Organisation et modulation du réseau neuronal de la respiration chez la lamproie

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Organisation et modulation du réseau neuronal de la respiration chez la lamproie

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Résumé

Les mécanismes neuronaux contrôlant la respiration sont présentement explorés à l'aide de plusieurs modèles animaux incluant le rat et la grenouille. Nous avons utilisé la lamproie comme modèle animal nous permettant de caractériser les réseaux de neurones du tronc cérébral qui génèrent et modulent le rythme respiratoire. Nous avons d'abord caractérisé une nouvelle population de neurones, dans le groupe respiratoire paratrigéminal (pTRG), une région du tronc cérébral essentielle à la genèse du rythme respiratoire chez la lamproie. Les neurones de cette région sont actifs en phase avec le rythme respiratoire. Nous avons montré que ces neurones possèdent une arborisation axonale complexe, incluant des projections bilatérales vers les groupes de motoneurones du tronc cérébral qui activent les branchies ainsi que des connexions reliant les pTRG de chaque côté du tronc cérébral. Ces résultats montrent que le pTRG contient un groupe de cellules qui active les motoneurones respiratoires des deux côtés et qui pourrait être impliqué dans la synchronisation bilatérale du rythme respiratoire.

Nous avons ensuite étudié les mécanismes neuronaux par lesquels le rythme respiratoire est augmenté en lien avec l'effort physique. Nous avons montré que la région locomotrice du mésencéphale (MLR), en plus de son rôle dans la locomotion, active les centres respiratoires pendant la nage, et même en anticipation. Les neurones de la MLR projetant vers les centres locomoteurs et respiratoires sont ségrégés anatomiquement, les neurones localisés plus dorsalement étant ceux qui possèdent des projections vers les centres respiratoires. Nous avons aboli la contribution de la partie dorsale de la MLR aux changements respiratoires en injectant des bloqueurs des récepteurs glutamatergiques localement, sur des préparations semi-intactes. Nous avons montré que lors d'épisodes de

nage, une majeure partie de l'effet respiratoire est abolie par ces injections, suggérant un rôle prépondérant des neurones de cette région dans l'augmentation respiratoire pendant la locomotion.

Nos résultats confirment que le rythme respiratoire est généré par une région rostrolatérale du pons de la lamproie et montrent que des connexions des centres locomoteurs arrivent directement à cette région et pourraient être impliquées dans l'augmentation respiratoire reliée à l'effort physique.

Mots-clés : respiration, locomotion, lamproie, modulation, CPG, tronc cérébral, pons, mésencéphale, contrôle descendant

Abstract

The neural control of breathing is currently investigated on multiple animal models such as frogs and rats. We have used the lamprey as an experimental model to characterize the brainstem neural networks involved in the genesis and modulation of the respiratory rhythm. We have first characterized a new population of respiratory neurons in the paratrigeminal respiratory group (pTRG). The pTRG is a region that was shown to be essential to respiratory rhythmogenesis in lampreys. We have shown that the pTRG contains a group of neurons with complex axonal arborisations, including bilateral projections to the motoneuron pools of the brainstem that activate gills, as well as bilateral projections connecting the pTRGs on the two sides of the brainstem. These results suggest that pTRG neurons could participate in the descending control of respiratory motoneurons as well as the bilateral synchrony of the respiratory rhythm.

We have then studied the neural mechanisms by which respiration is increased during locomotion. We have shown that the mesencephalic locomotor region (MLR), in addition to its role in controlling locomotion, also increases breathing during locomotion. Neurons in the MLR are anatomically segregated, those projecting to the respiratory centers being located more dorsally. We have abolished the contribution of the dorsal part of the MLR to respiratory changes by injecting glutamate receptor blockers locally in semi-intact preparations. We have shown that during swimming episodes, a major part of the respiratory effect is dependent on the dorsal part of the MLR.

Our results confirm that the respiratory rhythm is generated by a rostrolateral region in the pons of lampreys and show that connections from locomotor centers can

directly activate this region. These connections could be implicated in the increase of breathing activity related to locomotion.

Keywords : respiration, locomotion, lamprey, modulation, CPG, brainstem, pons, mesencephalon, descending control

Table des matières

Liste des tableaux

Tableau de l'article 2

Liste des figures

Figures de l'article 1

Figures de l'article 2

Figures de l'article 3

Liste des abréviations

AMPA : Acide 2-amino-3-(5-methyl-3-oxo-1,2- oxazol-4-yl)-propanoique

AP-5 : Acide (2R)-amino-5-phosphonovalerique

ARRN : Noyau réticulaire rhombencéphalique antérieur

BötC : Complexe Bötzinger

CNQX : 6-cyano-7-nitroquinoxaline-2,3-dione

CO_2 : Gaz carbonique

CPG : Générateur central de patron

cVRG : Groupe respiratoire ventral caudal

DRG : Groupe respiratoire dorsal

EPSC : Courant post-synaptique excitateur

EPSP : Potentiel post-synaptique excitateur

GABA : Acide gamma-aminobutyrique

I_{CAN} : Conductance cationique non-spécifique activée par le calcium

I_{NaP} : Conductance persistante sodique

IX : Noyau moteur glossopharyngien

KF : Noyau Kölliker-Fuse

LPBr : Région latérale parabrachiale

Mes : Mésencéphale

Mlf : Fascicule longitudinal médian

MLR : Région locomotrice du mésencéphale

MN : Motoneurone

mV : Racine motrice du trijumeau

NMDA : Acide N-Methyl-D-aspartique

NOMA : Noyau octavomoteur antérieur

O_2 : Oxygène

pFRG : groupe respiratoire parafacial

PreBötC : Complexe pré-Bötzinger

PRG : Groupe respiratoire pontique

Pro : Prosencéphale

PRRN : Noyau réticulaire rhombencéphalique postérieur

PT : Tubercule postérieur

pTRG : Groupe respiratoire paratrigéminal

RTN : Noyau rétro-trapèzoïde

rDV : Racine descendante trigéminale

rVRG : Group respiratoire ventral rostral

sep : Couche sous-épendymaire

VII : Noyau moteur facial

VRG : Groupe respiratoire ventral

X : Noyau moteur vague

XII : Noyau hypoglosse

Bien souvent et à toutes les époques, on a comparé les êtres vivants aux machines, mais c'est de nos jours seulement que l'on peut comprendre la portée et la justesse de cette comparaison.

Étienne-Jules Marey, *La machine animale: locomotion terrestre et aérienne*, 1878.

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Introduction

La vie animale repose sur un ensemble de mécanismes biologiques qui opèrent dans les tissus biologiques et les organes. L'une des caractéristiques qui regroupe ces mécanismes est leur besoin en énergie. La respiration est l'une des activités motrices essentielles à la machinerie biologique qui produit cette énergie. Elle permet à la fois de faire entrer de l'oxygène dans l'organisme et d'expulser du gaz carbonique dans l'environnement. Sans ces échanges essentiels entre le sang et l'environnement, les tissus biologiques perdent leur fonctionnalité et périssent très rapidement. Aristote (350 A.D.) illustrait ainsi le lien péremptoire unissant la respiration à la vie:

> *This explains why life and death are bound up with the taking in and letting out of the breath […] the last act when life comes to a close is the letting out of the breath, and hence its admission must have been the beginning of the process.*

Il y a peu d'actes moteurs qui soient d'une part aussi automatiques et réguliers que la respiration et d'autre part autant adaptables aux différentes situations auxquelles l'organisme est confronté. La respiration s'effectue sans effort, automatiquement, et souvent inconsciemment. Chez les mammifères, la génération du rythme respiratoire est assurée par un réseau qui s'étend de la moelle épinière au pons, incluant le bulbe rachidien, qui semble contenir le cœur du générateur de rythme (von Euler, 1979; von Euler, 1983; Feldman et Del Negro, 2006). Ces neurones s'activent de manière rythmique tout au long de la vie, assurant le maintien des contractions des muscles respiratoires et les échanges gazeux qui en résultent. Dans la première partie de cette thèse, un bref historique du domaine de la physiologie respiratoire sera présenté; nous aborderons

ensuite l'état des connaissances sur les mécanismes neurobiologiques qui génèrent la respiration. Nous décrirons les recherches effectuées sur les groupes d'interneurones et de motoneurones du tronc cérébral et de la moelle épinière qui participent à la genèse du patron respiratoire et au contrôle des muscles impliqués dans la respiration.

Par la suite, nous aborderons les mécanismes neuronaux par lesquels la respiration est modifiée en lien avec les activités physiques. En effet, plusieurs activités ont un impact sur le rythme respiratoire, incluant la course, la marche ou même la préparation à l'exercice (Dejours et al., 1955; Tobin et al., 1986; Decety et al., 1993; Mateika et Duffin, 1995). Ces adaptations sont sans doute cruciales à la survie animale, puisque la quantité d'oxygène disponible est l'un des principaux facteurs limitant l'endurance lors d'efforts prolongés (Boutellier et Piwko, 1992). Un chapitre de livre dont je suis le premier auteur a été publié en 2010 a été ajouté à l'introduction. Ce chapitre comporte une revue de question décrivant les différentes hypothèses sur les mécanismes physiologiques responsables des modifications du rythme respiratoire reliées à l'effort.

Historique de la physiologie respiratoire

La compréhension de la respiration et la guérison des maladies respiratoire figurent parmi les préoccupations majeures des médecins de l'antiquité. Certains remèdes dédiés aux problèmes respiratoires et certains outils visant à accéder aux voies respiratoires étaient déjà accessibles aux égyptiens il y a plus de 5000 ans (Ocklitz, 1997; Aboelsoud, 2010). Les découvertes en chimie et en biologie du 18^{e} et du 19^{e} siècle ont

permis d'identifier l'oxygène (O_2) et le gaz carbonique (CO_2) comme éléments centraux de la production d'énergie par les animaux. Les scientifiques de l'époque constatèrent que la transformation de l'oxygène en gaz carbonique par les êtres vivants est très semblable à celle qui alimente les feux; qu'il s'agit d'une combustion par l'oxygène (Lavoisier, 1774; Lavoisier, 1862). On suggéra alors que la couleur rouge du sang après son passage par les poumons est due à sa charge en oxygène, et que l'oxygène pourrait se lier au sang par un phénomène similaire à celui de sa liaison aux métaux, leur donnant une couleur rouge (par exemple lors du phénomène de l'oxidation ou rouille). Ces prédictions visionnaires furent confirmées par la découverte de l'hémoglobine, la protéine sanguine responsable du transport de l'oxygène dans le sang, qui possède un noyau de fer responsable de la liaison avec l'oxygène et qui est responsable de la couleur rouge du sang lorsqu'oxygénée (Hoppe-Seyler, 1866; Perutz et al., 1960).

Le renouvellement de la réserve d'oxygène du sang dépend d'un apport constant en air à la surface des poumons. Les animaux possèdent une machinerie musculaire permettant d'échanger l'air des poumons avec l'environnement. Au 17^{e} siècle, il était déjà établi que les mouvements respiratoires étaient contrôlés par le système nerveux (Capuanus, 1683; Boerhaave, 1715). Les travaux subséquents montrèrent l'importance de certaines régions du tronc cérébral et des nerfs crâniens dans l'activation des muscles respiratoires (Breuer, 1868; Lumsden 1923a, 1923b). C'est par ces nerfs que les réseaux de neurones du tronc cérébral qui génèrent la respiration transmettent le patron d'activité aux muscles respiratoires.

Parallèlement aux premières identifications des sites du tronc cérébral nécessaires à la genèse du rythme respiratoire, certains scientifiques se sont penchés sur

sa régulation biochimique. Des travaux pionniers montrèrent que le CO_2 et l'O_2 contenus dans les vaisseaux sanguins modulent fortement la respiration (Pflüger, 1868; Haldane et Priestley, 1905; Dejours, 1962). L'intuition porta plusieurs physiologistes de l'époque à croire que ces gaz activaient directement les centres respiratoires, hypothétiquement dotés de chémorécepteurs. Cependant, cette intuition fut réfutée partiellement par l'identification et la charactérisation de chémorécepteurs de l'O_2 dans l'artère carotide, près du coeur (Gesell, 1939; Holgert et al., 1995).

D'autres études montrèrent tout de même que des chémorécepteurs du CO_2 et de l'acidité du liquide céphalo-rachidien étaient localisés dans le tronc cérébral et qu'ils pourraient aussi jouer un rôle dans les modifications respiratoires (Schmidt, 1932; Loeschcke, 1982; Loeschcke et al., 1958). Bien qu'il existe toujours des débats sur la localisation de ces chémorécepteurs (Guyenet et al., 2008), la région du noyau rétrotrapézoïde semble être critique pour la détection de l'acidité dans le liquide céphalo-rachidien. Des cellules gliales de cette région peuvent détecter des variations de pH et augmenter l'activité respiratoire (Gourine et al., 2010).

Au début du 20^{e} siècle, les travaux pionniers d'August Krogh et de Johannes Lindhard décrivirent les liens qui existent entre l'exercice et la respiration. Ces auteurs montrèrent que lorsque des sujets humains débutent une activité physique, la respiration est augmentée immédiatement (Krogh et Lindhard, 1913). La rapidité avec laquelle les changements respiratoires se produisent suggère que les mécanismes chémorécepteurs ne sous-tendent pas cet effet, car les produits de l'activité musculaires doivent mettre un certain temps avant de se rendre aux centres respiratoires ou à l'artère carotide, où les chémorécepteurs sont localisés, alors que les effets respiratoires, eux, sont instantanés.

Krogh et Lindhard proposèrent donc que des connexions nerveuses pourraient sous-tendre l'augmentation de la respiration au début de l'exercice. Cette hypothèse constitue l'une des trois hypothèses concernant les modifications de la respiration pendant l'exercice. La première, celle de la chémoréception, stipule que les modifications respiratoires lors de l'activité physique seraient produites par la détection de CO_2 et d'O_2 par les chémorécepteurs périphériques et centraux. La deuxième, celle du feedback neuronal périphérique, stipule que des détecteurs d'activité musculaire localisés en périphérie induiraient l'augmentation de la respiration pendant l'exercice par le biais de retours sensoriels. Une revue détaillée du débat autour de ces trois hypothèses est ajoutée à l'introduction comme premier article (Gariépy et al., 2010).

Mes travaux de thèse de doctorat portent sur les réseaux de neurones qui génèrent le rythme respiratoire et qui le modulent pendant la locomotion. Dans les sections qui suivent, je présenterai donc un bref aperçu des connaissances actuelles sur les réseaux de neurones qui génèrent le rythme respiratoire chez les mammifères ainsi que chez la lamproie.

Réseaux neuronaux de la respiration chez les mammifères

Muscles et motoneurones respiratoires

La respiration est effectuée par des muscles de l'abdomen, du thorax et par certains muscles des voies respiratoires supérieures. Le muscle inspiratoire principal chez les mammifères est le diaphragme. Les motoneurones phréniques, qui contrôlent le diaphragme, sont localisés dans la moelle épinière entre les segments C3 à C7 (Webber et

al., 1979; Yates et al., 1999; Mantilla et Sieck, 2011). Les motoneurones phréniques sont activés par des connexions provenant des régions respiratoires du bulbe dont le complexe Bötzinger (von Euler, 1983; Merrill et Fedorko, 1984; Rekling et al., 2000).

Les muscles intercostaux participent aussi à la respiration et sont activés par des motoneurons localisés entre les segments C7 à T13 (Giraudin et al., 2008) . Les muscles abdominaux participant à la respiration sont quant à eux activés par des motoneurones des segments T8 à L2 et sont activés pendant la phase expiratoire (Giraudin et al., 2008; Abdala et al., 2009; Iizuka, 2011). Les motoneurones spinaux contrôlant les muscles intercostaux reçoivent des afférences du noyau rétroambiguus et cette voie est facilitée par l'activation de récepteurs sensibles à l'étirement dans les poumons (DiMarco et al., 1981; Boers et al., 2006).

Régions du tronc cérébral impliquées dans la respiration

Chez les mammifères, le rythme respiratoire est composé d'une phase inspiratoire pendant laquelle les muscles respiratoires augmentent le volume des poumons, et d'une phase expiratoire pendant laquelle les poumons se rétractent et expulsent l'air vers l'extérieur. L'expiration est constituée d'une période passive (E1) puis d'une période active (E2) pendant laquelle les muscles expiratoires de la cage thoracique et de l'abdomen contribuent à l'expulsion de l'air (Richter et al., 1986; Bianchi et Pásaro, 1997). Des travaux pionniers du début du 20^{e} siècle ont permis d'identifier l'étendue du réseau de neurone nécessaire pour générer le rythme respiratoire. Lumsden (1923c) a effectué des lésions du tronc cérébral partant du mésencéphale

jusqu'au bulbe rachidien dans le but d'identifier les sites nécessaires à la genèse du rythme respiratoire. Il constata que des lésions du pons induisaient des changements marqués du rythme respiratoire. Il désigna le centre pontique contribuant à la respiration « centre pneumotaxique ». Après retrait du pons, le bulbe rachidien pouvait toujours générer deux types de patrons rythmiques: l'apnée, constituée d'inspirations prolongées, et l'halètement, lors duquel les respirations sont brèves, saccadées, et séparées par des pauses de durée variable. Lumsden suggéra donc la présence d'un centre apneustique et d'un centre d'halètement dans le bulbe rachidien.

La terminologie de Lumsden est maintenant très peu utilisée par les auteurs contemporains parce que les régions anatomiques où l'on a identifié des neurones respiratoires ont été caractérisées plus en détail. De plus, comme nous allons le voir dans ce qui suit, une importante partie des auteurs contemporains considèrent que le rythme respiratoire généré au niveau du bulbe, en absence du pons, constitue une version légèrement modifiée de l'eupnée mais qu'il s'agit bel et bien du même rythme, alors que d'autres considèrent qu'il s'agit d'halètement tel que décrit par Lumsden.

Le groupe respiratoire ventral

Le groupe respiratoire ventral (VRG) comprend le complexe Bötzinger, le complexe pré-Bötzinger (PreBötC), le groupe respiratoire ventral rostral (rVRG), localisé aux alentours du noyau ambiguus et le groupe respiratoire ventral caudal (cVRG), localisé aux alentours du noyau rétroambiguus (Figure 1) (Song et al., 2000). Le PreBötC est considéré comme le centre générateur de l'inspiration puisqu'il est actif pendant

l'inspiration et que sa lésion abolit la respiration (Smith et al., 1991). Le cVRG et le complexe Bötzinger contiennent des neurones dont l'activité augmente pendant l'expiration ainsi qu'une sous-population active pendant l'inspiration (Merrill, 1977; Ezure et al., 2003). L'abolition de l'activité dans le complexe Bötzinger arrête l'activité respiratoire spontanée (Mutolo et al., 2002). Les neurones du complexe Bötzinger possèdent des projections excitatrices vers les neurones reliés à l'expiration dans le cVRG et dans la moelle épinière (Bongianni et al., 1990). Plus spécifiquement, des stimulations antidromiques dans le noyau rétroambiguus, qui fait partie du cVRG, ainsi que dans la moelle épinière, activent les neurones du complexe Bötzinger de manière antidromique (Figure 1) (Fedorko et Merrill, 1984). Des connexions diffuses vers le côté contralatéral et vers le noyau Kölliker-Fuse du pons ont aussi identifiées par marquage anatomique (Ezure et al., 2003). En plus des connexions excitatrices, le complexe Bötzinger envoie des connexions inhibitrices vers les régions plus caudales du VRG et la moelle épinière (Anders et al., 1991; Song et Shao, 2000). Les neurones du rVRG sont actifs pendant l'inspiration et projettent vers les motoneurones phréniques (Rybak et al., 2007; Huang et Goshgarian, 2009).

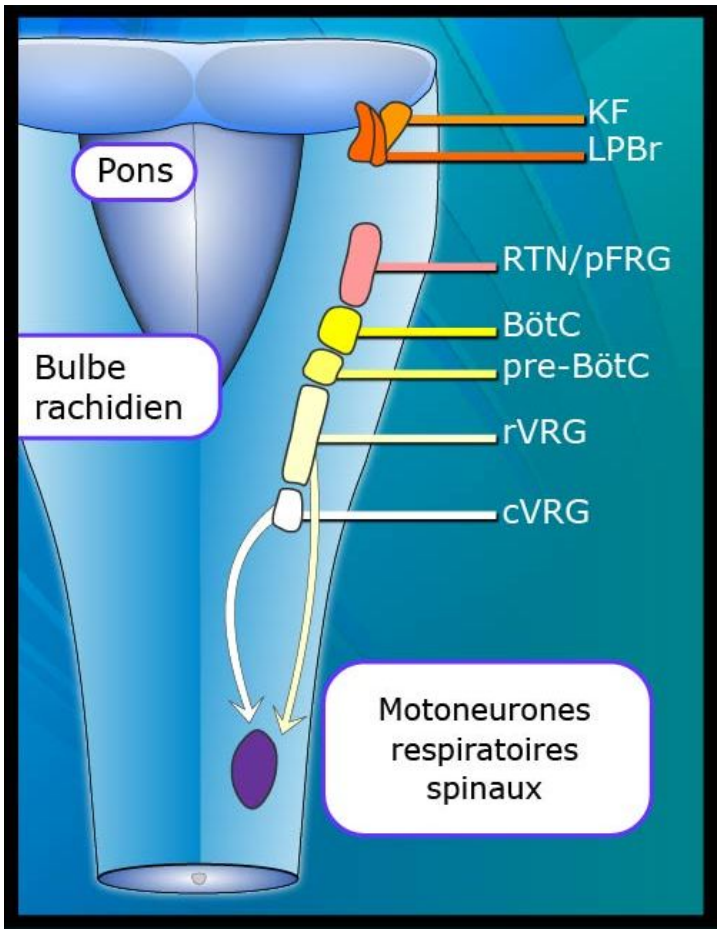

Figure 1: Localisation des centres respiratoires chez les mammifères. Une série de noyaux localisés dans le bulbe génèrent le rythme respiratoire et leurs neurones projettent vers les motoneurones spinaux qui activent les muscles respiratoires. De plus, des régions du pons sont impliquées dans la modulation et la transition entre les phases expiratoires et inspiratoires. BötC, Complexe Bötzinger; cVRG, Groupe respiratoire ventral caudal; KF, Noyau Kölliker-Fuse; LPBr, Région latérale parabrachiale; pre-BötC, Complexe pré-Bötzinger; RTN/pFRG, Groupe respiratoire parafacial; rVRG, Groupe respiratoire ventral rostral.

Mécanismes cellulaires de la rythmogénèse

Les activités rythmiques générées par les neurones en lien avec des comportements comme la locomotion, la respiration, ou la mastication ont en commun qu'elles sont constituées de différentes phases et qu'une alternance entre ces phases doit être produite. Les mécanismes de genèse de la respiration ne sont pas encore décrits au même niveau de détail que d'autres générateurs de rythme. Par exemple, les réseaux de genèse de la locomotion chez la lamproie (Grillner, 2003; Grillner et al., 2008; Grillner et Jessell 2009) et ceux du ganglion stomato-gastrique du homard (Marder et Calabrese, 1996; Weimann et al., 1997; Marder et al., 2005) ont été utilisés pour étudier en détail les

mécanismes cellulaires et de réseau qui permettent la genèse et la modulation des activités rythmiques. Certains principes généraux semblent s'appliquer à l'ensemble des générateurs de rythme étudiés, entre autres le fait que des courants ioniques rythmogènes soient impliqués, qu'ils soient activés par des propriétés intrinsèques ou par des connexions synaptiques (Harris-Warrick, 2010).

La respiration repose sur un ensemble de propriétés membranaires des neurones du tronc cérébral et sur des connexions synaptiques les reliant. Il y a toujours un débat concernant l'importance relative de ces deux types de mécanismes dans la genèse du rythme. Le PreBötC est considéré par plusieurs comme principal site de genèse de l'inspiration chez les mammifères. Il est localisé légèrement caudalement par rapport au noyau moteur facial (VII). La destruction de cette région abolit le rythme respiratoire enregistré sur des préparations de tronc cérébral isolé (Smith et al., 1991). De plus, de fines tranches de cette région maintenues *in vitro* peuvent générer un rythme semblable au rythme inspiratoire (Smith et al., 1991). Ce type d'isolation a permis d'exposer les neurones de cette région pour des enregistrements à l'aide d'électrodes de patch, permettant de mieux comprendre le rôle de certaines conductances neuronales dans la genèse du rythme. Les bouffées inspiratoires du PreBötC peuvent être ralenties ou bloquées par des antagonistes des récepteurs AMPA, des antagonistes des récepteurs glutamatergiques métabotropes, ainsi que par des bloqueurs des conductances cationiques non-spécifiques activées par le calcium (I_{CAN}) (Otsuka et al., 1994; Pantaleo et al., 2005; Pace et al., 2007; Pace et Del Negro, 2008; Mironov, 2008). Il semble donc que des propriétés synaptiques et intrinsèques soient nécessaires au maintien du rythme. Ces observations constituent la base sur laquelle repose l'hypothèse de « groupe pacemaker ».

