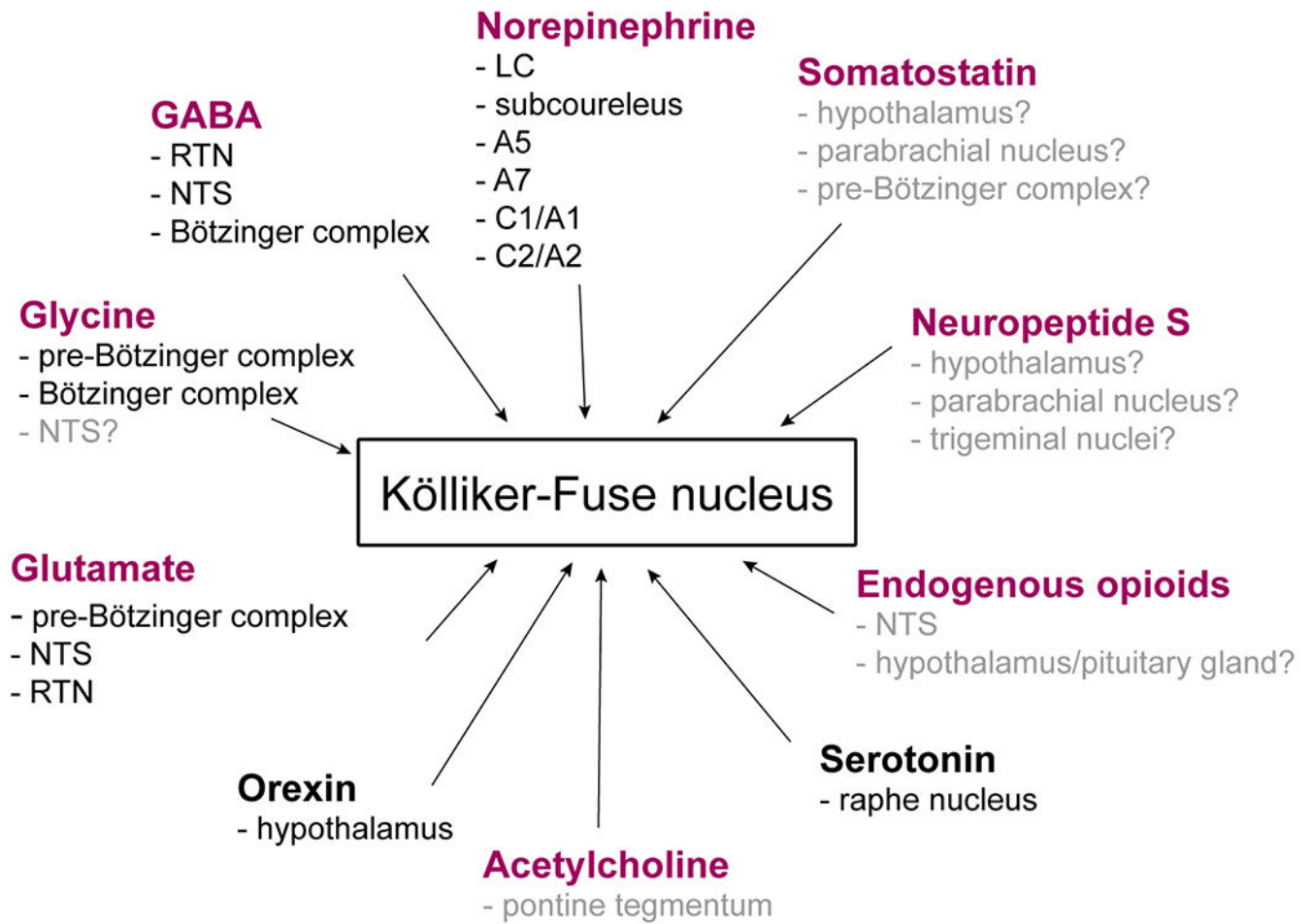


Figure 2.

Neurochemical identity and origin of afferent projections to KF. Purple font indicates neurotransmitters and neuropeptides produced locally in the KF. Origins listed in gray font indicate potential neuropeptide and neurotransmitter release from regions with known anatomical projections to the KF. The specific neurochemical identity of these projections has not functionally addressed.