L'hypothèse du groupe pacemaker stipule que la genèse des bouffées inspiratoires repose sur l'activation des I_{CAN} qui sont eux-mêmes activés par une entrée de calcium induite par des récepteurs métabotropes et ionotropes du glutamate (Rubin et al., 2009). Finalement, la bouffée inspiratoire est terminée par une combinaison de déactivation de certaines conductances et par l'activation de conductances potassiques.

Cette hypothèse possède plusieurs similarités avec d'autres modèles de genèse de rythme précédemment discutés. Par exemple, la genèse du rythme locomoteur chez la lamproie repose aussi sur une combinaison de propriétés intrinsèques et de connexions synaptiques. Le générateur central de patron (CPG) locomoteur de la lamproie est constitué par un réseau d'interneurones excitateurs interconnectés, et la bouffée locomotrice repose sur une activation de récepteurs glutamatergiques qui mène à une entrée de calcium, ce qui active des conductances potassiques dépendantes du calcium, qui terminent la bouffée excitatrice (Wallén et Grillner, 1987; Cangiano et Grillner, 2005; Grillner, 2006). La différence entre ces hypothèses et celle du groupe pacemaker repose sur le rôle prépondérant accordé aux I_{CAN} dans cette dernière (Rubin et al., 2009). Malheureusement le bloqueur des I_{CAN} utilisé dans les études sur le PreBötC, l'acide flufénamique, peut affecter la genèse de rythme par un effet non-spécifique sur d'autres conductances, tel que montré chez la lamproie (Wang et al., 2006). Il est donc difficile d'accorder un rôle essentiel et prépondérant aux I_{CAN} basé seulement sur l'effet de l'acide flufénamique sur le rythme. Récemment cependant, il a été montré que les vagues calciques dans les dendrites des neurones du PreBötC précèdent la dépolarisation du soma. Ces résultats ouvrent donc la possibilité que l'entrée de calcium puisse être causale à la dépolarisation des neurones, et non l'inverse, ce qui pourrait indiquer l'importance

des conductances activées par le calcium comme les I_{CAN} pour initier l'activité inspiratoire (Del Negro et al., 2011).

D'autres hypothèses sont aussi considérées pour expliquer la genèse du rythme respiratoire dans le PreBötC. Il a été montré qu'une faible proportion des neurones du PreBötC ont des propriétés *pacemakers* – c'est-à-dire qu'ils peuvent générer des bouffées rythmiques en absence du reste du réseau respiratoire, dont l'activité est bloquée par du CNQX. Ces neurones sont divisés en 2 catégories: ceux qui continuent de générer un rythme en présence de cadmium, utilisé comme bloqueur de canaux calciques, et ceux dont l'activité est abolie par le cadmium (Thoby-Brisson et Ramirez, 2001; Del Negro et al., 2002; Mellen et Mishra, 2010). Ces neurones pourraient être les premiers à s'activer et pourraient entraîner le reste du réseau respiratoire par des connexions excitatrices (Butera et al., 1999; Ramirez et Viemari, 2005). Les neurones pacemakers non-sensibles au cadmium sont dits de type I_{NaP} car leurs propriétés pacemakers sont abolies par le riluzole, bloqueur des I_{NaP}. Le riluzole abolit l'activité respiratoire en tranches, mais certains auteurs ont critiqué l'hypothèse selon laquelle les neurones pacemakers de type I_{NaP} sous-tendraient la rythmogenèse, suggérant plutôt que c'est par une diminution du niveau d'excitation général du PreBötC que le riluzole a un effet sur la respiration (Del Negro et al., 2005; Del Negro et al., 2008).

Finalement, les neurones du PreBötC font partie d'un réseau très étendu incluant d'autres régions du bulbe et du pons aussi impliquées dans la respiration. Bien que le PreBötC puisse générer un rythme lorsqu'isolé, plusieurs auteurs croient que des mécanismes de réseau impliquant les connexions entre le PreBötC et le pons ou le groupe respiratoire parafacial (pFRG) sous-tendent la genèse du rythme respiratoire en condition

normale (St-John et Paton, 2004; Smith et al., 2007; Abdala et al., 2009; Smith et al., 2009).

Le groupe respiratoire dorsal

Le groupe respiratoire dorsal (DRG) est localisé dans le bulbe près du tractus solitarius et a été identifié chez le chat et par la suite chez le rat (Monteau et Hilaire, 1991; de Castro et al., 1994). Il contient des neurones qui sont actifs pendant l'inspiration (Hilaire et Duron, 1999). Certains de ces neurones sont sensibles à l'étirement des poumons (Ezure et Tanaka, 2000). Une technique d'imagerie a été développée pour visualiser l'activité respiratoire dans la partie dorsale du bulbe, mais la correspondance entre les activités respiratoires enregistrées et le DRG reste à clarifier (Onimaru et Homma, 2005). Chez le rat, le DRG occupe un rôle dans le réflexe Breuer-Hering, qui consiste en un allongement de l'expiration et un raccourcissement de l'inspiration suite à un étirement des poumons (Bonham et McCrimmon, 1990). Des stimulations de la moelle épinière induisent des décharges antidromiques dans les neurones du DRG, démontrant la présence de projections monosynaptiques descendantes (Lipski et al., 1983, Duffin et Lipski, 1987; de Castro et al., 1994; Tian et Duffin, 1998). Des enregistrements pairés combinés à des cross-corrélations suggèrent que ces neurones pourraient être activés par des neurones inspiratoires du VRG (Tian et Duffin, 1998).

Le pons

Les régions du pons contenant des neurones reliés à la respiration sont regroupées sous le terme de groupe respiratoire pontique (PRG) et correspondent au centre pneumotaxique de Lumsden. Deux noyaux constituent ce groupe: le noyau Kölliker-Fuse et le noyau parabrachial. Des lésions de ces noyaux réduisent l'effet d'une baisse d'oxygène (O_2) ou d'une augmentation de gaz carbonique (CO_2) sur l'activité respiratoire et augmentent la potentialisation à court-terme de la respiration lors d'expositions intermittentes au CO_2, suggérant un rôle dans la régulation de la respiration par les mécanismes de chémoréception (St-John, 1975; Mizusawa et al., 1995; Bonis et al., 2010a; Boon et Milsom 2010). Le noyau Kölliker-Fuse est influencé par les systèmes glutamatergiques et cholinergiques et est aussi connu pour son rôle dans le contrôle des états de sommeil. Il exerce des effets sur la respiration directement ainsi qu'indirectement par le biais de son effet sur le sommeil (Boon et Milsom, 2008; Bonis et al., 2010b). Il a été montré que dans certaines conditions, l'abolition de la transmission synaptique excitatrice dans ce noyau mène à l'apnée (Song et al., 2010). Ces études suggèrent donc un rôle du noyau Kölliker-Fuse dans la modulation de l'activité respiratoire.

En plus leurs rôles dans la modulation du rythme, certains auteurs ont suggéré que les régions respiratoires du pons pourraient jouer un rôle dans la formation du patron respiratoire. D'abord, tel qu'indiqué précédemment, Lumsden (1923c) avait montré que le patron respiratoire qui est généré après lésion du pons n'a pas toutes les caractéristiques de l'eupnée. Ensuite, il a été montré que des stimulations et des lésions dans le noyau parabrachial modifient le passage de l'expiration à l'inspiration ou vice-versa, suggérant un rôle dans la transition de phase (Cohen, 1971; von Euler et al., 1976;

von Euler, 1977; St-John et Zhou, 1991; Alheid et al., 2004; Rybak et al., 2008; Arata et al., 2010). Certains auteurs proposent un rôle encore plus important pour le pons. Julian F.R. Paton et Walter M. St.-John soutiennent que le pons est une région essentielle pour la genèse du rythme respiratoire normal et que le rythme respiratoire qui est étudié au niveau du bulbe dans les préparations *in vitro* est de l'halètement (Abdala et al., 2009; St-John, 2009). La question à savoir si le pons ne joue qu'un rôle modulateur ou s'il s'agit d'une région essentielle à la genèse du rythme respiratoire demeure donc sujette à débat.

Respiration chez la lamproie

Chez la lamproie, la respiration s'effectue sous l'eau par les branchies. La phase expiratoire consiste en une contraction rapide des branchies qui expulse l'eau qu'elles contiennent. Cette phase de courte durée est suivie d'une phase inspiratoire pendant laquelle une augmentation du volume des branchies mène à une entrée d'eau (Rovainen, 1977). Contrairement aux mammifères, la phase inspiratoire est passive et ne nécessite pas de contraction musculaire. Les motoneurones qui contrôlent les mouvements des branchies sont localisés dans le tronc cérébral. Les noyaux moteurs faciaux (VII), glossopharyngiens (IX) et vagues (X) contiennent les motoneurones qui activent les branchies pendant la phase expiratoire (Rovainen, 1977; Thompson, 1985; Guimond et al., 2003).

Plusieurs études suggèrent qu'une région rostrale et latérale du pons est nécessaire à la genèse du rythme. D'abord, des lésions séparant cette région des motoneurones respiratoires abolissent l'activité respiratoire dans les noyaux moteurs

(Kawasaki, 1979; Thompson, 1985; Martel et al., 2007). De plus, des injections d'antagonistes des récepteurs glutamatergiques dans cette région abolissent le rythme, alors que des agonistes l'accélèrent (Martel et al. 2007; Mutolo et al., 2007). La région porte maintenant le nom de groupe respiratoire paratrigéminal (pTRG), à cause de sa position latérale par rapport au noyau moteur du trijumeau. Il a été montré que des applications de riluzole ou d'acide flufénamique, qui bloquent les courants I_{NaP} et I_{CAN}, ralentissent le rythme respiratoire. Un co-application des deux substances abolit le rythme respiratoire, mais celui-ci peut être réactivé par la substance P (Mutolo et al., 2010). Ces expériences reproduisent des expériences effectuées chez les mammifères (Del Negro et al., 2005) et soulèvent la possibilité que les mécanismes qui sous-tendent la genèse respiratoire pourraient être semblables. Cependant, il reste à établir si les générateurs respiratoires chez la lamproie et chez les mammifères sont homologues. Plusieurs travaux sont effectués en ce sens. D'une part, la caractérisation des gènes impliqués dans le développement de ces réseaux (Bouvier et al., 2010; Gray et al., 2010; Champagnat et al., 2011) et d'autre part la compréhension du fonctionnement des générateurs de rythme chez différents vertébrés comme les poissons, les amphibiens et les mammifères (Wilson et al., 2006) pourraient permettre une comparaison et une compréhension de l'évolution des réseaux de neurones générant la respiration chez les vertébrés.

Pour mon projet de doctorat, nous avons utilisé le modèle de la lamproie pour comprendre l'organisation des réseaux neuronaux générant la respiration et les mécanismes par lesquels elle est modulée. Les réseaux de neurones qui génèrent la respiration sont moins connus que chez les mammifères puisque les efforts de recherche en neurophysiologie respiratoire ont été plus importants chez les mammifères ces

dernières années. Cependant, les réseaux de neurones du tronc cérébral et de la moelle épinière qui génèrent la locomotion sont mieux caractérisés que chez tout autre vertébré. De plus, la lamproie possède un système nerveux central qui peut être isolé en entier *in vitro*, permettant d'accéder à des groupes de neurones pour enregistrer des neurones individuels à l'aide d'électrodes de type *patch*. Ceci constitue un avantage majeur par rapport aux préparations de mammifères qui doivent généralement être coupées en fines tranches pour accéder aux neurones. Cette procédure est malheureusement accompagnée d'un désavantage; elle abolit la plupart des connexions nerveuses entre les centres nerveux qui contrôlent les comportements. En démarrant ce projet dans le laboratoire de Réjean Dubuc, nous avons effectué le pari qu'une connaissance des neurones du tronc cérébral impliqués dans la respiration pourrait d'abord être obtenue et que les connexions entre les centres locomoteurs et respiratoires pourraient ensuite être identifiées. Ce projet s'est concrétisé en 5 contributions scientifiques et une description détaillée de ma contribution à chacun de ces travaux suit.

Énoncé de problème

Les travaux de ma thèse portent sur deux questions actuelles concernant les réseaux neuronaux de la respiration. D'abord, les mécanismes permettant la sychronisation bilatérale du ryhtme respiratoire sont toujours inconnus, et la connectivité détaillée des neurones reliés à la respiration reste à établir autant chez les mammifères que chez la lamproie. Nous utilisons donc le modèle de la lamproie pour caractériser les projections des neurones du générateur de la respiration.

Deuxièmement, les mécanismes neuronaux qui sont responsables de l'augmentation de l'activité respiratoire pendant l'exercice sont aussi toujours inconnus. Nous utilisons des préparations de lamproie où les centres nerveux responsables de la locomotion et de la respiration sont intacts et fonctionnels, ce qui nous permet d'identifier les connexions qui existent entre les centres locomoteurs et respiratoires et d'évaluer leur rôle dans les changements respiratoires reliés à la locomotion.

Description des contributions

Les deux premières contributions effectuées au cours de ma formation aux cycles supérieurs en sciences neurologiques sont des articles auxquels j'ai participé comme co-auteur et ne sont pas inclus dans ma thèse.

1. L'article Martel et al., 2007, publié dans *Neuroscience*, décrit des travaux qui nous ont permis de localiser chez la lamproie les régions du tronc cérébral qui sont essentielles à la genèse des deux types de patrons respiratoires: la respiration normale et les longues bouffées qui apparraissent à une fréquence plus basse. Ma contribution à cet article fut d'effectuer des enregistrements intracellulaires des motoneurones respiratoires montrant une absence de rythme respiratoire normal suite à des lésions du pTRG.

2. Dans l'article Gravel et al. (2007), publié dans *Neuroscience*, nous montrons que des modifications respiratoires accompagnent la locomotion lors de la nage de fuite chez la lamproie. Ma contribution à cet article fut d'analyser les données de changements respiratoires pendant la locomotion et d'effectuer des lésions de la moelle épinière et du mésencéphale montrant que les augmentations respiratoires reliées à la locomotion reposent sur un réseau localisé dans le rhombencéphale.

Les trois autres contributions sont des articles que j'ai écrits comme premier auteur et sont inclus dans la thèse. Le premier est une revue de question et les deux autres sont des articles de recherche pour lesquels j'ai conçu et effectué les expériences décrites, en collaboration avec les co-auteurs.

3. Le premier article ajouté à ma thèse (Article 1) est inclus dans l'introdution (Gariépy et al., 2010). Il s'agit d'un chapitre du livre *Breathe, Walk and Chew: The*

Neural Challenge publié dans *Progress in Brain Research* dans lequel nous décrivons les différentes hypothèses pouvant expliquer la modulation du rythme respiratoire pendant l'exercice. Cette revue de question est ajoutée à l'introduction après la présente section. Le chapitre présente les arguments appuyant l'existence de plusieurs mécanismes physiologiques participant à l'augmentation de la respiration en lien avec les activités physiques.

4. L'article Gariépy et al. (2012a) (Article 2), publié dans *Journal of Comparative Neurology*, contient une description des neurones du groupe respiratoire paratrigéminal (pTRG). Nous avons montré que ces neurones projettent à la fois vers les motoneurones respiratoires ainsi que vers le pTRG controlatéral.

5. L'article Gariépy et al. (2012b) (Article 3), publié dans *Proceedings of the National Academy of Sciences of the United States of America*, montre qu'une sous-population de neurones localisée dans la région locomotrice du mésencéphale (MLR) envoie des projections directes vers les centres respiratoires et que ces neurones jouent un rôle essentiel dans les changements respiratoires reliés à la locomotion chez la lamproie.

The interactions between locomotion and respiration

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Abstract

Respiration is a vital motor activity requiring fine-tuning to adjust to metabolic changes. For instance, respiration increases in association with exercise. In this chapter, we review the mechanisms underlying respiratory changes during exercise. Three specific hypotheses were proposed. First, the chemoreception hypothesis suggests that chemoreceptors located centrally or peripherally modify breathing by detecting metabolic changes in arterial blood or cerebrospinal fluid. Second, the central command hypothesis stipulates that central neural connections from brain motor areas activate the respiratory centers during exercise. Third, the neural feedback hypothesis stipulates that sensory inputs from the contracting limb muscles modulate the respiratory centers during exercise. We present evidence from the literature supporting possible contributions from these three mechanisms. This review also addresses future research challenges relative to respiratory modulation during exercise.

Keywords

Locomotion, respiration, brainstem, lamprey, hyperpnea, chemoreception, feedback.

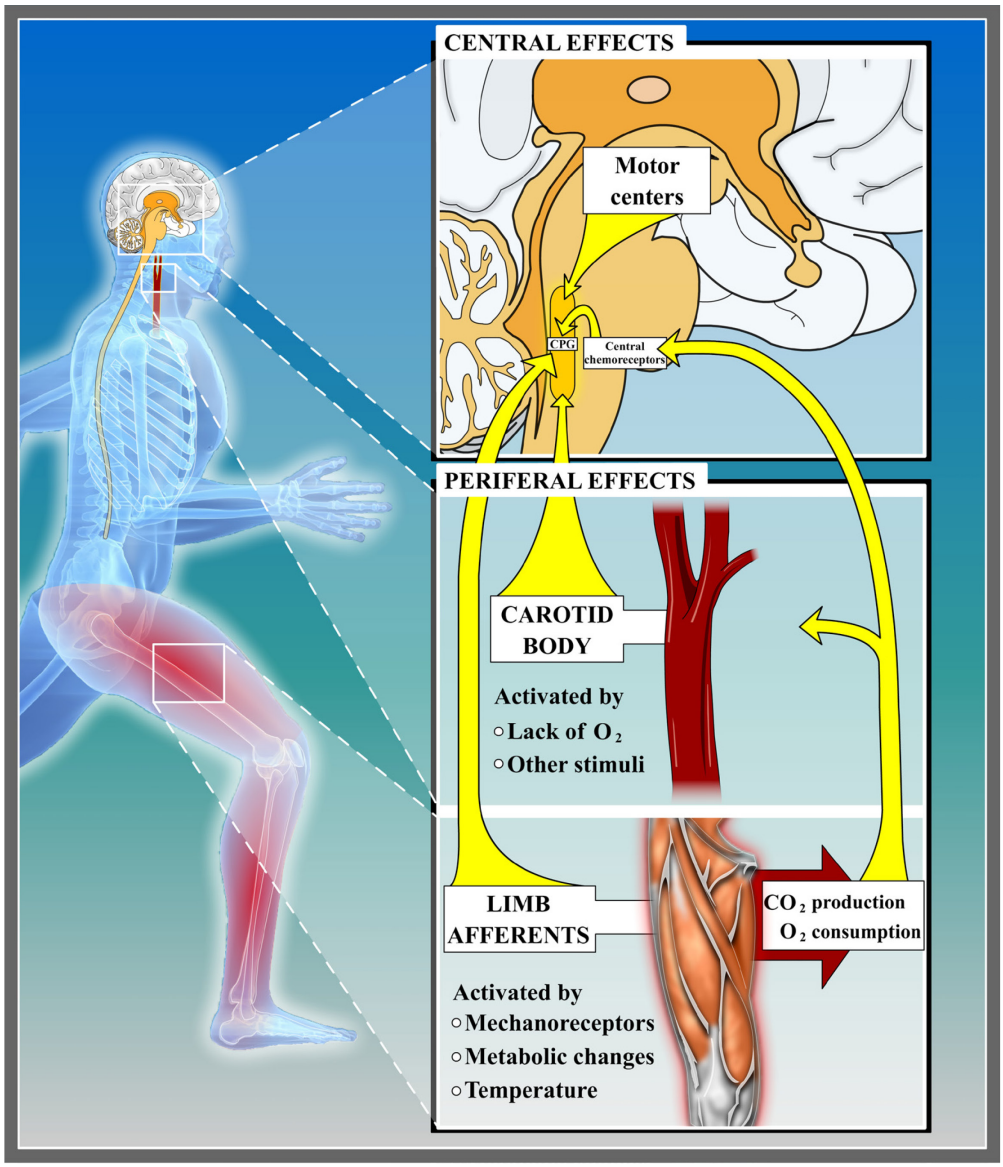
Introduction

Mastication, locomotion, and respiration are rhythmical motor activities patterned by neural networks referred to as central pattern generators (CPGs). The CPGs responsible for each of these three rhythmical activities interact with each other in order to generate motor output that is consistent with specific biomechanical and physiological constraints. For instance, swallowing is associated with a pause in respiratory output to prevent food from being directed through the respiratory system (Matsuo et al., 2007; McFarland et al., 1994; Miller, 1986). Another example of such an interaction is the way by which respiration increases in frequency and depth to compensate for increased needs for gas exchanges during exercise. In this chapter, we focus on how exercise modulates breathing.

Respiration is a rhythmic motor activity that is maintained throughout life to allow gas exchanges between the blood and the environment. Despite its automatic nature, it is strongly influenced by motor and mental activities. Exercise elevates the total demand of oxygen (O_2) and increases the production of carbon dioxide (CO_2). To compensate for this, the rate and depth of breaths increase during exercise. These changes usually correlate with the intensity of the effort (see Mateika and Duffin, 1995). Moreover, in some animal species running at high speed, there is a 1:1 coupling, that is, there is a respiratory cycle for each locomotor cycle.

Physiologists have been intrigued with the question relative to the mechanisms underlying increases in respiration during exercise for a long time. They initially hypothesized that chemical changes in the blood such as an increase of partial pressure of

CO_2, a decrease of partial pressure of O_2, or acidification were the important signals that triggered increased activity of the respiratory centers (Foster, 1866; Haldane and Priestley, 1905; Morat and Doyon, 1900; Volkman, 1841; Winterstein, 1911). Krogh and Lindhard (1913) took a whole different view and proposed that respiratory adjustments during locomotion were due to central neural connections from the motor centers responsible for initiating movements to the respiratory centers in the brainstem. Another view was adopted by others and it was hypothesized that somatic afferent fibers carried excitation to the respiratory centers during exercise (Haouzi et al., 2004b; Morin and Viala, 2002; Viala, 1997; Zuntz and Geppert, 1886). A recent published debate summarizes the arguments in favor of the central command and the peripheral feedback mechanism (Haouzi, 2006; Waldrop and Iwamoto, 2006). Taken together, the hypothesized mechanisms of respiratory modulation during exercise can be regrouped in (1) the chemoreception hypothesis, (2) the central command hypothesis, and (3) the peripheral nervous feedback hypothesis (A1 Figure 1). It is now generally accepted that each of these mechanisms might play complementary roles in modulating respiration during exercise. In this chapter, we will review part of the literature supporting these three hypotheses by focusing on debates that have driven scientific works in each of these fields.



A1 Figure 1: **Graphical representation of the three groups of hypotheses explaining respiratory modulation during exercise**. Chemoreceptors located in the carotid body and the central nervous system can detect gas partial pressure changes, acidity changes, or other metabolic changes in the blood or cerebrospinal fluid. The carotid body projects to the brainstem, where respiratory centers are located, through the sinus nerve, a branch of the glossopharyngeal nerve. Central commands are hypothesized to come from various regions of the brain. The premotor and motor cortex, the brainstem locomotor centers, and the spinal cord CPGs have been hypothesized to project directly or indirectly to the respiratory centers. Somatic afferents have also been hypothesized to send projections to the respiratory CPG. These afferents carry signals related to mechanoreception, changes in temperature, and metabolic changes in skeletal muscles. These signals could in turn increase respiration during exercise and could also be responsible for locomotor–respiratory coupling.

The chemoreception hypothesis

The influence of CO_2 and O_2 on breathing has been known since the demonstration that increasing the CO_2/O_2 ratio in inspired air of human subjects increases their breathing rate (Haldane and Priestley, 1905; Pflüger, 1868). It was originally hypothesized that CO_2 or O_2 concentrations were directly detected by central respiratory centers. This remained the dominant view during the first quarter of the twentieth century (see Heymans, 1963; Remmers, 2005). However, new findings in the 1920s and 1930s challenged this hypothesis. It was found that respiratory effects induced by O_2 deprivation were abolished by cutting the carotid sinus nerve, which innervates the carotid body known today to contain chemoreceptor cells (Heymans and Heymans, 1927). This stressed a contribution of peripheral receptors. Corneille Heymans received the Nobel Prize in Physiology or Medicine in 1938 for this discovery. However, the breathing rate was still increased by CO_2 inhalation (Schmidt, 1932). These observations led to the conclusion that the carotid body detected deficits in O_2, whereas CO_2 was detected centrally. However, long periods of hypoxia also induce a respiratory decrease which is attributed to central effects since it is not associated with a change in the firing of the carotid sinus nerve (Vizek et al., 1987). This was confirmed recently using genetic removal of Task2 potassium channels in the ventral medulla which abolishes this decrease (Gestreau et al., 2010). The original view of chemoreception was further subjected to debate with the proposal of Winterstein (1911, 1949). His reaction theory stated that chemoreceptors detect H+ ions concentration (pH) rather than gas concentrations. Perfusion of the subarachnoid space with solutions of varying pH and constant CO_2 concentration was shown to increase respiration proportionally to the reduction of pH. In contrast, perfusion

with solutions of constant pH and increasing CO_2 concentrations had no effect (Guyenet et al., 2008; Loeschcke, 1982; Loeschcke et al., 1958). They even produced a small depression of respiration, which supported the crucial role of acidity in modulating breathing.

The mechanisms responsible for the detection of gas concentrations/pH changes by the peripheral chemoreceptors are not fully understood. There is evidence that a decrease in potassium leak currents would result in a depolarization of the carotid body's type I cells (chemoreceptors) (Buckler, 1997; Gonzalez et al., 1994; López-Barneo et al., 1988, 2001; Wyatt and Peers, 1995). The disruption of mitochondrial processes can mimic the effect of hypoxia on those currents (Wyatt and Buckler, 2004). Recent hypotheses stipulate that an AMP-activated protein kinase or reactive oxygen species generated in low-oxygen conditions could constitute the hypoxia-detecting mechanisms (Dinger et al., 2007; Gonzalez et al., 2007; Wyatt and Evans, 2007).

The mechanisms by which the central chemoreceptors operate are also not fully understood. Some researchers propose that chemoreception is achieved by different types of neurons distributed in the brain; others support a specialized chemoreception theory which stipulates that some specific brain nucleus such as the retrotrapezoid nucleus is responsible for chemoreception (for review, see Guyenet et al., 2008). In accordance with specialized chemoreception, a recent study showed that glial cells of the retrotrapezoid nucleus can detect changes in pH (Gourine et al., 2010).

The involvement of central chemoreceptors in exercise hyperpnoea

As indicated above, there is strong evidence for the presence of chemoreceptors in the central nervous system. However, their contribution to the respiratory changes during exercise is still not fully established. Changes in gas concentrations or acidity were examined in the brainstem cerebrospinal fluid near during exercise. Exercise in horses maintained for 9 min neither reduced the pH nor increased the partial pressure of CO_2 in cerebrospinal fluid. On the contrary, there was a slight increase in pH and a decrease in the partial pressure of CO_2 (Bisgard et al., 1978). These results suggest that possible changes that would be detected by central chemoreceptors do not occur during exercise and as such, these results do not support an important role for central chemoreception in the modulation of respiration during exercise. Reductions in pH or increases of the partial pressure of CO_2 in the cerebrospinal fluid can be elicited by other methods such as addition of CO_2 to inspired air, occlusion of respiratory pathways, and injection of NaHCO3 in the blood (Eldridge et al., 1984). These methods do increase respiratory activity showing that central chemoreception likely contributes to adjustment of respiration in different physiological states. However, there is no evidence indicating that such control would occur during locomotion. A role of central chemoreceptors for increasing respiration during longer exercise periods or in other animal species cannot be excluded, but it is not supported by currently available data.

The involvement of peripheral chemoreceptors in exercise hyperpnoea

Duke et al. (1952) showed that the discharge of the carotid body nerve fibers is not modified by breathing CO or CO_2 when these gases are mixed with pure O_2. Because of this, investigators began using 100% O_2 inhalation as a tool to suppress the contribution of peripheral chemoreceptors in the modulation of respiration (for review, see Dejours, 1962). Using this technique, it was found that the peripheral chemoreceptors could be responsible for about 15% of the respiratory drive at rest (Dejours et al., 1957, 1958). This technique was also used to suppress the effect of peripheral chemoreceptors during exercise (Jeyaranjan et al., 1987; Wasserman et al., 1979; Whipp et al., 1992). In these studies, respiratory changes are maintained after breathing 100% O_2 during exercise, but the breathing rate is reduced by 15–20%. These results were interpreted as an indication that peripheral chemoreceptors contributed to a minor part of the respiratory adjustments during exercise. The contribution of the peripheral chemoreceptors in the carotid body to exercise hyperpnoea was also supported by data obtained from humans with bilateral carotid body resection. Although there was still a very significant respiratory response to exercise in these individuals, it was reduced as compared to that of control subjects (Honda, 1985).

Other chemoreceptor mechanisms

The lack of strong evidence indicating that CO_2, O_2, or pH at the peripheral and central level could be responsible for a major part of the respiratory effect during exercise prompted researchers to propose that some other factor, yet unknown, could be

responsible for the regulation of respiration (Mitchell et al., 1958; Sinnott, 1961). K+ ions released by muscles during exercise could modulate respiration (Paterson et al., 1989). Intra-arterial injections of KCl near the carotid body increases ventilation through an action of peripheral chemoreceptors (Band et al., 1985). However, experiments using sinusoidal variations in work rate showed that the concentration of K+ ions in the blood was not strongly correlated with the variations in ventilation (Casaburi et al., 1995). It was also proposed that release of catecholamines or adenosine as well as changes in blood osmolarity could play a role in the changes in respiratory activity during exercise (for review, see Mateika and Duffin, 1995). Some of these mechanisms were shown to affect respiration in nonphysiological conditions, but there is no direct evidence that they could be responsible for a major part of respiratory increases during active locomotion.

The central command hypothesis

Krogh and Lindhard (1913) initially proposed that neural mechanisms might be responsible for at least the early respiratory effect observed during locomotion. They showed that respiratory changes occurred at latencies of less than a second after the onset of locomotion in human subjects. They argued that such abrupt changes in respiration could not be induced by variations in the CO_2 content of the blood since blood from the contracting muscles could not reach the respiratory centers in such a short time. Ranson and Magoun (1933) then showed that stimulation of the hypothalamus of decorticated cats generated locomotion associated with increases in respiration. These results were pivotal to indicate that the control of breathing and locomotion might be colocalized in

similar regions of the brain. Eldridge et al. (1981, 1985) performed similar experiments in anesthetized cats in which muscular contractions were blocked. In this paralyzed preparation, chemical or electrical stimulation of the hypothalamic locomotor region produces fictive locomotion, that is, the motor nerves normally involved in locomotion show patterns of rhythmic activation, but the muscles do not actually contract. In those conditions, the increase of respiratory activity was similar to that observed during active locomotion. The authors concluded that central commands originating from locomotor regions played a major role in activating respiratory centers during locomotion. The advantage of the experimental setup used by Eldridge et al. (1981, 1985) was the complete isolation of the central nervous component from any peripheral changes that would result from muscular contractions. It was also shown that stimulation of the dorsolateral spinal funiculus, the cervical spinal cord, or the medullary locomotor strip elicited bouts of locomotion that were also associated with similar increases in respiration (Romaniuk et al., 1994). These observations suggested that either a wide variety of locomotion-inducing neural structures project to respiratory centers, or these structures converge to a single locomotor region, which in turn projects to respiratory centers. Another important issue relative to the central command hypothesis is the diversity of motor behaviors other than locomotion that influence respiration. For instance, hand gripping (Williamson et al., 2002), cycling (Krogh and Lindhard, 1913), weight lifting (Ratamess et al., 2007), or leg movements (Decety et al., 1993) all influence respiration. It is unlikely that the putative projections from locomotor centers to respiratory centers discussed above will account for all these adjustments of respiration during motor

behavior. The central mechanisms involved in respiratory changes during these motor activities will need further investigation.

The central command is the only proposed mechanism capable of modifying respiration before any muscular contraction. It has been shown in subjects performing mental simulation of exercise that respiration and heart rate are increased proportionally to the intensity of the mentally simulated effort (Decety et al., 1993). Higher brain areas activated during imaginary efforts have been identified using positron emission tomography (Thornton et al., 2001). Also in humans, respiration is modified before the onset of movement when the subjects are instructed to start moving after a given delay (Tobin et al., 1986). The activation of locomotor regions of cats at a subthreshold intensity for eliciting locomotion could still induce respiratory effects (Eldridge et al., 1981, 1985). These findings support a role for a central command in producing anticipatory adjustments of respiration in association with exercise. However, Eldridge et al. (1981) also showed that the respiratory increase was maintained throughout the fictive locomotor bouts. This suggests that the central command may be responsible not only for anticipatory and early respiratory responses to movement but also for adjusting the respiratory output during the entire movement episode.

It was shown by Haouzi et al. (2004a) that the peaks of breathing activity do not occur synchronously with the peaks of locomotor activity in sheeps walking on a treadmill when sinusoidal variations of speed are imposed. Rather, the peaks of breathing activity were synchronous with the peaks of CO_2 output and this led the authors to propose that respiratory changes were, in major part, dissociated from the influence of locomotor centers (Haouzi, 2006). These observations neither obligatorily dismiss the

contribution of a central command nor ascribe a major part of the respiratory changes to peripheral mechanisms. There is a delay between the changes in locomotor frequency and respiratory activity, but whether this delay is caused by peripheral or central mechanisms still needs to be determined.

Localization of the central command

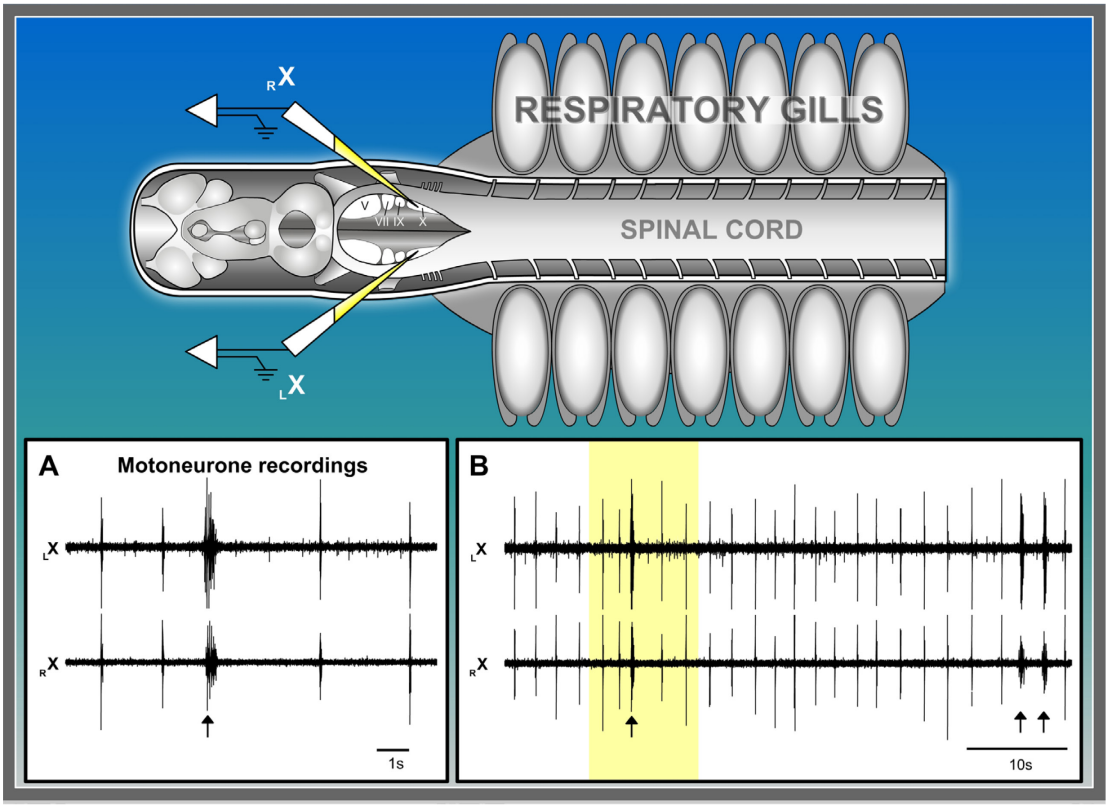
The mechanism proposed to account for the central effects of locomotion on respiration involves connections from supraspinal locomotor centers to respiratory regions (Eldridge et al., 1981; Waldrop and Iwamoto, 2006). An alternative hypothesis is that the locomotor CPGs in the spinal cord would provide inputs to the brainstem respiratory generator during locomotion. This hypothesis was proposed following the demonstration that pharmacological activation of the lumbar spinal cord increased respiration in an *in vitro* isolated brainstem–spinal cord preparation from rats (Morin and Viala, 2002). This paper addressed mainly the coupling between locomotion and respiration on a cycle to cycle basis. In addition, the authors suggested that the activation of locomotor CPGs could increase the frequency of the respiratory rhythm without coupling it to locomotion. The mechanisms by which the spinal locomotor networks can modulate the respiratory rhythm will need to be investigated in more details.

Recent studies on lampreys in our laboratory have provided strong arguments in favor of a supraspinal localization of the connectivity responsible for adjusting respiratory activity to locomotor output (Gravel et al., 2007). The central nervous system of lampreys displays a very similar organization to that of other vertebrate species including

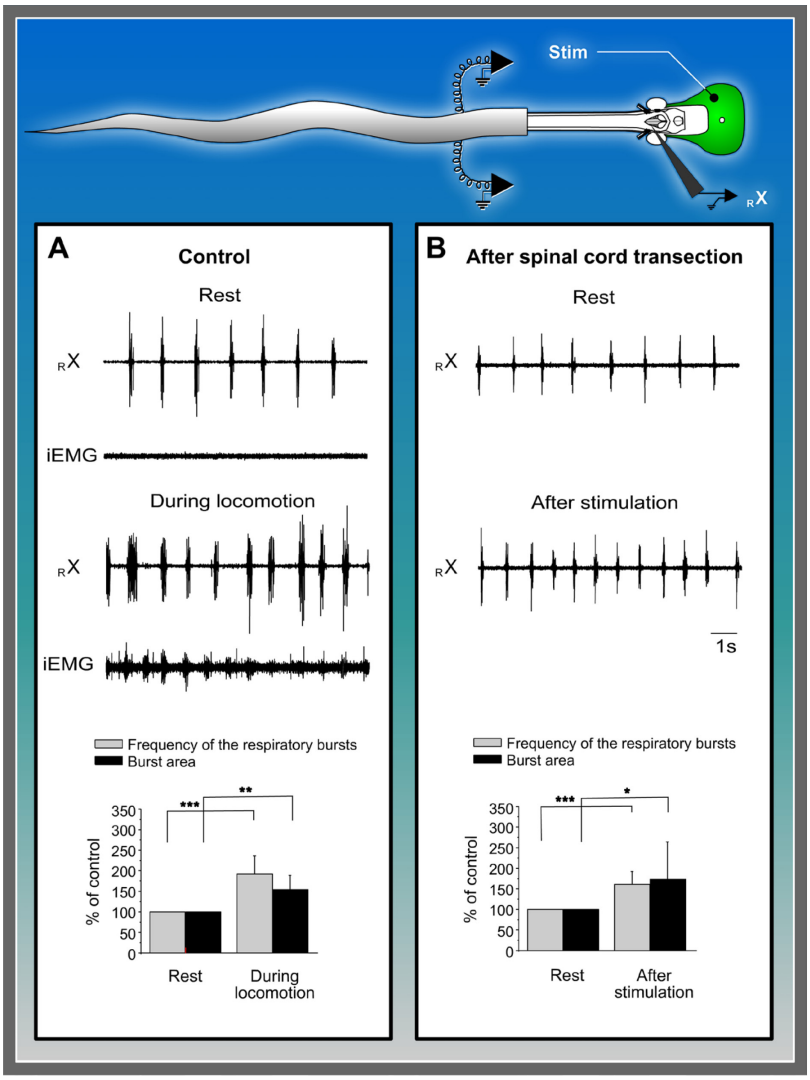
mammals. It contains far fewer neurons than that of mammals and the neurons are easily accessible to an array of neurobiological techniques, making the lampreymodel a very useful one to study the neural control of locomotion and respiration. Respiration in this animal consists in rhythmical contractions of gill muscles, which expels water fromthe gill baskets through the gill pores. Seven gill pores are present on each side of the body. Despite the considerable differences in the peripheral apparatus responsible for respiration compared to that of mammals, the general organization of the neural networks responsible for controlling both systems present important similarities. Respiratory rhythmogenesis in all vertebrates is accomplished by groups of cells in the hindbrain. In mammals, the rhythmic pattern activates different motoneuron pools including spinal motoneurons innervating the diaphragm, intercostal, and abdominal muscles (Bianchi and Pásaro, 1997; Giraudin et al., 2008). There are also brainstem motoneurons innervating muscles of the upper respiratory airways which also receive inputs from the respiratory generator. These motoneurons are located in the facial (VII), glossopharyngeal (IX), and vagal (X) nuclei (Bianchi and Pásaro, 1997). In lampreys, the respiratory motoneurons were also identified in the VII, IX, and X motor nuclei (Guimond et al., 2003; Martel et al., 2007; Rovainen, 1985; Thomspon, 1985). The rostrocaudal topography of respiratory motoneurons reflects the rostrocaudal innervations of gill muscles (Guimond et al., 2003). The respiratory rhythm can be recorded in the *in vitro* isolated brainstem with or without the gills attached (A1 Figure 2) and with or without the tail attached (A1 Figure 3). In the 1980s, the organization of the respiratory networks was examined in the *in vitro* isolated brainstem of lampreys. Lesions and intracellular recordings suggested that a region located rostrolaterally to the trigeminal motor nucleus was essential for respiratory

rhythmogenesis (Rovainen, 1985; Russell, 1986; Thomspon, 1985). This region is referred to as the paratrigeminal respiratory group (pTRG; Mutolo et al., 2007). It was also suggested that there would be a more caudal respiratory generator in the region corresponding to the medulla oblongata of mammals (Kawasaki, 1979, 1984; Thomspon, 1985). It was recently found using lesions and local drug applications that the pTRG is responsible for the generation of the regular, fast respiratory pattern (see small bursts in A1 Figure 2), whereas the caudal part of the brainstem generates large bursts that occur more sporadically (Martel et al., 2007;Mutolo et al., 2007, 2010). These large bursts are believed to play a role in emptying the gills from particles (see arrows in A1 Figure 2). One advantage of lampreys as a model is the ability to use semi-intact preparations, in which the brain and rostral spinal cord are exposed whereas the tail of the lamprey is left intact, capable of swimming freely in a deeper part of the recording chamber (A1 Figure 3a). Brainstem regions can thus be recordedwhile the animal displays active swimming. Fictive respiration can be recorded from respiratory motoneurons in the facial (VII), glossopharyngeal (IX), or vagal (X) motor nuclei (Guimond et al., 2003). To elicit bouts of swimming, sensory stimulation was applied by touching skin on the dorsal surface of the head or the tail. This type of stimulation is known to induce locomotion through the activation of the sensory inputs to reticulospinal cells (Antri et al., 2009; Viana di Prisco et al., 1997, 2000). We found that locomotion was associated with marked increases in the frequency and area of respiratory bursts (A1 Figure 3b). A complete transverse section at the level of the obex was performed to separate the spinal cord from the brainstem. Sensory stimulation did not induce locomotion anymore, but the effects on respiration were preserved (A1 Figure 3c). These effects were of slightly lesser

magnitude than those observed when muscle contractions occurred (91.5 ± 43.8% increase of respiratory frequency in control vs. 60.1 ± 31.3% after spinal transection for frequency of respiratory bursts; A1 Figure 3b and c). These results suggest that an important part of the central component of the respiratory changes during locomotion relies on brainstem connections and that active locomotion is not essential to augment respiration. We are currently investigating electrophysiologically and anatomically neuronal projections from locomotor regions to respiratory regions in the brainstem of lampreys.



A1 Figure 2: **Respiratory activities recorded in a semi-intact lamprey preparation with gills**. Top: Sketch of the preparation with gill baskets attached and recording electrodes placed over the right ($_R$X) and left ($_L$X) vagal motor nuclei. (a) Neurographic recordings showing the fast (small bursts) and slow (arrow, large bursts) respiratory rhythms. (b) Same records as in (a) but on a longer time scale. (Martel et al., 2007)



A1 Figure 3: **Locomotion-induced respiratory increases in a semi-intact lamprey preparation with tail attached**. Top: Sketch of the preparation. The tail is left intact and free to swim in a deep part of the bath. EMGs are placed in each side of the tail to record locomotor activity. The brainstem is exposed and an extracellular recording electrode is placed over the right vagal nucleus ($_RX$) to record respiratory activity. Mechanical stimulation (light touch) of the head induces a bout of swimming in this preparation. (a) Respiratory activity and EMG recordings at rest and during locomotion. Bottom: Statistical analyses on the changes in frequency and area of respiratory bursts in four semi-intact preparations. (b) Respiratory activity after complete lesion of the spinal cord at rest and after applying the same mechanical stimulus used in (a). Bottom: Statistical analyses on the changes in frequency and area of the respiratory bursts in four semi-intact preparations after removal of the spinal cord. (Gravel et al., 2007)

In conclusion, a central command component of respiratory modulation has been identified. The respiratory adjustments are preserved in the absence of chemical changes in the blood or cerebrospinal fluid and afferent activity resulting from muscular contractions. Furthermore, we have shown that connections in the brainstem might play an important role in respiratory modulation during locomotion (Gravel et al., 2007). Despite the strong evidence for the existence of a central command, cellular and network mechanisms responsible for respiratory increases during locomotion still need to be identified.

The peripheral nervous feedback hypothesis

It was proposed that peripheral nervous feedback from the contracting muscles contributed to the increase of respiratory activity during exercise (Haouzi et al., 2004b; Mateika and Duffin, 1995; Zuntz and Geppert, 1886). This hypothesis is supported by data indicating that stimulation of ventral roots causing contractions of hind limb muscles in anesthetized dogs and cats induces respiratory changes (Comroe and Schmidt, 1943). Cutting the dorsal roots abolished the respiratory response, suggesting that somatic afferents were involved (McCloskey and Mitchell, 1972). A fast respiratory response also occurred when limbs were passively moved both in awake and asleep humans (Ishida et al., 1993). It was proposed that the signal from the exercising limbs was carried by type III and IV afferents, which can be activated by movement, accumulation of exercise by-products, local inflammation, and rise in muscle temperature (for review, see Haouzi et al., 2004b). For instance, the receptors located in the skeletal muscles could detect local

vascular responses, which would provide an estimation of metabolic changes and thus excite respiratory centers proportionally to CO_2 production (Haouzi and Chenuel, 2005).

The importance of the peripheral feedback mechanisms during exercise is difficult to ascertain because experimental manipulations aiming at isolating peripheral feedback from other mechanisms do not always do so. For instance, when limbs are passively moved or electrically stimulated, the observed respiratory changes may also be due to changes in awareness, wakefulness, behavioral state, or different cognitive processes. Such interactions between the respiratory effects of passive limb movements and cognitive processes have been described in human subjects. Bell and Duffin (2004) showed that respiratory changes induced by passive limb movements were greatly reduced when the subjects solved a computer puzzle. These observations could be viewed as an indication that cognitive processes can suppress part of the respiratory changes induced by peripheral inputs. It is well documented in other motor systems that central processes gate sensory transmission during movements (Chapman et al., 1988; Clarac et al., 2000; Sillar, 1991; for review, see Rossignol et al., 2006).

Locomotor–respiratory coupling and peripheral afferents

Another aspect of respiratory adjustments during locomotion is the coupling that occurs between the two rhythms at high speed of locomotion. Locomotor–respiratory coupling is the synchronization of the phases of the respiratory rhythm and the locomotor rhythm during locomotion. It is proposed that this coupling is necessary to keep harmonized contractions between muscles responsible for locomotion and respiration and

avoid biomechanical conflicts that would result in inefficient muscular contractions or energy losses (Viala, 1997). This coupling was reported by Bannister et al. (1954) in humans running on a treadmill and was also shown to occur during pedaling on a bicycle using cross-correlation analysis (Bechbache and Duffin, 1977). It was originally observed that locomotor cycles were integer-multiples of breathing cycles, leading to locomotor–respiratory coupling ratios of 1:1, 2:1, 3:1, or 4:1. These observations were confirmed by some (Hill et al., 1988; Paterson et al., 1987), whereas others did not find coupling between the two rhythmic motor activities (Kay et al., 1975). Other types of locomotor–respiratory coupling such as 5:2 and 3:2 were also reported, although 2:1 seemed to be the dominant ratio observed in humans (Bramble and Carrier, 1983). Locomotor–respiratory coupling was seen more often during running than during cycling (Bernasconi and Kohl, 1993). Moreover, it was more frequent in experienced than nonexperienced runners (Bramble and Carrier, 1983). However, several studies made on nonexperienced runners have also found a coupling (see Viala, 1997).

The coupling between respiratory and locomotor activities was also observed in cats running on a treadmill (Iscoe, 1981). It was predominantly a 1:1 ratio. Other studies on different quadruped species have shown that 1:1 locomotor–respiratory coupling occurs mostly when the animals were galloping or hopping but also, in some occasions, during trotting (Baudinette et al., 1987; Bramble and Carrier, 1983; Kawahara et al., 1989; Young et al., 1992a).

The piston mechanism

One mechanism proposed to explain locomotor–respiratory coupling is the visceral piston mechanism (Bramble and Carrier, 1983). There would be a passive coupling betweenlocomotion and respiration due to the visceral mass rhythmically pushing on lungs during locomotion. This hypothesis was supported by kinematic data in dogs showing that oscillations of the visceral mass were pushing the diaphragm during locomotion, thus driving ventilation independently from muscular contractions (Bramble and Jenkins, 1993). In horses, a similar phenomenon caused by back flexion was observed (Young et al., 1992b). However, it was suggested that this passive biomechanical phenomenon might be less likely to occur in humans due to their upright position during walking (Viala, 1997). As reviewed in the following sections, evidences have now been obtained indicating that although such passive mechanisms could be present in some animals, there are also neural components actively coupling respiratory muscle contractions to the locomotor rhythm.

The spinal cord CPGs

The locomotor networks of the spinal cord are believed to play at least a partial role in coordinating respiration and locomotion in different coupling ratios. It was reported in different animal preparations that paralysis does not prevent locomotor–respiratory coupling (Corio et al., 1993; Funk et al., 1992b; Perségol et al., 1988; Viala et al., 1987). This indicates that central connections are sufficient to couple the respiratory rhythm to fictive locomotion in these experimental conditions and such mechanisms

might also be important to couple respiration to active locomotion. Morin and Viala (2002) showed that pharmacologically activating the lumbar locomotor generator was not sufficient to induce locomotor–respiratory coupling *in vitro*. This suggests that the activation of the cervical locomotor generator or the activation of both generators might be necessary to entrain respiration. The relative importance of this coupling mechanism compared to the other mechanisms remains open to investigation.

Supraspinal influences

Some studies have raised the possibility that cognitive processes could be involved in locomotor–respiratory coupling. For instance, Bechbache and Duffin (1977) showed that subjects pedaling at frequencies determined by a metronome were more likely to develop locomotor–respiratory coupling than subjects who are asked to follow a certain speed using a speedometer. These results suggest that respiratory muscles could be entrained by acoustic stimuli. Lower limb movements are known to be entrained by auditory inputs (Brown et al., 2006; Rossignol and Jones, 1976). Similar mechanisms could thus be responsible for the coupling of respiration to locomotion. Moreover, Bramble and Carrier (1983) reported that locomotor–respiratory coupling occurred earlier during locomotion and was stronger in experienced runners compared to inexperienced runners. This suggests that locomotor–respiratory coupling can be learned or acquired by training.

Studies made on decerebrate preparations in which locomotor–respiratory coupling is preserved have provided arguments against a contribution of forebrain

structures to the coupling (Corio et al., 1993; Funk et al., 1989; Viala, 1997). These studies showed that locomotor–respiratory coupling can occur without cortical areas and therefore that subcortical and peripheral mechanisms are involved. However, to our knowledge, no attempt was made to determine the relative importance or to completely exclude the contribution of cognitive processes to locomotor–respiratory coupling in humans, in which the locomotor–respiratory coupling patterns are more varied and often consist in subharmonics (2:1, 4:1) rather than the predominantly pure phaselock (1:1) observed in many animal preparations.

The peripheral nervous feedback

In mammals, repetitive stimulation of muscle and cutaneous afferents entrain respiration in different ratios (Howard et al., 1969; Iscoe and Polosa, 1976). Single stimulation can also reset the respiratory rhythm when applied during expiration (Kawahara et al., 1988). Passive wing flapping has been shown to entrain respiration in the Canada goose (Funk et al., 1992a). It was shown that low-threshold stimulation of sensory pathways from hindlimb muscles can effectively reset respiration in neonatal rats (Morin and Viala, 2002). These data suggest that there is connectivity between somatic afferents and the respiratory centers and that these connections can entrain the respiratory rhythm. It is thus possible that during locomotion, the rhythmical activation of these afferents entrain respiration in the different locomotor–respiratory coupling ratios that are known. However, as for the other proposed mechanisms, the relative importance of this mechanism compared to others during active locomotion is still unknown.

Conclusion

Modulation of respiration during exercise has been the center of a century-old debate opposing supporters of the chemoreception hypothesis, the central command hypothesis and the peripheral nervous feedback hypothesis. Most likely, the respiratory adjustments during locomotion result from a combination of these mechanisms since no isolating paradigm has successfully shown that either of these mechanisms alone could explain all the adjustments that occur during exercise. Respiratory centers are being increasingly well described in terms of connectivity and cellular properties, and animal models in which these properties can be studied have become available, including some where locomotor centers are still present and functional (Gravel et al., 2007; Smith and Feldman, 1987). Using these preparations, it will likely be possible to identify in a near future the neural cellular mechanisms responsible for respiratory adjustments during locomotion.

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Résultats

Bilateral connectivity in the brainstem respiratory networks of lampreys.

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Abstract

This study examines the connectivity in the neural networks controlling respiration in the lampreys, a basal vertebrate. Previous studies have shown that the lamprey paratrigeminal respiratory group (pTRG) plays a crucial role in the generation of respiration. By using a combination of anatomical and physiological techniques, we characterized the bilateral connections between the pTRGs and descending projections to the motoneurons. Tracers were injected in the respiratory motoneuron pools to identify pre-motor respiratory interneurons. Retrogradely labeled cell bodies were found in the pTRG on both sides. Whole-cell recordings of the retrogradely labeled pTRG neurons showed rhythmical excitatory currents in tune with respiratory motoneuron activity. This confirmed that they were related to respiration. Intracellular labeling of individual pTRG neurons revealed axonal branches to the contralateral pTRG and bilateral projections to the respiratory motoneuronal columns. Stimulation of the pTRG induced excitatory postsynaptic potentials in ipsi- and contralateral respiratory motoneurons as well as in contralateral pTRG neurons. A lidocaine HCl (Xylocaine) injection on the midline at the

rostrocaudal level of the pTRG diminished the contralateral motoneuronal EPSPs as well as a local injection of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and (2R)-amino-5-phosphonovaleric acid (AP-5) on the recorded respiratory motoneuron. Our data show that neurons in the pTRG send two sets of axonal projections: one to the contralateral pTRG and another to activate respiratory motoneurons on both sides through glutamatergic synapses.

Keywords

Respiration, neuroanatomy, retrograde labeling, anterograde labeling, bilateral, descending command, whole-cell patch recordings.

Introduction

Respiration is a vital motor function controlled by neurons of the brainstem and spinal cord in vertebrates. Identifying the connections between the different respiratory regions is a focus of research in several animal models including fish, amphibians, and mammals. It has been argued that the respiratory oscillators from different classes of vertebrates could be homologous (Vasilakos et al., 2005; Wilson et al., 2006; Kinkead, 2009), but a better understanding of the neuronal networks underlying respiratory rhythmogenesis is needed for comparative analysis. This study focuses on the neural networks controlling respiration in the lamprey, a basal vertebrate.

In mammals, respiration is ensured by the contraction of respiratory muscles that are activated by motoneurons located in the spinal cord and brainstem. The spinal cord contains motoneurons activating the diaphragm, the intercostal muscles, and the abdominal muscles involved in breathing (Dobbins and Feldman, 1994; Giraudin et al., 2008). The brainstem contains motoneurons activating muscles of the upper respiratory pathways through the facial (VII), glossopharyngeal (IX), vagal (X), and hypoglossal (XII) nerves (Hwang et al., 1988; Bianchi and Pásaro, 1997). In lampreys, all respiratory motoneurons are located in the motor nuclei of cranial nerves VII, IX, and X (Rovainen, 1977; Guimond et al., 2003), none being located in the spinal cord (Martel et al., 2007).

In this study, we investigated the bilateral projections of neurons in the respiratory generator as well as their descending projections to the respiratory motoneurons in lampreys. In mammals, the neural areas that generate the respiratory pattern have been explored in several studies. These areas are located in the brainstem

and include the pre-Bötzinger complex (preBötC), the retrotrapezoid nucleus/parafacial respiratory group (RTN/pFRG), and the Kölliker-Fuse nucleus (Smith et al., 1991; Onimaru and Homma, 2003; Del Negro et al., 2005, 2008; Feldman and Janczewski, 2006; Janczewski and Feldman, 2006; Onimaru and Homma, 2006; Barnes et al., 2007; Pace et al., 2007; Rubin et al., 2009; Abdala et al., 2009; Mörschel and Dutschmann, 2009; Feldman, 2010; Pagliardini et al., 2011). Anatomical studies have revealed abundant interconnections between these areas as well as with other areas of the brainstem involved in the neural control of respiration, including the caudal and rostral ventral respiratory groups and the Bötzinger complex (Smith et al., 1989; Tan et al., 2010).

Researchers have also identified crossing connections between the brainstem respiratory centers (Onimaru et al., 1993; Bouvier et al., 2010; Tan et al., 2010), and some were shown to play a crucial role in synchronizing the respiratory rhythm on both sides (Bouvier et al., 2010). The ventral respiratory group and the Bötzinger complex also send efferent projections to the brainstem and spinal cord areas that contain respiratory motoneurons, and some of these connections cross and might participate in the bilateral synchronization of the respiratory rhythm (Feldman et al., 1985; Ellenberger and Feldman, 1988; Janczewski and Karczewski, 1990; Goshgarian et al., 1991; Dobbins and Feldman, 1994; Alheid et al., 2002; Ezure et al., 2003; Li et al., 2003; Duffin and Li, 2006; Tarras-Wahlberg and Rekling, 2009).

The studies performed in mammals have revealed some of the interconnections between the brainstem respiratory areas. However, an understanding of the interactions between the properties of single neurons and their connectivity will likely be obtained

from preparations in which the recording of single neurons is made possible while preserving the entire central respiratory network intact. Some of the important questions that remain relate to the extent of the role of individual neurons in the network. For instance, could individual neurons send crossing connections as well as project to the respiratory motononeurons, or are there separate neuronal populations with each type of projection? We have addressed this question by using a brainstem preparation of lamprey.

A region of the rostro-lateral rhombencephalon, the paratrigeminal respiratory group (pTRG), has been identified as necessary for respiratory rhythmogenesis in lampreys (Kawasaki, 1979; Thompson, 1985; Martel et al., 2007; Mutolo et al., 2007; Mutolo et al., 2010). In this study, we have identified a population of pTRG neurons sending projections to the brainstem motor nuclei involved in breathing using retrograde labeling. In addition, single-cell recording and labeling allowed us to confirm that these pTRG neurons were related to respiration and that they sent axonal branches to both the respiratory motoneurons and the contralateral pTRG. Some of the implications of these results with regards to the evolution of the neural systems controlling breathing in vertebrates are discussed.

Materials and Methods

Experiments were performed on 57 postmetamorphic and adult sea lampreys, Petromyzon marinus that were collected from the Great Chazy River (Champlain, NY), from Lake Huron (ON, CAN), and from Morpion Stream (Ste-Sabine, QC, Canada). The

animals were kept in aerated water at 7°C. All surgical and experimental procedures conformed to the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care and Use Committee of the Université de Montréal and the Université du Québec à Montréal. Care was taken to minimize the number of animals used and their suffering.

Anatomical experiments

The connections between the pTRG of both sides of the brain and the descending connections from the pTRG to the respiratory motoneurons were examined by using the tracers biocytin (Sigma, St. Louis, MO) and Texas Red dextran amines (3,000 MW, Invitrogen/Molecular Probes, Eugene, OR). Animals were first deeply anesthetized with MS-222 (Sigma; 100 mg/liter in fresh water), and then their brainstem was completely isolated *in vitro*, with the exception of the underlying cranium that was kept for pinning and support. During the dissection and experiments, the preparations were kept at 7–9°C in continuously renewed oxygenated Ringer's solution with the following composition (in mM): NaCl, 130; KCl, 2.1; CaCl2, 2.6; MgCl2, 1.8; HEPES, 4; dextrose, 4; NaHCO3, 1. The pH was adjusted to 7.4 with NaOH.

The brain tissue at the site of the tracer injection was first lesioned under visual guidance with the tip of a glass micropipette to allow the local neuronal processes to pick up the tracer (Glover et al., 1986). Crystals of tracers were inserted to dissolve in the lesioned area. To label the motoneurons, nerves VII, IX, and X were cut peripherally, and crystals of tracer were deposited on the cut end of the nerves. The preparations were kept

overnight in Ringer's solution to allow tracers to be transported. They were then fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.1 M, pH 7.4 with 0.9% NaCl) for 24 hours at 4°C, transferred to 20% sucrose in phosphate buffer (0.1 M, pH 7.4) for cryoprotection, and cut transversally or parasagittally in a cryostat (25-µm thickness).

The sections were collected on ColorFrost Plus slides (Fisher Scientific, Fair Lawn, NJ) and left to dry on a warming plate at 37°C overnight. The next day, the sections were either mounted directly with Vectashield (Vector, Burlingame, CA) or incubated as follows to reveal the biocytin: three 10-minute rinses with PBS, a 60-minute incubation in streptavidin conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA) diluted 1:200 in PBS at room temperature, three rinses with PBS, a quick rinse in distilled H2O, and air-drying for 15 minutes. These slides were then mounted with Vectashield. The size of the retrogradely labeled pTRG neurons was measured with a 40× objective and a micrometric scale incorporated in the ocular of the fluorescence microscope. Only neurons with a clearly visible nucleus were measured along their longest axis. To provide a general idea of the range of neuronal diameters, we sampled 101 pTRG neurons in three animals.

Electrophysiological experiments

The animals were anesthetized and dissected similarly to anatomical experiments. The brainstem preparation was pinned down onto silicone elastomer

(Sylgard) at the bottom of an experimental chamber continually perfused with cold Ringer's solution at a rate of ~4 ml/min.

Extracellular recordings of respiratory motoneurons were made by using glass electrodes filled with Ringer's solution and placed on the surface of the X nucleus (tip diameter ≈5 μm). The electrodes were connected to an AC amplifier (model 1800, A-M Systems, Sequim, WA; low cut-off: 100 Hz; high cut-off: 500 Hz). Respiratory motoneurons were recorded intracellularly with sharp glass microelectrodes (4 M KAc; 80–120 MΩ). The signals were amplified by an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA; sampling rate: 10 kHz). The motoneurons were impaled by directing the tip of the electrode to the IX/rostral X motor nuclei. In some experiments, the pTRG was electrically stimulated with single pulses (1–10 μA) by using glass-coated tungsten microelectrodes (0.8–2 MΩ) at a rate of 0.05 Hz.

To determine the localization of the descending pathways from the pTRG to respiratory motoneurons, injections of lidocaine HCl (Xylocaine 2%, AstraZeneca, Mississauga, ON, Canada) were made with a micropipette by using a Picospritzer (General Valve, Fairfield, NJ). In some experiments, the glutamatergic receptor blockers 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 1 mM) and (2R)-amino-5-phosphonovaleric acid (AP-5; 500 μM) were injected on intracellularly recorded respiratory motoneurons. In all cases, the inactive dye Fast Green was added to the drug solution to monitor the size and exact location of the injections. The size of the injections was estimated visually to be no more than 300 μm in diameter. The duration of pressure injections was adapted to the flow of the drug solution out of the micropipette, varying from several seconds to minutes.

Whole-cell recordings from pTRG neurons were also performed to characterize the activity of pTRG neurons and their projections in the brainstem. As a first step, Texas Red dextran amines were injected in the X nucleus *in vivo* to retrogradely label the pTRG neurons to be recorded the following day. A 2-mm2 opening was made on the top of the cranium to expose the caudal brainstem of the anesthetized animal. The tracers were introduced in the X nucleus, and the incision was then closed with Vetbond (3M Animal Care Products, St. Paul, MN). The animal was then returned to a small nursery aquarium filled with oxygenated Ringer's solution for the night at room temperature, and an *in vitro* isolated brainstem preparation was performed as described above on the following day. In order to access the pTRG cells with patch electrodes, part of the dorsal hindbrain was removed by using a vibratome. The alar plate lateral to the V motor nucleus, the dorsal part of the V motor nucleus, the dorsal isthmic region, and the optic tectum were removed. The cells were targeted under an Eclipse FN-1 microscope (Nikon Instruments, Melville, NY) equipped for fluorescence. The patch solution contained (in mM): cesium methane sulfonate 102.5, NaCl 1, MgCl2 1, EGTA 5, HEPES 5, and 0.1% biocytin for intracellular labeling of the entire axonal arborization of the recorded cell. The pH was adjusted to 7.2 with CsOH and pipettes were pulled to a tip resistance of 5 MΩ. Patch recordings were made in whole-cell voltage-clamp mode (−70 mV) with a model 2400 amplifier (A-M Systems).

Data acquisition and analyses

Data were acquired via a Digidata 1322A interface using Clampex 9 software (Axon Instruments) for computer analysis. Excitatory postsynaptic potentials (EPSPs) were analyzed by using Spike2 version 5.19 (Cambridge Electronic Design, Cambridge, UK) and homemade scripts. Averages from 20 to 100 EPSPs per animals were used for comparing control, effect, and washout conditions.

Data in the text and figures are given as means ± standard deviation. Statistical analyses were carried out by using SigmaStat v3.5 (Systat Software, Chicago, IL). A Student's t-test was used to compare the means of two groups. A Mann-Whitney test was used when the compared distributions did not respect the assumptions of normality of distribution or equality of variance. Repeated measures ANOVA was used when more than two groups were compared. Distributions were considered statistically significant when the value of P was less than 0.05.

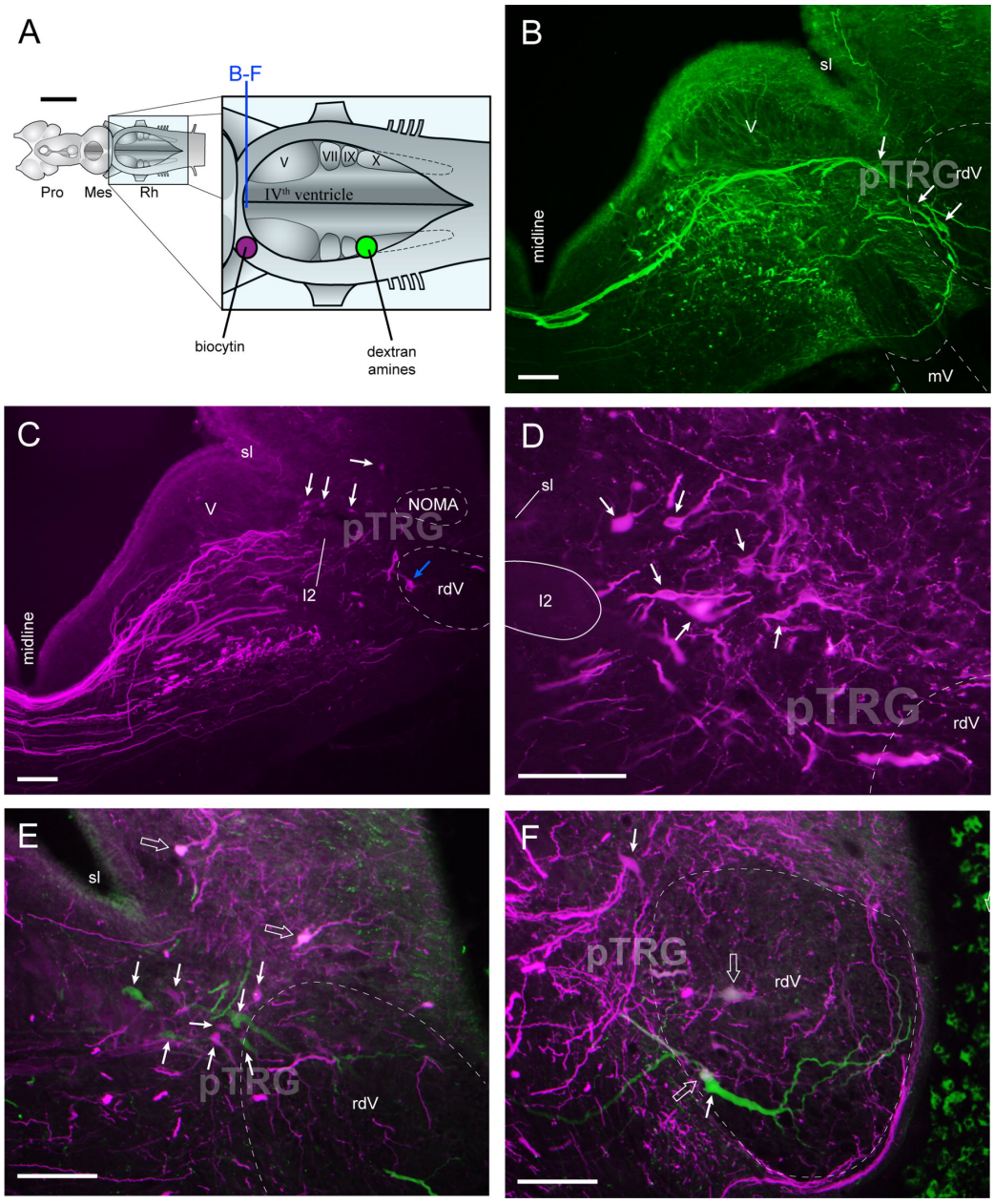
The sections were observed and photographed with an E600 epifluorescence microscope equipped with a DXM1200 digital camera (Nikon Canada, Mississauga, ON, Canada) using fluorescein isothiocyanate (FITC) or Texas Red filter sets. The figures were designed and assembled using CorelDraw X4 (Corel, Ottawa, ON, Canada) and Illustrator CS4 (Adobe Systems, San Jose, CA) software. Photoshop CS4 software (Adobe) was used to make small adjustments when photomicrographs taken with separate fluorescence filter sets were merged. All purely red-green images were transferred to magenta-green in Photoshop to make our work accessible to those who have red-green color blindness.

The axonal projections of each injected neuron were analyzed in detail by using fluorescence microscopy. A 3D reconstruction was made from one typical pTRG neuron for illustrative purposes by using Neurolucida (MBF Neuroscience, Williston, VT). In addition to the contours of the brainstem, the MRRN, PRRN, V, VII, IX, X motor nuclei, and pTRG were also outlined based on the location of the cell bodies in each nucleus.

Results

Anatomical tracing experiments

Neuronal tracers were injected in the pTRG, the respiratory motoneuronal pools, and the respiratory nerves in order to identify the connections between the pTRGs on both sides and their descending projections to respiratory motoneurons. A first series of experiments was undertaken to retrogradely label the pTRG neurons that project to respiratory motoneurons by injecting tracers unilaterally in the respiratory motoneuronal pools (A2 Figure 1A; n = 21). In a subset of these experiments, an additional injection of fluorescent dextran amines or biocytin was made in the ipsilateral (n = 6) or contralateral (n = 6) pTRG areas in order to identify putative crossing projections between the pTRGs of both sides of the brain.



A2 Figure 1: **Morphology and location of neurons in the paratrigeminal respiratory group (pTRG) that project to the contralateral pTRG, the contralateral respiratory motoneuron pool, or both.** A: Schematic representation of a dorsal view of the brain of the adult sea lamprey illustrating injection sites in the pTRG (magenta) and respiratory motoneurons (green). B: Photomicrograph of a cross section illustrating retrogradely labeled neurons (arrows) in the pTRG area after an injection of biocytin in the contralateral respiratory motoneuron pool. A population of large neurons with remarkable dendritic extensions was located in the contralateral pTRG and had a characteristic large-diameter axon that crossed the midline at the level of the cell body. Smaller cells were also present lateral to the trigeminal motor nucleus (V) and close to the sulcus limitans

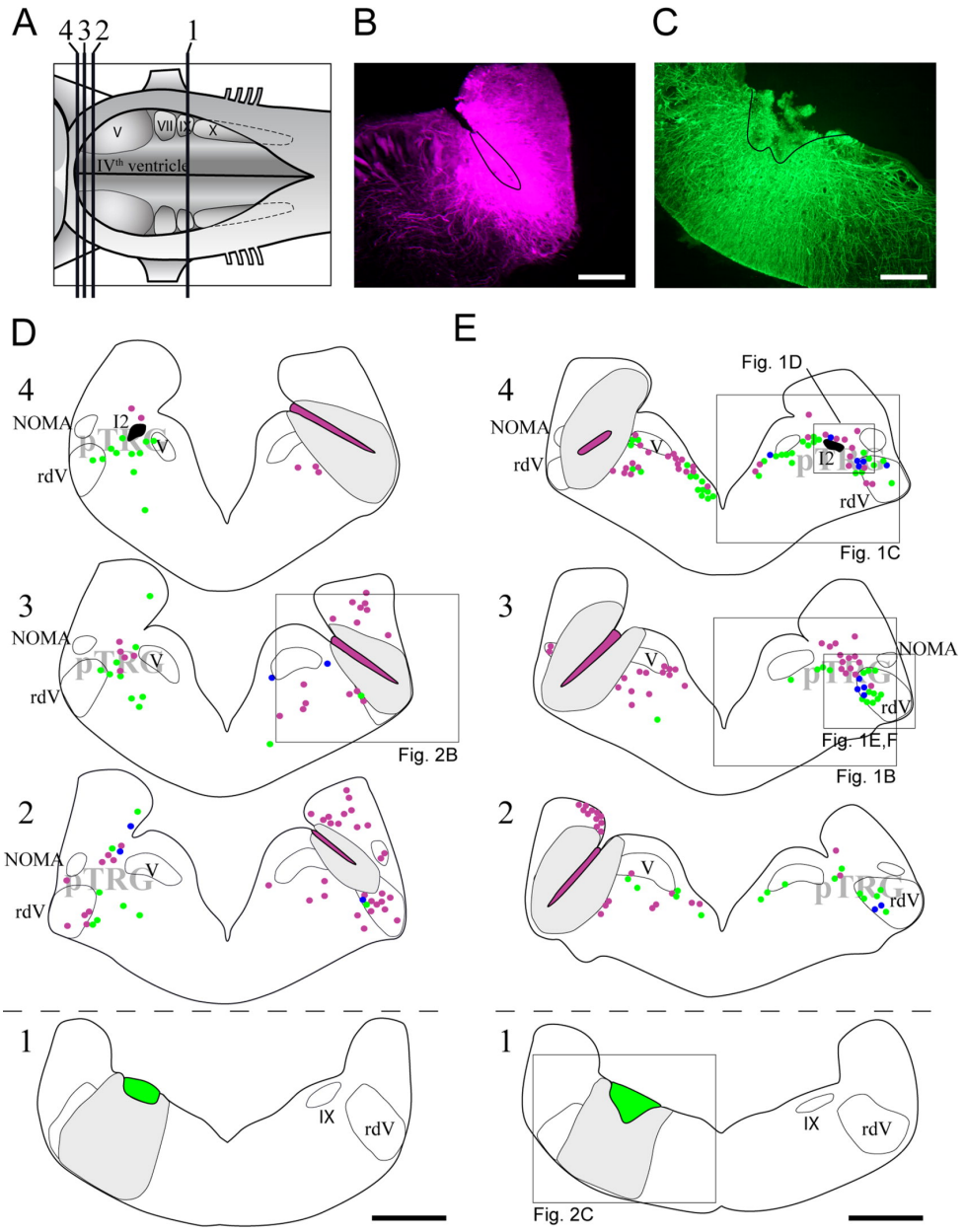
(sl). C: Photomicrograph of a cross section at the level of the anterior octavomotor nucleus (NOMA). A population of small cells (white arrows), located close to the sulcus limitans (sl), was labeled after tracer injections in the contralateral pTRG. A number of large rdV cells, similar to those described in B, were also labeled (blue arrow). D: Higher power photomicrograph of pTRG neurons labeled after an injection in the contralateral pTRG. Note the presence of numerous anterogradely labeled varicose fibers. E,F: Higher power photomicrographs of the contralateral pTRG area, in animals that received pTRG (magenta) and respiratory motoneuron (green) tracer injections on the same side. In the contralateral pTRG, double-labeled neurons (open arrows) were consistently found (E,F). Many neurons were also single-labeled (filled arrows). In D–F, the midline is also to the left. I2, isthmic Müller cell 2; Pro, prosencephalon; Mes, mesencephalon; mV, trigeminal motor root; Rh, rombencephalon; rdV, trigeminal sensory root. Scale bar = 2 mm in A; 100 μm in B–F.

Neurons in the pTRG were labeled from unilateral motoneuronal injections. A population of large cells (cell bodies from 25 to 40 μm) was labeled in the pTRG, close to the medial and dorsal borders of the trigeminal sensory root (rdV; A2 Figure 1B–F, 2D,E). These neurons were found both ipsilaterally and contralaterally to the motoneuronal injection (A2 Figure 2). They had at least one very large dendrite that arborized within the rdV (A2 Figure 1E, F),and a very conspicuous axon that crossed the midline at the level of the cell body (A2 Figure 1B). Smaller cells were also found more dorsally in the ipsilateral and contralateral pTRG (A2 Figure 1E, green cells and open arrows). These neurons were located laterally to the V motor nucleus, close to the sulcus limitans. The cells were either round, bipolar, or multipolar and the largest diameter of their soma varied from 10 to 23 μm (hydrated tissue).

The additional injection in the pTRG also labeled many small to medium-sized neurons (10 to 23 μm) in the contralateral pTRG area (A2 Figure 1C,D; magenta and blue dots in the pTRG area in A2 Figure 2D,E). Some larger and more ventrolaterally located cells were also labeled. These cells were very similar to the ones described above after

injections in the motor nucleus (A2 Figure 1C, blue arrow). It is also worth noting that numerous labeled fibers crossed the midline. Some of these fibers were likely anterogradely labeled as they displayed many varicosities in the contralateral pTRG area and seemed to terminate among the cell bodies retrogradely labeled from the motoneuronal injection (A2 Figure 1D). Double-labeled neurons (neurons that were labeled by the pTRG and the motoneuronal injections) were found in the pTRG whether the injected pTRG was located ipsilaterally or contralaterally to the injected motoneuronal pool (A2 Figure 1E,F,, open arrow; blue dots in pTRG area in A2 Figure 2D,E). These results suggest that single cells in the pTRG could send projections both to the pTRG on the opposite side and to the respiratory motoneuronal column.

Another series of anatomical experiments was designed to reveal the anterograde projections from the pTRG area to the respiratory motoneurons (A2 Figure 3A; n = 3). Biocytin was injected on the proximal stump of the cut nerves VII, IX, and X on both sides. Respiratory motoneurons were intensively labeled, as seen on parasagittal sections (magenta cells, A2 Figure 3B). The apical dendrites of the motoneurons arborized heavily in the subependymal layer (sep in A2 Figure 3). In the same preparations, FITC-conjugated dextran amines were injected into the pTRG area on one side only, to visualize the descending projections from the pTRG (A2 Figure 3, green). The majority of the fibers arising from the pTRG area were seen ipsilateral to this injection, with a similar but smaller distribution on the contralateral side (A2 Figure 3C2,D2). A large number of fibers coursed longitudinally within the subependymal layer (A2 Figure 3B,C2), and varicosities were seen close to the apical dendrites of the respiratory motoneurons (A2 Figure 3C).



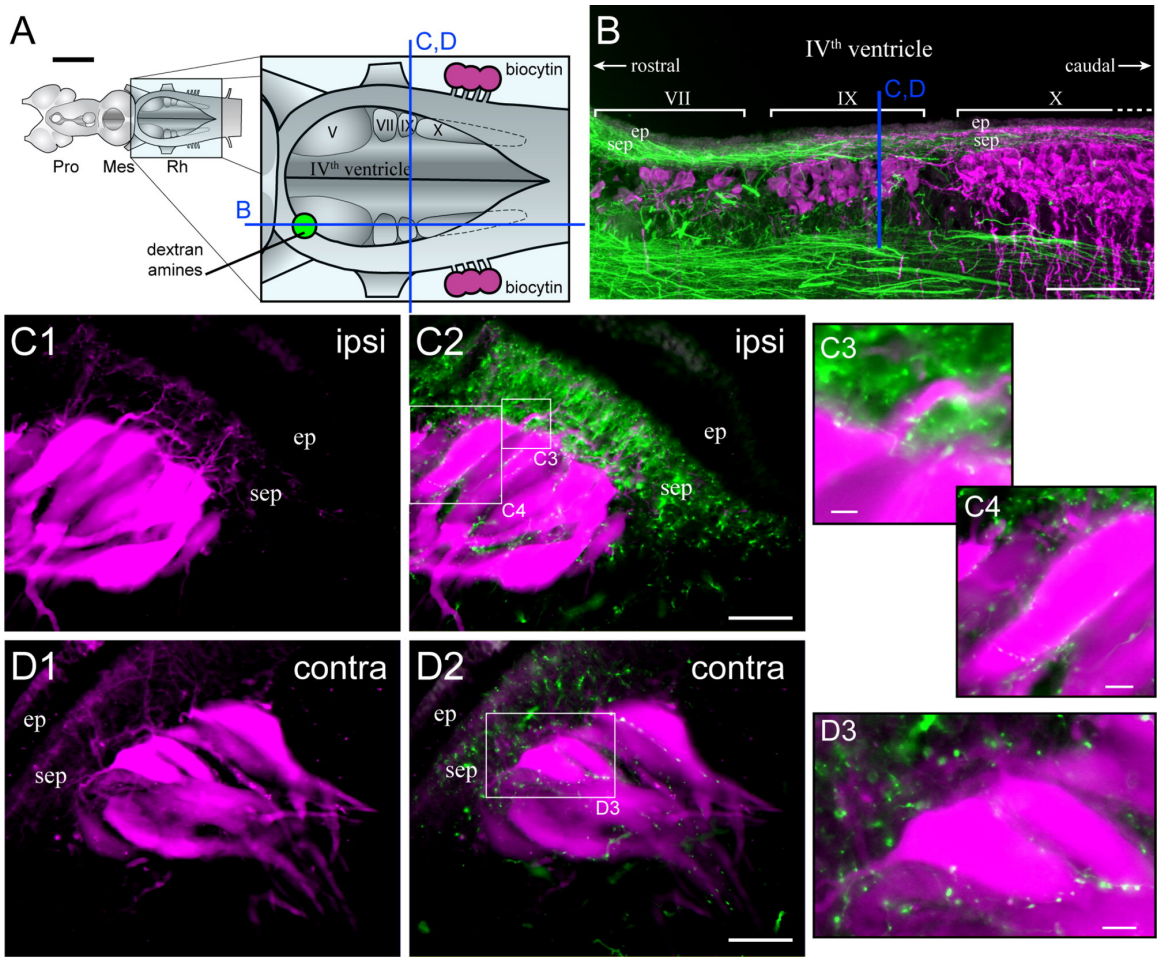
A2 Figure 2: **Distribution of retrogradely labeled neurons in the pTRG area after tracer injections in the pTRG and in the respiratory motoneurons.** A: Schematized representation of a dorsal view of the rhombencephalon where the levels of the cross sections in D and E are indicated. Line 1 passes through the level of the injection in the motor nucleus, whereas lines 2–4 pass through the level of the pTRG. B,C: Photomicrographs illustrating examples of injection sites with Texas Red dextran amines (B, converted to magenta; see Materials and Methods) and biocytin (C, revealed with green streptavidin-Alexa Fluor 488). The area where the neural tissue was damaged by the injection pipette is delineated by a black line. D,E: Schematized cross sections from two typical preparations that received motoneuron and pTRG injections on opposite sides (D) or on the same side (E). The retrogradely labeled neurons shown on each illustrated

section were sampled from three adjacent sections and pooled together into one. One section was skipped between each sampled section, to make sure that no cell was counted twice. This inevitably leads to an underestimation of the number of cells, but their distribution was the main interest. Double-labeled neurons appear in blue. The grayed areas around the injection sites represent regions where analysis was impossible due to the high intensity of fluorescence (see B,C). The areas filled with green or magenta correspond to the area where the tissue was damaged by the pipette. Rectangles refer to the location of photomicrographs in B and C, and in A2 Figure 1. I2, isthmic Müller cell 2; NOMA, octavomotor nucleus; rdV, trigeminal sensory root. Scale bars = 200 μm in B,C; 500 μm in D,E.

Other fibers, some of larger caliber, coursed longitudinally in the tegmentum ventral to the motoneurons, close to the motoneurons' basal dendrites (A2 Figure 3B, especially clear to the right of the photograph due to the angle of the section relative to the distribution of the motor nuclei as seen in A). These ventral fibers had fewer varicosities, but some were in close proximity to the motoneurons' basal dendrites (A2 Figure 3C2,D2). Some anterogradely labeled fibers entered the cell body layer of the motoneuron populations (A2 Figure 3B,C2,C4,D2,D3). Overall, the anatomical experiments support the hypothesis that some pTRG neurons project directly to the contralateral pTRG and to the respiratory motoneuronal nuclei.

Electrophysiological characterization of the descending pTRG projections

To further characterize the bilateral descending projections from the pTRG to the respiratory motoneurons, individual respiratory motoneurons were recorded intracellularly, while the pTRG was stimulated on both sides. Increasing stimulation intensities (1–10 μA) were used until a maximal intensity was reached that just induced



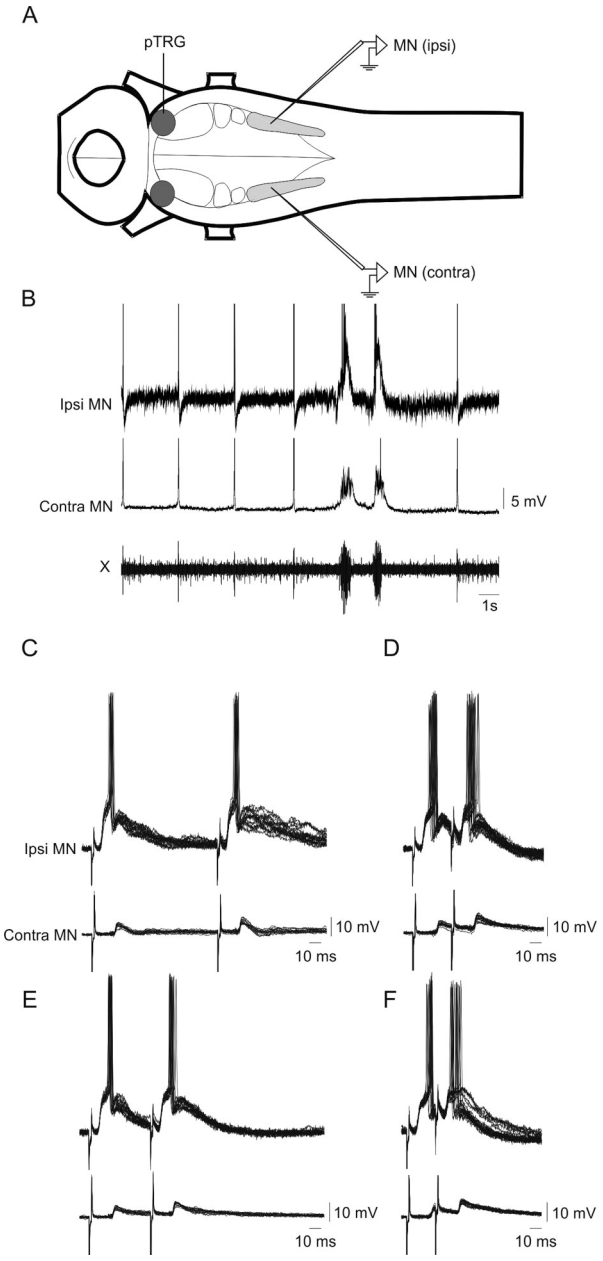
A2 Figure 3: **Projections from the pTRG area to respiratory motoneurons.** A: Schematic representation of a dorsal view of the brain of the adult sea lamprey. To the right, the enlarged rhombencephalic region shows the location of the trigeminal (V), facial (VII), glossopharyngeal (IX), and vagal (X) motor nuclei at the bottom of the IVth ventricle. B: Photomicrograph of a parasagittal section through the respiratory motoneuron populations in a preparation that received a unilateral injection of FITC-dextran amines (green) into the pTRG. The motoneurons were labeled with biocytin (revealed in red with streptavidin-Alexa Fluor 594; displayed here in magenta) by injections of the three respiratory nerves peripherally. The illustrated section comes from the same side as the pTRG injection. Fibers from the pTRG area run longitudinally in the subependymal layer (sep) and in the tegmentum, ventral to the cell bodies of the motoneurons. Some of the fibers turn ventrally or dorsally to enter the motoneuron layer. C,D: High-power photomicrographs of glossopharyngeal respiratory motoneurons as they appear on cross sections. C1,D1: Motoneurons were clearly labeled on both sides of the brain (ipsi, ipsilateral; contra, contralateral to the pTRG injection). Note the arborization of apical dendrites in the subependymal layer and basal dendrites in the tegmentum. C2,D2: Superimposed photomicrographs illustrating the proximity of fibers descending from the pTRG (green) and the dendrites and cell bodies of motoneurons. Elements in white are not double-labeled but result from the superimposition of the varicosities

labeled from the pTRG that are in close proximity to the respiratory motoneurons. C3,C4,D3: The boxed areas in C2 and D2 were enlarged to better show the green varicosities around motoneuronal elements. ep, ependymal layer; Pro, prosencephalon; Mes, mesencephalon; Rh, rombencephalon; V, trigeminal motor nucleus; VII, facial motor nucleus; IX, glossopharyngeal motor nucleus; X, vagal motor nucleus. Scale bar = 2 mm in A; 200 μm in B; 50 μm in C2 (applies to C1,C2) and D2 (applies to D1,D2); 5 μm in C3; 10 μm in C4,D3).

action potentials. This facilitated the quantitative analysis of the postsynaptic potentials (A2 Figure 4, 5). All of the recorded respiratory motoneurons displayed larger EPSPs in response to stimulation of both contralateral and ipsilateral pTRGs. The latency was generally longer for contralateral stimulation (10.2 ± 3.8 vs. 7.4 ± 4.0 ms; paired t-test; $P < 0.05$; $n = 4$). The maximum slope of the response onset was slightly larger for contralateral stimulation (1.35 ± 0.57 vs. 1.07 ± 0.23 mV/ms; paired t-test; $P < 0.05$; $n = 4$).The area of EPSPs varied from one preparation to another and the exact location of the stimulating electrodes in the pTRG. However, there was no significant difference between responses to contralateral and ipsilateral stimulation when data from all preparations were pooled (176.9 ± 141.3 mV/ms contra; 233.7 ± 134.5 mV/ms ipsi; paired t-test; $P = 0.28$; $n = 4$).

Because the stimulating electrodes in the pTRG on each side may have been positioned at different locations in the pTRGs, paired intracellular recordings were made from a respiratory motoneuron on each side while the pTRG was stimulated on one side ($n = 2$; A2 Figure 4). As in the previous experiment, the latency of the EPSPs was longer in the motoneuron contralateral to the stimulation (13.7 ± 3.1 vs. 4.8 ± 0.6 ms; two-tailed t-test; $P < 0.001$; $n = 2$). The EPSPs in both motoneurons followed high frequencies of

stimulation with little attenuation (A2 Figure 4C–F, 10–40 Hz), suggesting a fast and efficient connection.



A2 Figure 4: **Characterization of bilateral descending inputs from the paratrigeminal respiratory group (pTRG) to pairs of simultaneously recorded respiratory motoneurons on the two sides of the brainstem.** A: Schematic representation of the lamprey brainstem. A respiratory motoneuron is recorded simultaneously from the two sides of the brain and a stimulating electrode is placed in the pTRG on one side. B: Recordings of spontaneous on-going fictive respiratory activity from a pair of motoneurons (MN) located on each side. The intracellularly recorded respiratory bursts are in phase with those recorded extracellularly from the vagal motor nucleus (X). C–F: Intracellular excitatory responses to paired pulse stimulation with increasing frequency (C, D, E, and F correspond to 10, 20, 30, and 40 Hz, respectively). MN, motoneuron; X, vagal motor nucleus; ipsi, ipsilateral; contra, contralateral.

Localization of descending inputs from the pTRG to the respiratory motoneurons

In order to localize the trajectory of the crossing descending projections from the pTRG to respiratory motoneurons, Xylocaine was injected to block neural activity at different locations along the presumed axonal tract projection linking the pTRGs and the respiratory motoneurons (A2 Figure 5A). Some of the injections were made along the midline at the rostrocaudal level of the pTRG. These injections dramatically decreased the motoneuronal response to stimulation of the contralateral pTRG area (A2 Figure 4B; EPSP area reduction of 60.6 ± 22.5%; one-way repeated measures ANOVA; $P < 0.05$; seven cells, seven preparations). After washout, the EPSPs recovered (A2 Figure 5B; 77.1 ± 27.9% of control; one-way repeated measures ANOVA; $P > 0.05$). A Xylocaine injection made in the pTRG located ipsilaterally to the recorded respiratory motoneuron decreased the area of EPSPs elicited by stimulation of the contralateral pTRG (A2 Figure 5C; 47.0 ± 19.8% reduction; one-way repeated measures ANOVA; $P < 0.05$; five cells, five preparations). After washout, the EPSPs recovered (A2 Figure 5C; 95.5 ± 49.7% of control; one-way repeated measures ANOVA; $P > 0.05$).

To determine whether the reduction was due to an effect on synaptic connections between the two pTRGs or to blocking action potentials in axons traveling laterally in the contralateral pTRG before descending to motoneurons, CNQX and AP-5 were injected instead of Xylocaine. These injections also led to decreases in the area of EPSPs, suggesting that a synaptic relay was present (A2 Figure 5D; 59.5 ± 20.3% reduction; one-way repeated measures ANOVA; $P < 0.05$; four cells, four preparations). After washout, the EPSPs did not recover (A2 Figure 5D; 40.6 ± 29.9% of control; one-way repeated measures ANOVA; $P < 0.05$). These results suggest that the pTRG of one side of the

brain sends bilateral descending inputs to respiratory motoneurons and that a part of the crossing connection relays in the contralateral pTRG.

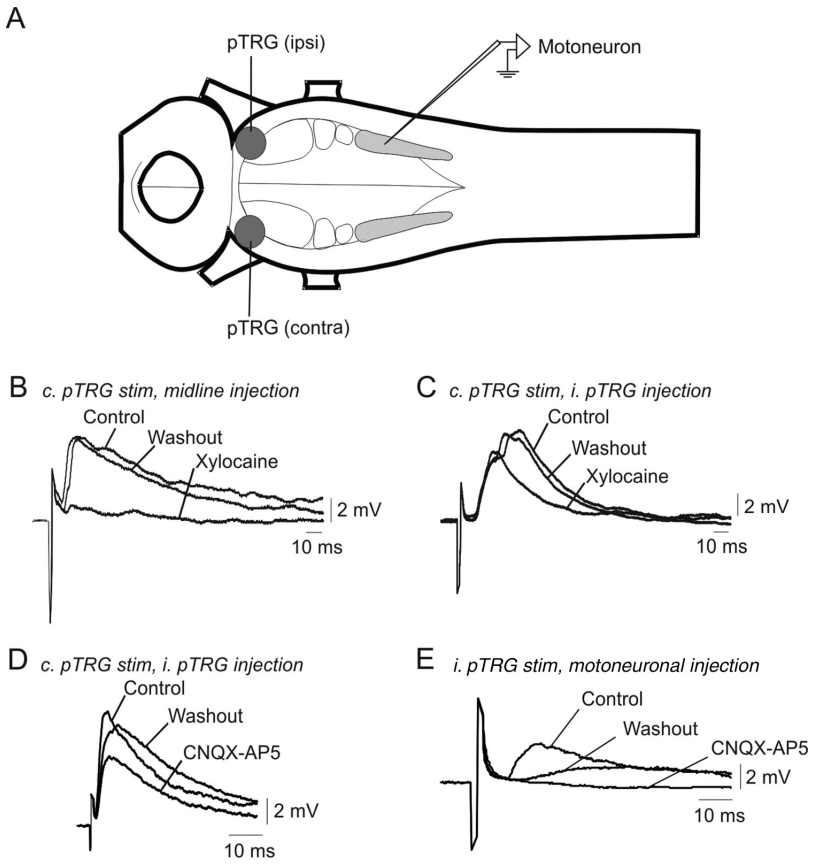
Effects of blocking ionotropic glutamatergic receptors on the recorded motoneurons

To identify the neurotransmitter responsible for the excitatory inputs from the pTRG to respiratory motoneurons, a mixture of CNQX/AP5 was injected over the IX and X motor nuclei. This abolished the respiratory bursts recorded extracellularly from the motoneurons. The effect on EPSPs elicited in respiratory motoneurons in response to the ipsi- or contralateral pTRG varied from complete abolition to a large reduction (A2 Figure 5E; 70.2 ± 30.2% reduction; one-way repeated measures ANOVA; $P < 0.05$; seven cells; seven preparations). The EPSPs recovered during the washout period (84.0 ± 24.7% of control; one-way repeated measures ANOVA; $P > 0.05$).

Anatomical characterization of pTRG neurons at the single-cell level

Previous anatomical experiments allowed us to examine the projections at a population level. Because of the large number of labeled fibers, it is impossible to follow the course of the axonal projections from individual neurons. To circumvent this problem, we patch-recorded individual pTRG neurons and injected them intracellularly with biocytin (A2 Figure 6; n = 8). To focus on the pTRG neurons projecting to respiratory motoneurons, Texas Red dextran amines were injected in the X motor nucleus 24 hours before the electrophysiological experiment. The labeled neurons were then

targeted under a fluorescence microscope for whole-cell patch recordings. Under voltage-clamp, these neurons displayed rhythmically occurring inward currents that preceded the respiratory bursts recorded extracellularly from the motoneurons (A2 Figure 6A).



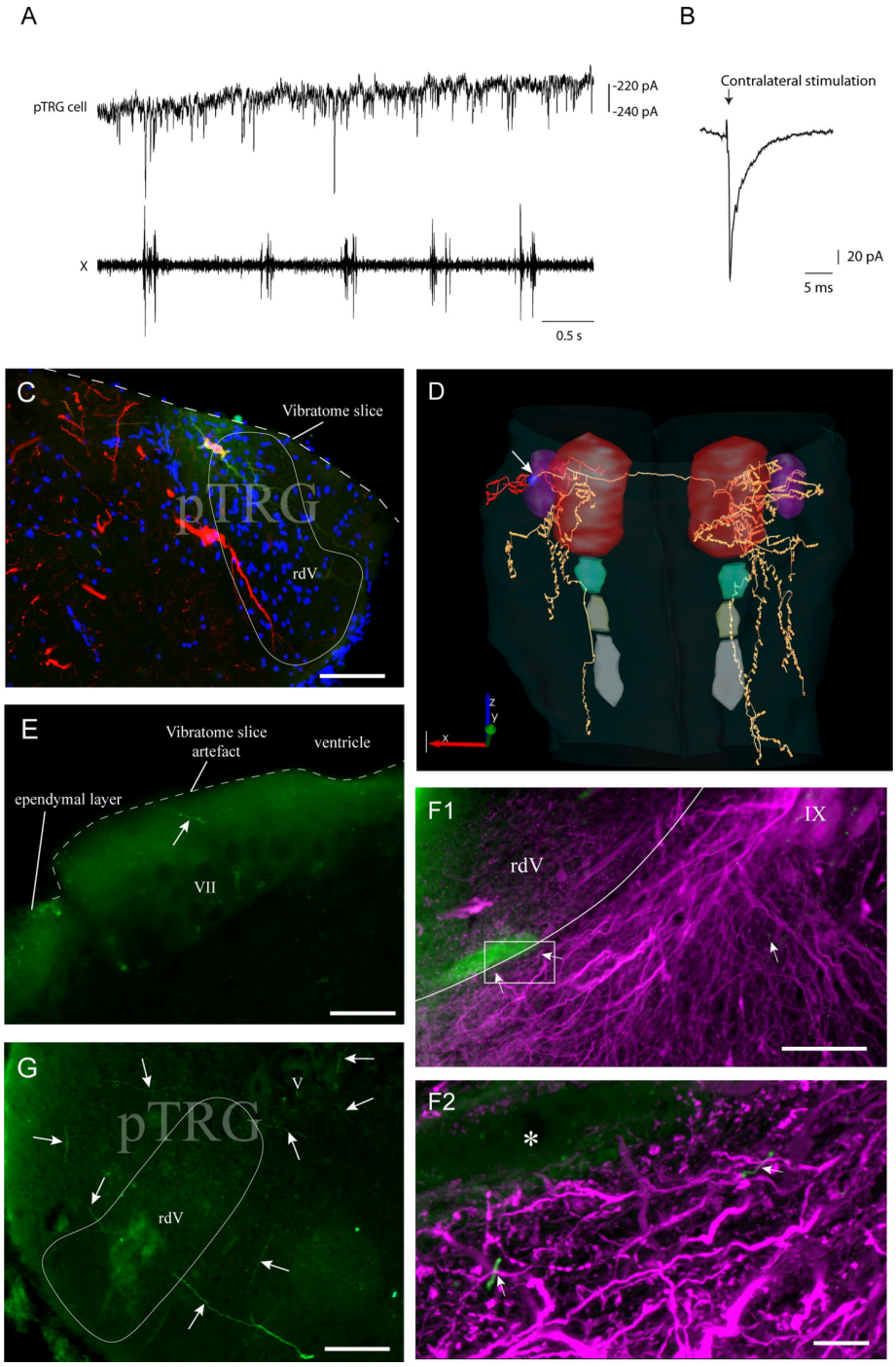
A2 Figure 5: **Electrophysiological characterization of the descending connections from the paratrigeminal respiratory group (pTRG) to the respiratory motoneurons.** A: Schematic representation of the lamprey brainstem with the pTRG and respiratory motoneurons represented on both sides. B: Average of the EPSPs recorded from a respiratory motoneuron following electrical stimulation of the contralateral pTRG. Xylocaine was applied along the midline at the pTRG level. C: Average of the EPSPs recorded from a respiratory motoneuron following electrical stimulation of the contralateral pTRG. Xylocaine was applied in the ipsilateral pTRG. D: Average of the EPSPs recorded from a respiratory motoneuron following a stimulation of the contralateral pTRG. CNQX (1 mM) and AP-5 (500 μM) were applied in the ipsilateral pTRG. E: Intracellular recording of a respiratory motoneuron during stimulation of the ipsilateral pTRG. CNQX and AP-5 were injected directly over the recorded motoneuron. ipsi, i, ipsilateral; contra, c, contralateral.

To test electrophysiologically whether these neurons received inputs from the contralateral pTRG, electrical stimulation of the latter was performed (n = 3), and large inward currents were induced in the recorded pTRG neuron (A2 Figure 6B). After the recorded cells were filled with biocytin, a relatively constant pattern of projections was observed. Table I shows the regions of the brainstem where each of the recorded neurons (n = 8) projected fibers with varicosities. Most neurons projected to the contralateral pTRG as well as bilaterally to the VII, IX, and X respiratory motoneuron nuclei (A2 Figure 6C-G). Two neurons had unilateral projections on the ipsi- and contralateral sides, respectively (Table I). These results indicate that the pattern of projection of individual neurons in the paratrigeminal respiratory group (pTRG) is similar to that seen with population labeling: six of eight recorded neurons displayed projections to the contralateral respiratory-generating area and bilateral projections to the respiratory motoneuronal columns.

Table I: Summary of the projections from the recorded pTRG neurons[1]

Neuron no.	c. pTRG	iVII	iIX	iX	cVII	cIX	cX
1	√	√	√	√	√	√	√
2	√	√	√	√	√	√	√
3	√	√	√	√	√	√	√
4		√	√	√			
5	√				√	√	√
6	√	√	√	√	√	√	√
7	√	√	√	√	√	√	√
8	√	√	√	√	√	√	√

[1]A checkmark indicates the presence of at least one branch containing varicosities in the structure or around the dendrites of the neurons located in the structure. pTRG, paratrigeminal respiratory group; VII, facial nucleus; IX, glossopharyngeal nucleus; X, vagal nucleus; i, ipsilateral; c, contralateral.

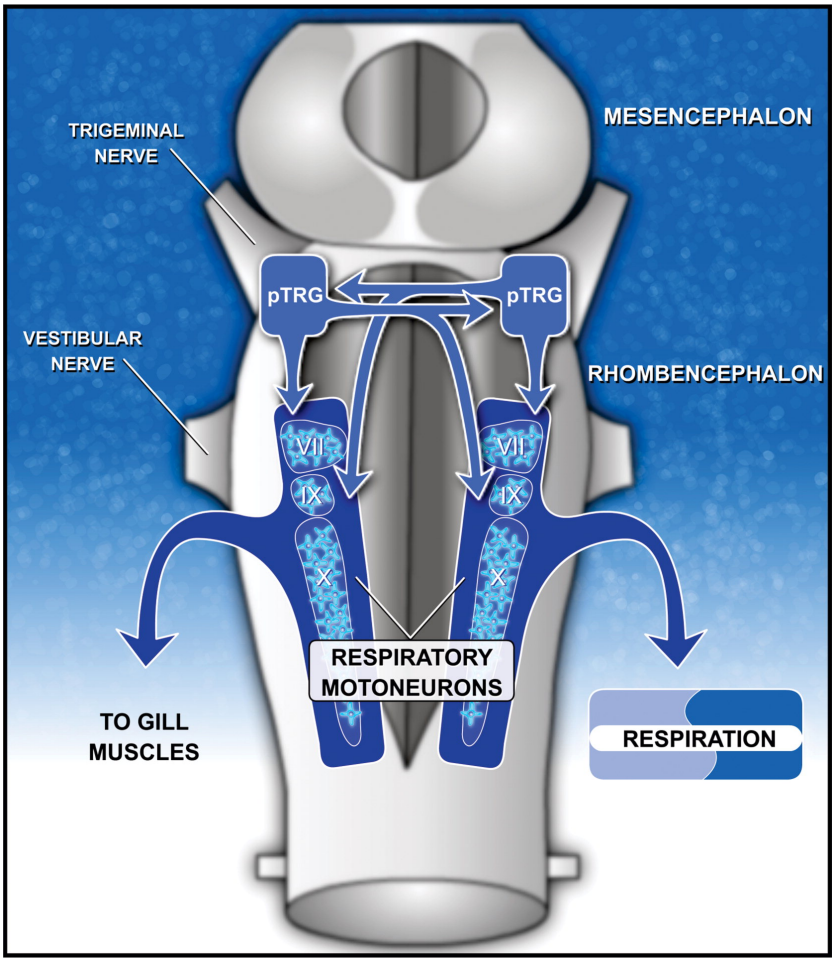


A2 Figure 6: **Electrophysiological and anatomical characterization of paratrigeminal respiratory group (pTRG) neurons.** A: Patch recording of a pTRG neuron in whole-cell voltage-clamp mode. The neuron displays inward currents in phase with the respiratory activity recorded extracellularly from the vagal motor nucleus (X). B: Average of the inward currents induced by electrical stimulation of the contralateral pTRG. C: Superimposition of three photomicrographs of the recorded pTRG neuron filled with biocytin (green) and retrogradely filled from an injection of dextran amines

(red) in the X nucleus. DAPI labeling, in blue, shows the location of DNA-containing cell nuclei. D: 3D reconstruction of the axonal projections of the recorded pTRG neuron seen from a dorsal view of the rhombencephalon. Varicosities and dendritic spines are represented as circles. The diameters of the axons, dendrites, and cell body (→) were amplified for better visibility. Dendrites, red arborizations; soma, blue; axons, yellow arborizations; V, red; VII, light blue; IX, light yellow; X, light gray; pTRG, purple. E: Photomicrograph illustrating axonal branches from the pTRG neuron innervating the ipsilateral facial motor nucleus (VII). F1: Photomicrograph illustrating axonal branches from the pTRG neuron around the dendrites of the IX nucleus. The outlined area corresponds to the photograph in F2. F2: Z-projection of 14 adjacent optic slices (0.5 μm each) obtained from a confocal microscope. A larger diameter axonal branch with no varicosity (green, left arrow) and a smaller diameter axonal branch with varicosities (green, right arrow) were located in close proximity to the basal dendrites of the IX motoneurons (magenta) labeled from the tracer injection in the adjacent motoneuron pool. *, blood vessel. G: Photomicrograph illustrating axonal branches from the pTRG neuron innervating the contralateral pTRG. → indicates axonal branches except in D. rdV, trigeminal sensory root. Scale bar = 100 μm in C; 500 μm in D (the three axes); 50 μm in E; 100 μm in G; 100 μm in F1; 10 μm in F2.

Discussion

This study shows that lamprey pTRG neurons possess a complex axonal arborization with projections to multiple brainstem sites involved in respiration. We identified three sets of projections from pTRG neurons: one set of projections to the contralateral pTRG, one to the ipsilateral respiratory motoneuronal column, and one to the contralateral motoneuronal column. One of the most salient results in this study is that individually labeled pTRG neurons were shown to possess these three sets of projections. These findings are represented in a schematic of the lamprey brainstem (A2 Figure 7).



A2 Figure 7: **Schematic representation of the newly identified connectivity in the respiratory networks of lampreys.** Our study shows that neurons in the paratrigeminal respiratory group (pTRG), located in the rostral rhombencephalon, send axonal arborizations to the contralateral pTRG and bilaterally to the respiratory motoneurons. VII, facial nucleus; IX, glossopharyngeal nucleus; X, vagal nucleus.

Bilateral connections

We found that neurons in the pTRG project to the contralateral pTRG. Furthermore, the electrophysiological experiments show that glutamatergic synapses in the contralateral pTRG are partly involved in the inputs to the contralateral respiratory motoneurons. This suggests that crossing glutamatergic connections at the pTRG level might influence the output of the respiratory generator to the respiratory motoneurons.

Xylocaine injections along the midline at the pTRG level had an even stronger effect than unilateral pTRG blockade. These injections almost completely abolished the EPSPs. One possible explanation for this is that the axons relaying the inputs to contralateral motoneurons cross the midline in a rather compact bundle. As they reach the other side, the axons spread out with some coursing down to project to respiratory motoneurons without reaching the contralateral pTRG. Other axonal projections would reach relay cells in the contralateral pTRG that in turn send their axons down to the respiratory motoneurons. This is consistent with the anatomical finding of a dense bundle of decussating axons at the midline that spreads out as it progresses laterally. Individually filled pTRG neurons also displayed separate axonal branches to the contralateral pTRG and bilateral projections to the respiratory motoneurons. The injections of Xylocaine in the pTRG might block one branch without blocking the descending branch that travels medially and projects to the motoneurons.

We have thus found anatomical and electrophysiological evidence for two sets of projections that might underlie the bilateral synchronization of respiratory activity: the decussating descending axons from the pTRG to the motoneurons of both sides and

decussating axons to the contralateral pTRG. This is similar to what was found in mammals: contralateral projections exist between respiratory-generating regions (Bouvier et al., 2010; Tan et al., 2010) and from respiratory-generating regions to respiratory motoneurons (Janczewski and Karczewski, 1990; Goshgarian et al., 1991; Duffin and Li, 2006; Tarras-Wahlberg and Rekling, 2009). Some Bötzinger complex neurons were found to project to both the ipsilateral and contralateral brainstem (Ezure et al., 2003), but whether individual pre-BötC and pFRG neurons possess similar complex axonal arborizations remains to be determined. In our study, we found that most pTRG neurons possess projections both to the contralateral respiratory-generating area and to the motoneuronal columns. Overall, six of eight individually labeled cells displayed the two types of projections. This proportion may be an underestimation as the vibratome might have cut a part of the axonal arborization for some of the labeled neurons.

The requirement for a strong coordination between the two sides of the brain is not exclusive to respiratory systems. For instance, in feeding systems, interneurons were found to synchronize bilaterally the networks underlying suckling in neonatal rats (Koizumi et al., 2009). Locomotor networks of the spinal cord also need bilateral coordination, with the particularity that this coordination is often in anti-phase (Fagerstedt et al., 2000; Grillner, 2003; Mentel et al., 2008; Brocard et al., 2010).

Descending projections

This study shows that pTRG neurons send axonal branches that travel caudally to the VII, IX, and X nuclei. Previous studies have shown that gill contractions propagate

rostrocaudally in lampreys (Guimond et al., 2003) in such a way that muscles of the seventh gill pore were activated with a slight delay compared with muscles of the first gill pore. The major part of that delay was attributed to the longer X nucleus axons compared with those of the VII nucleus, from the soma in the brainstem to their respective gill muscles. However, recordings over respiratory motoneuron cell bodies showed that there was still a delay of 5 ms between the rostral and caudal motoneuronal pools. This could not be attributed to motoneuronal axon length, and the rostrocaudal course of axons originating from the pTRG neurons characterized in the present study might be responsible. A similar rostrocaudal delay was also observed between the respiratory motoneuronal pools of the spinal cord in rats (Giraudin et al., 2008). In the locomotor networks of the lamprey spinal cord, the spinal segments also display a rostrocaudal delay during forward locomotion. Ascending, descending, long, and short axonal projections as well as excitability gradients are at play (Cohen et al., 1992; Matsushima and Grillner, 1992; see also Kozlov et al., 2009). Whether some of these are involved in the respiratory rostrocaudal delay in lampreys remains to be determined.

We have shown that glutamate receptor blockers injected over respiratory motoneurons abolish their excitatory responses to pTRG stimulation. This indicates that connectivity between the pTRG and the brainstem respiratory motoneurons uses glutamate as a neurotransmitter. This is similar to mammals, in which both spinal and brainstem respiratory motoneurons are activated by excitatory glutamatergic synapses (Greer et al., 1991; Berger, 2000; Wang et al.,2002).

Comparative aspects relative to the brainstem respiratory networks in vertebrates

The localization of the pTRG premotor interneurons has important implications for understanding how respiratory networks have evolved in vertebrates. The pTRG has been previously identified in lampreys by showing that chemical or physical lesion of this region abolishes breathing (Rovainen, 1977; Kawasaki, 1979; Thompson, 1985; Martel et al., 2007; Mutolo et al., 2007, 2010). However, individual pTRG neurons had not been characterized anatomically. Four hypotheses may be considered regarding the fate of the pTRG during evolution: 1) it is homologous to the lung oscillator of amphibians, which in turn would be homologous to the pre-BötC of mammals; 2) it is homologous to the buccal oscillator of amphibians, which in turn would be homologous to the pFRG in mammals; 3) it is homologous to the medial parabrachial or Kölliker-Fuse nucleus; or 4) it was lost during evolution and was replaced by nonhomologous networks.

Because both the pFRG and the pre-BötC are located more caudally in the brainstem than the pTRG cells described in this study, the first two hypotheses would imply that the respiratory generator has been displaced from a rostral to a caudal position during evolution from the common ancestors of lampreys and other vertebrates to mammals. In support of the first hypothesis, some studies have shown similarities among the pTRG in lampreys, the lung oscillator in amphibians, and the pre-BötC in mammals. For instance, opiate sensitivity was shown in the pTRG in lampreys (Mutolo et al., 2007), the lung oscillator in amphibians (Vasilakos et al., 2005), and the pre-BötC in mammals (Gray et al., 1999). Furthermore, co-application of I_{NaP} and I_{CAN} current blockers (riluzole and flufenamic acid, respectively) in the pTRG of lampreys or the pre-BötC of rodents abolishes the respiratory rhythm. In both cases, the rhythm can be restarted by substance

P (Del Negro et al., 2005; Mutolo et al., 2010). However, in support of the second hypothesis, the pTRG of lampreys is functionally closer to the gill oscillator of amphibians because it produces gill movements.

Finally, in support of the third hypothesis, the pTRG is located in the lateral pons, similarly to the medial parabrachial and Kölliker-Fuse nuclei. The respiratory regions of the pons are believed to play a role in phase-switching in mammals (Cohen, 1971; von Euler et al., 1976; Arata et al., 2010) whereas some argue that they are necessary to generate eupnea (St. John, 2009). It is difficult to determine what happened to the pTRG of lampreys in evolution based solely on pharmacological and anatomical data. It was recently shown that the pre-BötC neurons are derived from a group of Dbx1-expressing progenitors (Gray et al., 2010), and further knowledge of the developmental origins of the pTRG neurons identified in this study might provide an answer. Nearby motor control structures such as the reticular nuclei and the mesencephalic locomotor region have been studied in several species and are thought to be homologous to corresponding mammalian structures (Cabelguen et al., 2003; Dubuc, 2009; Martin et al., 2011; Le Ray et al., 2011). It is thus likely that a better understanding of the respiratory-generating networks and their development in a wide range of species will allow us to address the question of homology between the respiratory oscillators in vertebrates.

Acknowledgements

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Specific neural substrate linking respiration to locomotion

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Abstract

When animals move, respiration increases to adapt for increased energy demands; the underlying mechanisms are still not understood. We investigated the neural substrates underlying the respiratory changes in relation to movement in lampreys. We showed that respiration increases following stimulation of the mesencephalic locomotor region (MLR) in an *in vitro* isolated preparation, an effect that persists in the absence of the spinal cord and caudal brainstem. By using electrophysiological and anatomical techniques, including whole-cell patch recordings, we identified a subset of neurons located in the dorsal MLR that send direct inputs to neurons in the respiratory generator. In semi-intact preparations, blockade of this region with 6-cyano-7-nitroquinoxaline-2,3-dione and (2R)-amino-5-phosphonovaleric acid greatly reduced the respiratory increases without affecting the locomotor movements. These results show that neurons in the respiratory generator receive direct glutamatergic connections from the MLR and that a

subpopulation of MLR neurons plays a key role in the respiratory changes linked to movement.

Keywords

Breathing, modulation, motor output, exercise, brainstem.

Introduction

At the onset of exercise in humans, ventilation increases abruptly. Further increases occur during the exercise bout and these changes are correlated with the intensity of the motor output (1). Chemoreception was proposed to induce these changes, as breathing air with high partial pressure of CO_2 was known to markedly increase respiration (2, 3). However, it was later demonstrated in several animal species that the CO_2 arterial partial pressure does not increase during moderate exercise, and it even decreases (2). Other substances such as O_2, H+ ions, and S-nitrosothiols are also known to modulate respiration (4–6), but there are not sufficient changes in these respiratory modulators to explain the respiratory increases during exercise (2, 7).

Alternatively, neural mechanisms have been proposed to contribute to the respiratory changes during exercise. For instance, sensory feedback from the contracting muscles could play a role (8, 9). However, respiratory increases also occur during mental simulation of movement as well as before the onset of exercise in humans (10, 11). Furthermore, it was shown that the stimulation of the forebrain and brainstem regions controlling locomotion increases respiratory activity in the absence of movement-related feedback in animal models (12–14). These results indicate that a neural feedback mechanism cannot be solely responsible for movement-related increases in respiratory activity; central connections between locomotor and respiratory areas in the brain are likely to play an important role (15). However, because the central neural connections were never identified, this central hypothesis remains criticized (16). For instance, whether the respiratory increases rely on direct projections from the brainstem locomotor

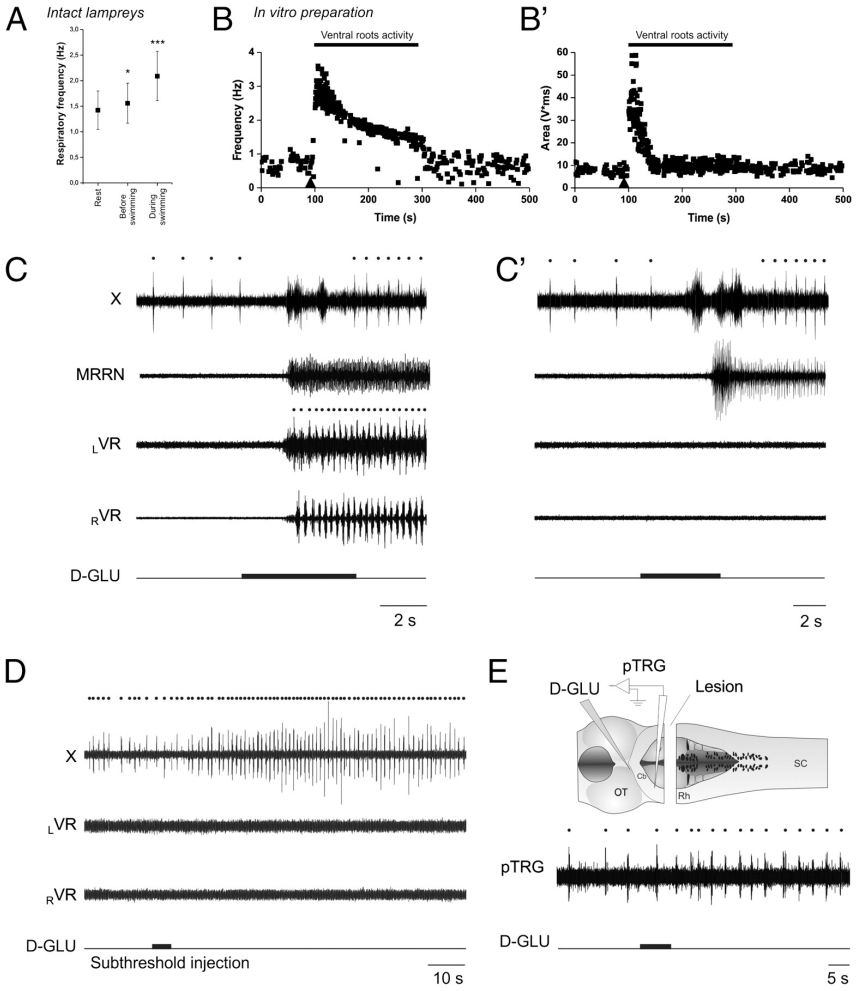
centers or on feedback from other parts of the locomotor networks such as the spinal cord remains undetermined.

The characterization of the neural mechanisms underlying the generation and modulation of respiration has advanced considerably as a result of newly developed *in vitro* mammalian preparations (17–26). Moreover, many identified brainstem neurons such as serotoninergic neurons, locus coeruleus neurons, and orexin neurons modulate breathing in accordance with behavioral states or CO_2 levels (27–29). However, the role of these modulatory mechanisms in the respiratory increases related to exercise is unknown. In this study, we used the *in vitro* brainstem–spinal cord preparation of the lamprey, in which it is possible to simultaneously assess locomotor and respiratory outputs while recording individual neurons in the neural networks controlling locomotion and respiration. The pre-Bötzinger complex is a medullary region critical for the generation of respiratory activity in mammals (30, 31). The lamprey respiratory generator is localized in a region of the pons (32) referred to as the paratrigeminal respiratory group (pTRG) (33), which presents pharmacological similarities with the pre-Bötzinger complex of mammals (34, 35). The neural centers controlling locomotion in lampreys have been very well documented (36–38). One such locomotor center is the mesencephalic locomotor region (MLR), located at the mesopontine border. It was shown to initiate and control the intensity of locomotion in a graded fashion (36), similarly to what was previously shown in mammals (39). It is thought to have a similar function in humans (40). By using kinematic, anatomical, and electrophysiological (A3 Supplementary Figure 1) approaches, we show that a group of neurons located in the dorsal part of the MLR projects to neurons in the pTRG. Blocking the dorsal part of the

MLR abolishes an important part of the respiratory changes linked to exercise. Our results suggest that a major part of the respiratory increases rely on a neural command emerging from a specific group of neurons in the MLR.

Results

We first observed the respiratory changes that occur in relation to movements in freely behaving lampreys (A3 Figure 1A; raw data provided in A3 Supplementary Figure 2). Respiratory increases were found to occur before the onset of movement (increase by 10.4 ± 10.0%; 1.55 ± 0.30 Hz vs. 1.42 ± 0.38 Hz in control; one-tailed paired t test, $P < 0.05$; n = 9, locomotor bouts; measured 6 s before movement) and became more important during movement (2.09 ± 0.48 Hz; one-tailed paired t test, $P < 0.001$; n = 9 locomotor bouts). These results reproduce well what was previously shown in mammals (10‑14). We then isolated the brainstem and spinal cord *in vitro* to see whether feedback from the contracting muscles was necessary for the respiratory increases (A3 Figure 1 B, B', and C). Similarly to what was shown in mammals (12–14), we found that chemical stimulation of the MLR by D-glutamate (2.5 mM) injections induces fictive locomotor activity, as recorded from the ventral roots, along with increases in respiration, despite the absence of feedback from the muscles. Respiratory frequency was increased by



A3 Figure 1: **Respiratory increases related to locomotion *in vivo* and *in vitro*.** (A) Analysis of the respiratory frequency at rest, 6 s before swimming, and during swimming *in vivo*. Analyses were made for a total of nine locomotor bouts in six animals. Raw traces are provided in A3 Supplementary Figure 2. (B and B') Time course of the instantaneous frequency (B) and area (B') of the respiratory bursts recorded from the vagal motoneuronal pool during fictive locomotion in the *in vitro* brainstem-spinal cord preparation. Triangle marks the beginning of D-glutamate injection in the MLR, which lasted 5 s. Raw traces for this fictive locomotor bout are provided in A3 Supplementary Figure 3. (C) Raw traces of the respiratory increases following D-glutamate (D-GLU, 2.5 mM) in the MLR in a control brainstem–spinal cord preparation. (C') Similar D-glutamate injection after removal of the spinal cord in the same preparation. In the absence of the spinal cord, the activity of the brainstem locomotor networks is monitored with an extracellular recording electrode placed over reticulospinal cells in the MRRN. (D) Effects of a D-glutamate MLR injection that was not large enough to elicit locomotor activity in a control brainstem–spinal cord preparation. Note that the increases in respiratory frequency remained even though the stimulation was not sufficiently strong to induce locomotion on the ventral roots. (E) Effects of MLR stimulation on respiratory frequency after removing the caudal half of the rhombencephalon. Dots indicate respiratory and locomotor cycles. X, vagal motor nucleus; $_{L}$VR, left ventral root; $_{R}$VR, right ventral root; SC, spinal cord; OT, optic tectum; Cb, cerebellar commissure; Rh, rhombencephalon. MRRN, middle rhombencephalic reticular nucleus.

91.4 ± 58.6% (0.89 ± 0.30 Hz in control to 1.57 ± 0.39 Hz; one-tailed paired t test, $P < 0.001$; n = 7 animals, n = 48 locomotor bouts; A3 Suplementary Figure 3 and SI Text). We also found that stimulation of the MLR with smaller injections of D-glutamate could induce respiratory changes without triggering locomotor activity (A3 Figure 1D). In these cases, the spread of the injections did not exceed 50 μm in diameter compared with 300 μm for the control injections (Materials and Methods). These results suggest a strong link between the locomotor and respiratory control networks that could operate in the absence of locomotor output; further experiments were carried out to identify the underlying neural substrate.

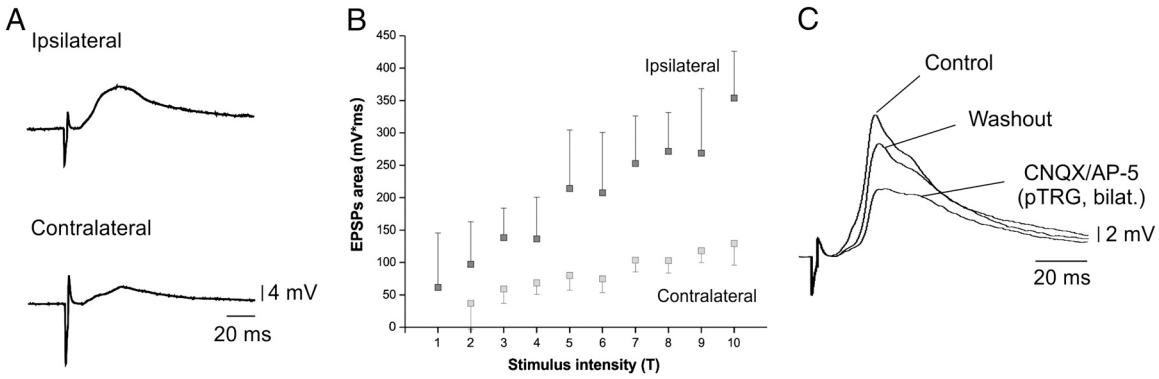
Localization of neural connections underlying respiratory changes

To test whether feedback from spinal locomotor networks was involved in the respiratory increases, the spinal cord was transected at the level of the obex in three animals. After removal of the spinal cord, stimulation of the MLR still induced similar increases in respiratory activity (control, increases of 116.8 ± 76.8%, n = 17 D-glutamate injections; transected, 120.6 ± 79.0%, n = 21 D-glutamate injections; two-tailed t test, P = 0.88; A3 Figure 1C and C'). Furthermore, removing all brainstem tissue caudal to the trigeminal motor nuclei (n = 3 preparations, n = 28 injections; A3 Figure 1E) did not prevent the respiratory changes in response to MLR stimulation. The respiratory rhythm recorded from the pTRG still increased by 68.7 ± 40.0% in response to MLR stimulation (0.28 ± 0.05 Hz in control, 0.48 ± 0.11 Hz after stimulation; one-tailed paired t test, $P < 0.001$; n = 28 injections). Although these respiratory increases

were still important, they were statistically smaller than those occurring in control condition ($P < 0.01$). It is noteworthy that the respiratory frequency was generally slower in the absence of the spinal cord and caudal brainstem; thus, the increases could only be compared in relative terms (i.e., as percentages). This reduction suggests that the respiratory generator receives an excitatory drive from neural areas located caudal to the trigeminal motor nucleus. Overall, the results from the lesion experiments suggest that an important part of the neural circuitry responsible for respiratory changes following MLR stimulation is located in the rostral part of the hindbrain.

Underlying neural connectivity

To further characterize the connections between the MLR and respiratory neurons, we recorded the synaptic responses of respiratory motoneurons to MLR stimulation ($n = 24$ preparations; single pulses, 2 ms duration; A3 Figure 2). In four of these experiments, intracellular recordings of respiratory motoneurons were made on both sides of the brainstem. The size of the area of excitatory postsynaptic potentials (EPSPs) was well correlated with the MLR stimulation intensity ($r = 0.81 \pm 0.08$; linear fits; $P < 0.001$ for each preparation and each side; $n = 627$ ipsilateral EPSPs; $n = 705$ contralateral EPSPs; A3 Figure 2A and B). The latency of the synaptic responses was significantly shorter in motoneurons recorded on the ipsilateral side of the MLR (10.0 ± 4.8 ms, $n = 4$ ipsilateral motoneurons; contralateral, 17.1 ± 7.4 ms, $n = 4$ contralateral motoneurons; one-tailed paired t test, $P < 0.05$).



A3 Figure 2: **EPSPs induced in respiratory motoneurons by MLR stimulation.** (A) Average EPSPs (10 traces) evoked in respiratory motoneurons located ipsilaterally and contralaterally to the stimulated MLR (2-ms pulse duration, 12 μA). (B) Plot of the area of EPSPs vs. the MLR stimulation intensity. The intensity of MLR stimulation was varied from threshold (T) level (1T) to 10 times threshold. (C) EPSPs recorded in respiratory motoneurons following MLR stimulation under control condition and after localized injection of CNQX and AP5 bilaterally in the pTRG.

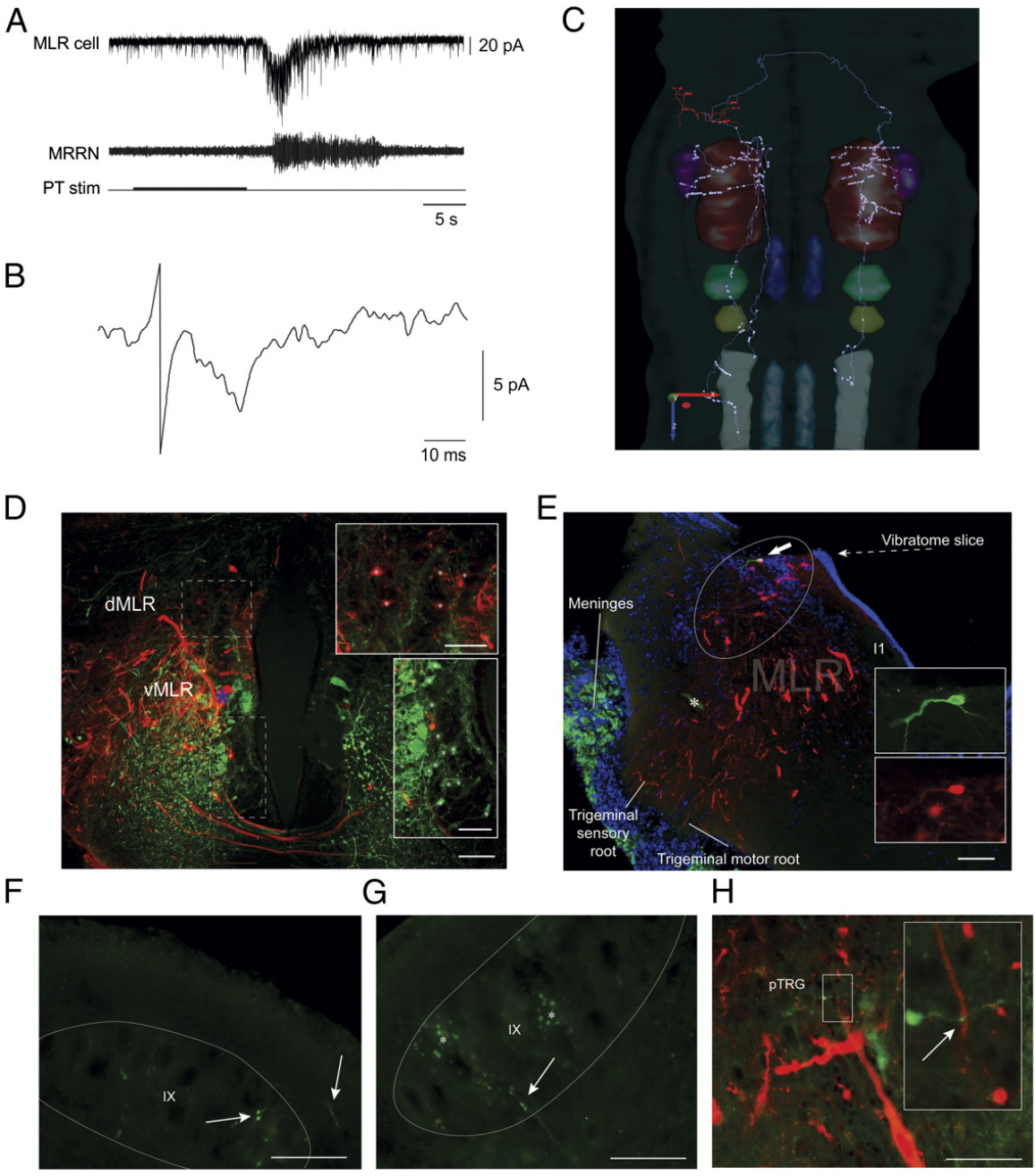
Glutamatergic receptor blockers [6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and (2R)-amino-5-phosphonovaleric acid (AP5); n = 5 preparations, n = 5 cells] were injected in the pTRG to determine if it acted as a relay for MLR inputs to respiratory motoneurons(A3 Figure 2C). Bilateral injections of CNQX (1 mM) and AP5 (500 μM) in the pTRG significantly reduced the area of EPSPs (reduction of 67.2 ± 10.0%; one-tailed paired t test; n = 5 motoneurons; $P < 0.001$). After washout, the area of EPSPs partially recovered (67.6 ± 22.2% of control; one-tailed paired t tests; n = 5 motoneurons; $P < 0.05$). These results suggest that there are synaptic connections between the MLR and the respiratory generator. Blocking the pTRG did not completely abolish the motoneuronal EPSPs, suggesting that monosynaptic connections from the MLR to the respiratory motoneurons could also be present. To further confirm that the MLR connects to the respiratory central pattern generator (CPG), we performed trains of electrical stimulation

in the MLR. We found that this induced resets of the respiratory rhythm (A3 Supplementary Figure 4).

Detailed connectivity between MLR neurons and respiratory generator

The preceding physiological experiments suggested that connections between the MLR and the respiratory CPG were present. Whether these connections were direct was not established, and we addressed this question by using whole-cell patch recordings of MLR and pTRG neurons. The first series of experiments consisted in recording from MLR cells. Dextran amines were injected in the IX nucleus and the pTRG (A3 Supplementary Figure 5) to label MLR cells that projected to the respiratory centers, and single MLR cells were targeted for patch recordings (n = 8 cells, n = 8 preparations). The posterior tuberculum (PT) is part of the supraspinal system controlling locomotion in lampreys and constitutes a powerful input to the MLR. It was shown to be a relay involved in the transformation of olfactory inputs into locomotor commands (41), and we used it to trigger activation of the brainstem locomotor networks. The PT was stimulated (5–10 μA; 3 Hz; 2-ms pulse duration; 10 s) and excitatory currents were induced in the MLR cells, concomitantly to middle rhombencephalic reticular nucleus (MRRN) activity, as would be expected for neurons activated during locomotion (A3 Figure 3A). The PT was also stimulated with single pulses at stronger intensities (10–15 μA) and short-latency excitatory postsynaptic currents (EPSCs) were elicited in MLR neurons (A3 Figure 3B). After the recorded MLR neurons were filled with biocytin, their axonal projections were examined and fibers were seen in the pTRG on both sides (A3 Figure 3C). The eight

labeled neurons had a very similar projection pattern. They typically sent one to three major branches ipsilaterally and one major axonal branch contralaterally that divided into smaller branches with varicosities. Interestingly, all cells sent varicosed fibers in the pTRG, with some fibers reaching the respiratory motoneurons nuclei on both sides (A3 Figure 3E–G). Some varicosities were observed in close proximity to pTRG neurons that had also been retrogradely labeled from injections in the respiratory motoneuronal pools (A3 Figure 3H). Interestingly, none of the MLR cells had significant axonal projections within the hindbrain reticular nuclei, known to relay locomotor commands to the spinal cord (36–38). To address this point further, fluorescent dextran amines were injected in the reticular formation and in the respiratory motor nuclei to label cells that would project to both structures. There were no double-labeled cells in the MLR, suggesting that separate populations of MLR neurons project to locomotor and respiratory structures. We found that MLR neurons projecting to the respiratory motor nuclei were generally located in the dorsal part of the MLR, whereas those projecting to the reticular formation were located more ventrally (A3 Figure 3D).

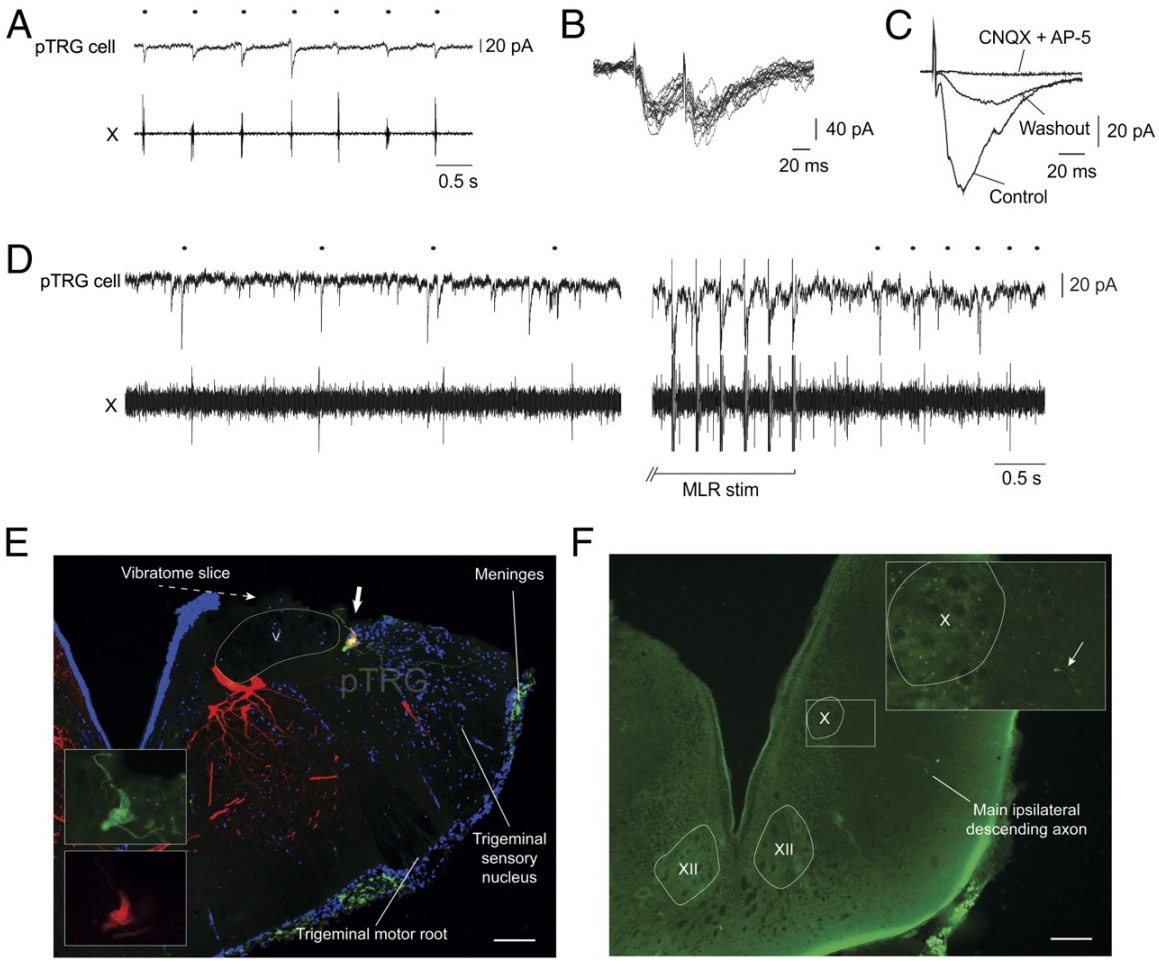


A3 Figure 3: **Electrophysiological and anatomical characterization of individual MLR neurons projecting to the respiratory nuclei.** (*A*) Electrophysiological recordings of a single neuron in the MLR in voltage-clamp whole-cell mode following stimulation of the PT. (*B*) Short latency EPSCs (average of 10 traces) evoked by PT stimulation. (*C*) Three-dimensional reconstruction of the axonal projections of the recorded neuron seen from a dorsal view. Varicosities and dendritic spines are represented as circles. The diameter of the axons, dendrites, and cell body were amplified for better visibility. The *y*-, *z*-, and *x*-axis scales represent 500 µm. Color correspondence: dendrites and cell body, light red; axons, light grey; V, dark red; VII, light green, IX, dark yellow; X, dark grey;

MRRN, dark blue; PRRN, light blue; pTRG, purple. (*D*) Superimposition of photomicrographs illustrating the populations of MLR neurons backfilled from injections in the respiratory motoneurons (red) and in the reticular formation (green). The dorsal (dMLR) and ventral (vMLR) parts of the MLR are indicated. The asterisk marks a reticulospinal cell that was labeled by the respiratory motoneuron injection. White asterisks (*Insets*) indicate MLR neurons. (Scale bars: 100 μm; *Insets*, 50 μm.) (*E*) Superimposition of three photomicrographs illustrating the whole-cell patch-recorded neuron intracellularly labeled with biocytin (green) among a population of other MLR neurons projecting to the respiratory motor nuclei (dextran amines, red) as seen on a cross section at the level of the MLR. DAPI labeling (blue) shows the location of DNA-containing cell nuclei in the region. I1 is a giant reticulospinal cell used as an anatomical landmark for the position of the MLR. Asterisk marks artifact of tissue folding. (Scale bar: 100 μm.) (*F*) Photomicrograph illustrating two axonal branches (arrows) from the labeled MLR neuron in the ipsilateral IX. (Scale bar: 50 μm.) (*G*) Photomicrograph illustrating an axonal branch (arrow) from the labeled MLR cell in the contralateral IX motor nucleus. Asterisk marks autofluorescent globular artifacts. (Scale bar: 50 μm.) (*H*) Photomicrographs illustrating an axonal branch coming from the MLR cell in the contralateral pTRG. A varicosity (arrow) can be seen in close proximity with a dendrite from a retrogradely labeled pTRG neuron (red). The larger photograph was taken under standard epifluorescence microscopy. *Inset*: Photomicrograph obtained with an Olympus FV1000 confocal microscope equipped with a 60× objective (oil, NA = 1.42). The photograph is a superimposition of two consecutive 0.5-μm optical slices taken from a *z*-stack of 14 photographs. (Scale bar: 50 μm.) PRRN, posterior rhombencephalic reticular nucleus; MRRN, middle rhombencephalic reticular nucleus; PT, posterior tuberculum; pTRG, paratrigeminal respiratory group; V, trigeminal motor nucleus; VII, facial motor nucleus; IX, glossopharyngeal motor nucleus; X, vagal motor nucleus.

We then examined the responses of neurons within the respiratory generator to MLR stimulation. We focused on pTRG neurons projecting to the respiratory motoneurons by first injecting dextran amines into the IX motor nucleus on one side. This allowed us to target pTRG neurons for whole-cell patch recordings (n = 6 cells, n = 6 preparations). A Vibratome cut was made dorsal to the pTRG to gain a better access to the cells. The recorded neurons displayed clear depolarizing currents in phase with the spontaneous respiratory activity (A3 Figure 4A). Electrical stimulation of the MLR induced short-latency EPSCs (7.8 ± 3.3 ms) that followed high-frequency stimulation (16 Hz; A3 Figure 4B), compatible with a monosynaptic connection in lampreys (42). In five

preparations tested, bath application of CNQX and AP5 greatly reduced the EPSCs (76.9% ± 20.7% reductions; one-tailed paired t test; n = 5 pTRG neurons; $P < 0.001$; A3 Figure 4C), and a partial recovery was obtained after washout in four cases (57.9 ± 17.1% of control; one-tailed paired t test; n = 4 pTRG neurons; $P < 0.01$ with effect, $P < 0.05$ with control). It is noteworthy that the bath application of CNQX and AP5 also abolished respiratory activity. In normal Ringer solution, when stimulating the MLR (1–10 μA; 3–5 Hz; 2-ms pulse duration; 10 s), the increases in frequency were present both in the pTRG cell and the respiratory motoneurons (A3 Figure 4D). The respiratory activity was generally weaker and slower in these preparations, presumably because of the Vibratome cut performed above the pTRG. Injection of the recorded pTRG cells with intracellular dye revealed descending axonal branches with varicosities in the respiratory motoneuron pools (A3 Figure 4E and F). Altogether, these experiments suggest that MLR neurons send monosynaptic glutamatergic inputs to respiratory pTRG neurons that in turn project to respiratory motoneurons. The anatomical findings suggest that a dorsal subpopulation of MLR cells could be involved in the modulation of respiration, whereas more ventral MLR cells project to reticulospinal cells that are involved in the control of locomotion.

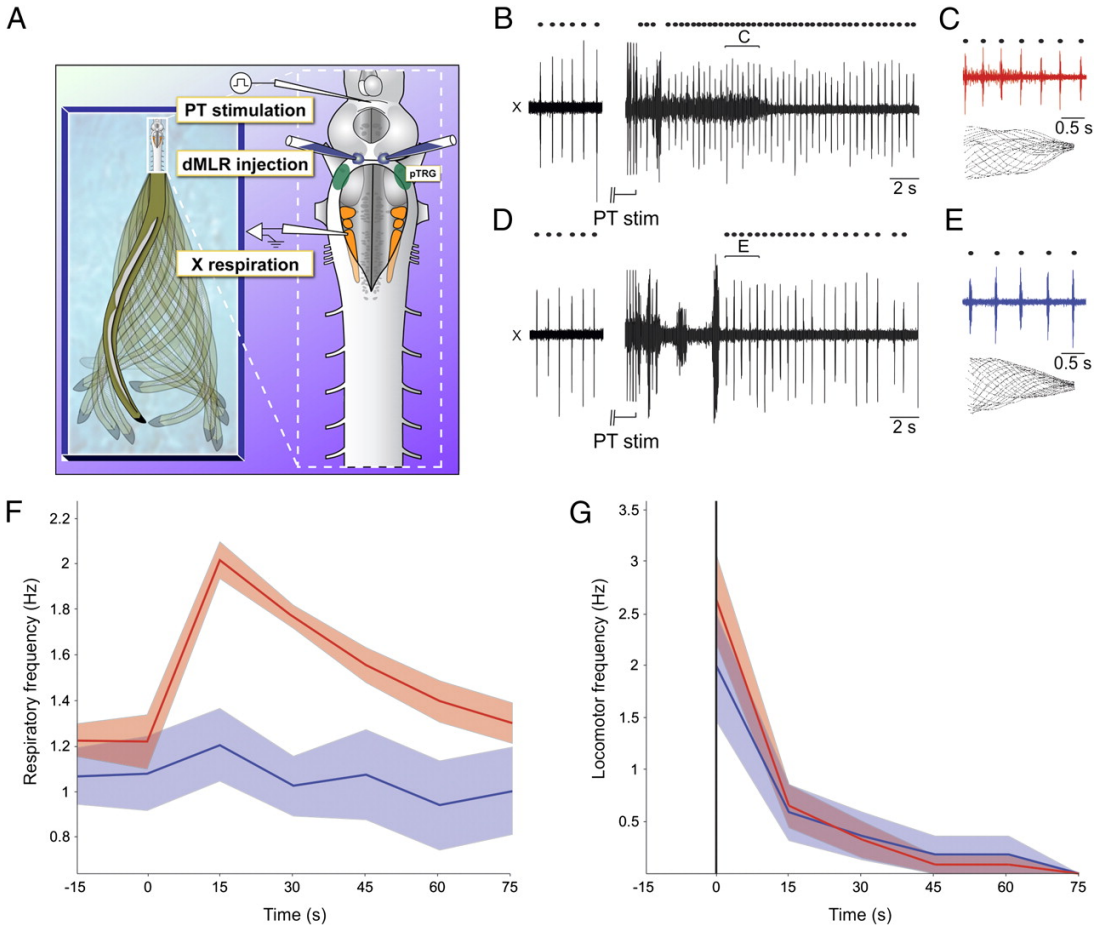


A3 Figure 4: **Electrophysiological and anatomical characterization of pTRG premotor respiratory interneurons.** (*A*) Voltage-clamp whole-cell recording of a neuron in the respiratory generator (i.e., pTRG). The neuron was retrogradely labeled from the IX respiratory motor nucleus. Excitatory currents occurred in-phase with respiratory activity recorded extracellularly from the vagal nucleus (X). (*B*) EPSCs induced by high-frequency (16 Hz) electrical stimulation of the MLR in a pTRG neuron. (*C*) Effect of a bath application of CNQX (50 μM) and AP5 (100 μM) on the MLR-induced EPSCs. (*D*) Electrical stimulation of the MLR induces excitatory currents and an increase of baseline currents in a pTRG interneuron, along with an increase of respiratory activity. (*E*) Superimposition of three photomicrographs of the recorded pTRG neuron filled with biocytin (green) and retrogradely filled from an injection of dextran amines (red) in the IX nucleus. DAPI labeling (blue) shows the location of DNA-containing cell nuclei. (Scale bar: 100 μm.) (*F*) Photomicrograph illustrating an axonal branch of the pTRG cell (arrow) in the ipsilateral X motor nucleus in the caudal brainstem. (Scale bar: 100 μm.) Dots indicate respiratory bursts. V, trigeminal motor nucleus; X, vagal motor nucleus; XII, hypoglossal motor nucleus.

Role of dorsal MLR in respiratory changes associated with locomotion

We have further examined the role of MLR neurons in modulating respiratory activity during active locomotion. Experiments were performed on semi-intact lampreys in which the brain and rostral spinal cord were first exposed and then the rostral forebrain removed, while the tail was left intact and free to swim in a deeper part of the bath (n = 5 preparations; A3 Figure 5A). In this preparation, stimulation of the PT (5–10 μA; 3–5 Hz; 2-ms pulse duration; 10 s) induced swimming that was associated with increases in respiratory activity (increases of 77.2 ± 56.5%; Wilcoxon signed-rank test, $P < 0.001$; n = 15 locomotor bouts; A3 Figure 5B, C, F, and G, red). Local injections of CNQX (1 mM) and AP5 (500 μM) were then performed in the dorsal part of the MLR to test whether this area was involved in the respiratory increase. The injections considerably reduced the respiratory increases associated with locomotion (increases of 16.9 ± 25.7%; Wilcoxon signed-rank test, $P < 0.05$ for the increases; Mann–Whitney rank-sum test, $P < 0.01$ for difference with increases in control; A3 Figure 5D–F, blue). The frequency of swimming was not significantly changed after the injection (2.63 ± 1.71 Hz in control vs. 2.00 ± 1.31 Hz after injection; Mann–Whitney rank-sum test, $P = 0.44$; n = 15 locomotor bouts in control, n = 6 locomotor bouts after injection; comparison made at time 0–15 s; A3 Figure 5G). To further support the contribution of MLR cell bodies in the respiratory effects and to determine whether they were sensitive to GABA, we also performed MLR stimulation with D-glutamate while applying GABA in the dorsal part of the MLR. The GABA injections considerably reduced the respiratory effects (n = 5 preparations; A3 Supplementary Figure 6). These experiments show that a major part of the respiratory

increases linked to movement rely on a specific dorsal region of the MLR and that blockade of this region affects respiratory activity without modifying locomotor activity.



A3 Figure 5: **Role of the dorsal MLR in respiratory modulation during locomotion.** (*A*) Schematic representation of the semi-intact preparation. (*B*) Respiratory increases following PT stimulation that induced locomotor movements. (*C*) Close-up of the respiratory bursts during locomotion (*Upper*) and drawings of tail movements induced by the PT stimulation (*Lower*). (*D*) Respiratory increase following PT stimulation after blockade of the dorsal part of the MLR (dMLR) with microinjections of CNQX (1 mM) and AP5 (500 µM). (*E*) Close-up of the respiratory bursts during locomotion (*Upper*) and drawings of tail movements induced by the PT stimulation (*Lower*). (*F*) Analysis of all PT stimulation in all lampreys (n = 5 preparations) in control situation (red, n = 15) and after blockade of the dorsal part of the MLR (blue, n = 6). Data were triggered around the beginning of locomotion (i.e., time 0) and were pooled in classes of 15 s. The shaded area around the curves corresponds to the SE of the mean. (*G*) Similar analysis for locomotor frequency. Dots indicate respiratory bursts. PT, posterior tuberculum; X, vagal motor nucleus.

Discussion

How respiration is modified to compensate for an increased energy demand during movement and exercise has been an important question in physiology that was investigated during the past century. Several lines of evidence have pointed to central neural connections as possible mechanisms (12–14), but the hypothesized neural connections were never identified. In this study, we show that a group of neurons dorsally located in the MLR sends direct inputs to pTRG neurons. Moreover, blocking excitatory synaptic transmission in the dorsal part of the MLR greatly reduces the respiratory increases associated with locomotion, despite the presence of active locomotor movements.

Monosynaptic connections from MLR neurons to neurons in the respiratory generator

In this study, we provide several lines of evidence indicating that the MLR projects to the respiratory CPG. MLR stimulation increases respiratory frequency and also resets the respiratory rhythm. As shown for other systems such as locomotor networks (43), this indicates that the CPG receives direct or indirect inputs from the stimulated region. We have identified a population of MLR neurons with direct projections to the pTRG and respiratory motoneurons by using retrograde labeling.

Projections from the MLR to cranial motor nuclei including some with respiratory functions were proposed to exist in cats and rats (44, 45), but direct connections to neurons in the respiratory generator remained to be identified. Interestingly, the MLR neurons projecting to respiratory areas did not project to the locomotor areas, as would be

expected if the same neurons would control respiration and locomotion in parallel. Therefore, our results suggest that respiration is controlled by a subpopulation of cells in the dorsal MLR. The fact that we could abolish a major part of the respiratory increase without affecting the locomotor pattern by blocking the dorsal part of the MLR also supports the idea that the MLR neurons controlling respiration and locomotion constitute separate populations. We also show that the population of dorsal MLR cells possess bilateral projections to the pTRG and respiratory motoneurons. This could contribute to the bilateral synchronization of the respiratory rhythm, which was shown to rely on crossing hindbrain respiratory neurons in mammals (46).

Functional significance

It is well documented that respiration increases before and during movement in many animal species, including humans, and that the respiratory changes are correlated to the intensity of the muscular effort (1, 2, 8, 10, 12–14). Classically, the fast respiratory response to exercise was thought to result from a neural component, whereas the late respiratory changes were thought to result from combined neural and peripheral components (1). In this study, we describe a direct connection between the MLR and the respiratory generator that could underlie both the early and late respiratory changes. Indeed, we found that blocking excitatory transmission in the dorsal MLR markedly reduced not only the early changes but changes throughout the locomotor bout. Moreover, the neural substrate we describe in the present study does not require sensory inputs to activate the respiratory centers, nor does it require the spinal cord, caudal part of

the hindbrain, or forebrain. Because the MLR initiates locomotion and controls the intensity of the locomotor output (36, 47), MLR neurons are presumed to encode information on the intended locomotor activity before it is relayed to the reticulospinal cells. This would make these neurons good candidates to adjust respiration before the movement is executed. This does not preclude possible additional contributions from sensory inputs to the respiratory adjustments. However, it is tempting to speculate that modifying respiration through direct projections from a brainstem center controlling locomotion could provide an advantage in terms of speed and precision of the respiratory increases. Other cells than those identified using single-cell recordings might be present in the dorsal MLR and might participate in the respiratory changes through other mechanisms than direct glutamatergic projections. However, whole-cell recording of neurons in the pTRG allowed us to show that most of the excitatory inputs from the MLR to these neurons are of very short latency and are abolished by glutamatergic receptor blockers.

It was proposed that the central command modifying respiration during exercise originated from the cerebral cortex (1, 48, 49) or the hypothalamus (12). Forebrain structures project directly or indirectly to the MLR (50). One possibility raised by the present study is that upstream locomotor centers could use the MLR as a relay to increase respiration during locomotion. We show that MLR cells are activated by stimulation of the PT, thought to be homologous to the substantia nigra of amniotes (51). We also show that blocking the dorsal part of the MLR while stimulating the PT nearly abolishes the respiratory increase associated with locomotion. This suggests that higher centers might necessitate the dorsal MLR to exert their respiratory effect. It was recently shown that the

lamprey possesses important parts of the basal ganglia circuitry that is present in mammals (52). A better understanding of the interconnections between this circuitry and the MLR neurons might shed light on how higher motor centers control multiple behaviors in parallel.

It is well documented that mental simulation of physical effort and preparation for effort in humans induce respiratory and heart rate increases (10, 11). Furthermore, it was shown by using functional MRI that the MLR is activated in human subjects in similar tasks (40). The respiratory changes following mental simulation of movement were proposed to result from the activation of specialized motor anticipation networks or from components of the normal motor control regions (53). Our results provide support to the latter view because respiratory changes clearly occurred upon stimulation of the MLR at subthreshold levels. Hypothetically, the MLR neurons responsible for the respiratory command might be activated earlier than those controlling locomotion. This is supported by the observation that the recorded MLR neurons displayed excitation several seconds before the onset of MRRN activity, known to be part of the locomotor command signal reaching the spinal cord to generate locomotion (54).

Rethinking the role of the MLR

The MLR has been traditionally described as a region receiving convergent inputs from higher brain regions. It was believed to integrate these inputs to induce and control locomotion by providing excitation to reticulospinal cells in the lower brainstem (36–38, 54, 55). Other studies showed that stimulating locomotor centers induces

increases in respiratory activity (12–14), but it was not known whether the respiratory effect was induced directly by MLR neurons or by a feedback from the spinal cord and caudal brainstem. Our results now show that the MLR contains a set of neurons with specific projections to the respiratory centers, and that this pathway increases respiration in the absence of the caudal brainstem and the spinal cord networks controlling locomotion. Another example of the divergent outputs of the MLR is the recently described parallel projection to a group of muscarinoceptive cells at the pontobulbar border, which increases the locomotor output (56). Thus, the view that emerges from recent studies, including the present study, is that MLR neurons transform locomotor commands coming from higher brain areas into multifunctional outputs controlling respiration and locomotion through independent subsets of neurons. This view also provides an interesting addition to an ongoing discussion about regional specificity in the brain. Basic motor and sensory functions are often believed to originate from highly specialized regions (57). Our results, on the contrary, indicate that the MLR, rather than being highly specialized in a single motor function, controls multiple functions with a common high-level goal: to allow animals to move in their environment while maintaining respiratory homeostasis.

Materials and methods

All surgical and experimental procedures conformed to the guidelines of the Canadian Council on Animal Care and were approved by the animal care and use committee of the Université de Montréal and the Université du Québec à Montréal. Care

was taken to minimize the number of animals used and their suffering. Experiments were performed on 102 postmetamorphic and spawning-phase adult sea lampreys, Petromyzon marinus, that were collected from the Great Chazy river (New York), Lake Huron (Ontario, Canada), and Morpion Stream (Ste-Sabine, QC, Canada). Some postmetamorphic animals were purchased from ACME Lamprey (Maine). The animals were kept in aerated water at 7 °C.

In vitro animal preparation

For patch recording experiments, a surgery was made 24 h preceding the experiment to inject Texas red–dextran amines in the IX nucleus *in vivo*. This was done by anesthetizing the animals in tricaine methanesulfonate (MS 222, 100 mg/L of fresh water) and then transferring the animal in cold oxygenated Ringer solution (8–10 °C, 100% O_2) of the following composition (in mM): 130 NaCl, 2.1 KCl, 2.6 CaCl2, 1.8 MgCl2, 4 Hepes, 4 dextrose, and 1 NaHCO3 (pH 7.4). A 2-mm2 flap window was opened on the top of the head to expose the caudal brainstem. Needles were introduced in the IX nucleus to inject crystals of the dye. The incision was closed with Vetbond after the injection procedure. The animal was then returned to a small nursery aquarium filled with oxygenated Ringer solution for the night at room temperature. This injection was designed to retrogradely label both the population of MLR and pTRG neurons projecting to the respiratory motoneurons.

For electrophysiological experiments, the animals were first anesthetized and transferred into Ringer solution as described earlier. A complete transverse section was

then performed approximately 2 cm caudal to the seventh gill pore, leaving approximately 15 myotomal segments (among ~100). The viscera and muscles were dissected out. The brain and spinal cord were exposed on their dorsal surface. The preparation was then pinned down onto silicone elastomer (Sylgard) at the bottom of an experimental chamber continually perfused with cold Ringer solution at a rate of approximately 4 mL/min. The cranial nerves were cut proximally to the brain to abolish possible sensory feedback. The brain tissue rostral to the mesencephalon was removed, except for experiments in which we stimulated the PT. In some experiments, a transverse section was made at the obex to isolate the brainstem from the spinal cord. To access the pTRG and MLR cells with patch electrodes, a Vibratome cut of the dorsal tissue of the pons and mesencephalon was performed in cold Ringer solution (1–3 °C). For MLR cell recordings, only the optic tectum and the dorsal isthmic region were removed. For pTRG cell recordings, the alar plate lateral to the V nucleus, the dorsal part of the V motor nucleus, the dorsal isthmic region, and the optic tectum were removed.

Electrophysiological experiments

Extracellular recordings of spinal ventral roots, respiratory motoneurons, and the pTRG were made by using glass electrodes filled with Ringer solution (tip diameter ~5 μm) and connected to a microelectrode AC amplifier (low cutoff, 100 Hz; high cutoff, 500 Hz; model 1800; A-M Systems; A3 Supplementary Figure 1).

Respiratory motoneurons were recorded intracellularly with sharp glass microelectrodes (4 M KAc; 80–120 MΩ). The signals were amplified through an

Axoclamp 2A amplifier (sampling rate, 10 kHz; Axon Instruments). The motoneurons were impaled by directing the tip of the electrode to the IX or the rostral X motor nucleus. Motoneurons always displayed rhythmical membrane potential oscillations that were synchronous with the respiratory output recorded from the motoneuronal pools with extracellular electrodes. Patch recordings of pTRG and MLR cells were made in whole-cell voltage-clamp mode (−60 to −70 mV) with a model 2400 amplifier (A-M Systems). The cells were targeted under an Eclipse FN-1 microscope (Nikon Instruments) equipped for fluorescence. Patch pipette solution contained (in mM) cesium methane sulfonate 102.5, NaCl 1, MgCl2 1, EGTA 5, and Hepes 5, and 0.1% biocytin. pH was adjusted to 7.2 with CsOH, and pipettes were pulled to a tip resistance of 5 MΩ.

The MLR was stimulated chemically or electrically. The PT was stimulated electrically. Chemical stimulation was performed by pressure-injecting the excitatory amino acid D-glutamate (2.5 mM; Sigma), diluted in Ringer solution at pH 7.4 through a glass micropipette using a Picospritzer (General Valve). The inactive dye, fast green, was added to the drug solution to visually monitor the size and exact location of the injections. In some experiments, the same equipment was used to inject the ionotropic glutamatergic receptor blockers CNQX (1 mM) and AP5 (500 μM) in the pTRG and in other brainstem sites. The size of the pTRG and MLR injections was assessed visually by measuring the spread of the dye, fast green, in the tissue with a dissecting microscope equipped with a calibrated ocular micrometer. The spread was less than 300 μm in diameter for those injections. Theoretically, the maximal injection volume can be estimated by using the equation that links the volume of a sphere to its diameter. As such, the maximal volume of these injections would be 14 nL. Bath application of CNQX (50

μM) and AP5 (100 μM) were also performed. After each D-glutamate injection in the MLR, a washout period was allowed for recovery (several minutes to >1 h). To obtain subthreshold effects on respiration (i.e., respiratory effects without locomotion), the MLR was first localized by performing injections that resulted in fictive locomotion. The Picospritzer pressure was then reduced to a level that remained subthreshold for inducing fictive locomotion. The spread of these injections was less than 50 μm in diameter. Based on the equation as described earlier, the maximal volume of these injections would be 0.1 nL. The spread of the dorsal MLR injections was also less than 50 μm in diameter (A3 Figure 5 and A3 Supplementary Figure 6).

Electrical stimulation of the MLR and PT was performed with glass-coated tungsten microelectrodes (0.8–2 MΩ). For experiments in which the spinal cord was removed, the activity of reticulospinal cells in the brainstem was monitored before and after the spinal cord lesion by using an extracellular electrode to verify that the effects of MLR stimulation on the locomotor networks were still present. The stimulation site (i.e., MLR) was marked by an electrolytic lesion made by passing a continuous negative current of 5 μA during 10 s in 16 preparations in which we measured electrically induced locomotion, respiratory rhythm resetting, and motoneuronal EPSPs in response to MLR stimulation.

Semi-intact preparations were used to apply electrophysiological techniques in the brainstem while observing active locomotor movements from the intact tail (A3 Figure 5). For this preparation, we dissected the brainstem similarly to the *in vitro* preparation, but the spinal cord and tail were left intact and free to swim in a deeper part

of the bath. The locomotor movements were analyzed using the same technique that was used for *in vivo* recordings (SI Materials and Methods).

Data acquisition and analyses

Data were acquired via a Digidata 1322A interface by using Clampex 9 software (Axon Instruments) for computer analysis. Respiratory and locomotor bursts were detected and analyzed by using homemade software. The instantaneous frequency of the respiratory activity was calculated as the inverse of the time interval between the beginnings of two consecutive respiratory bursts. The area was measured on a rectified and filtered version of the signal (root mean square, 10-ms bins). The area of the respiratory bursts, EPSPs, and EPSCs was normalized because of the variability between preparations. This normalization was obtained by multiplying values of area by 100 and dividing by the average measure in control situation for each individual lamprey.

The effects of MLR and PT stimulation on respiration were examined on 20 respiratory cycles before (i.e., control) and during fictive locomotion. The duration of the effect on frequency and area of the respiratory bursts was analyzed by using iterated t tests between a group of 20 control bursts and a moving window of 20 bursts after the stimulation. For *in vivo* experiments, anticipatory respiratory changes were characterized by comparing the frequency of respiratory bursts occurring during a window of 6 s preceding any movement with a set of bursts occurring at rest, more than 10 s before the beginning of locomotion. For semi-intact preparations, respiratory frequencies were

regrouped in classes of 15 s (i.e., 0–15 s, 15–30 s) and triggered on locomotion onset to facilitate the pooling of data from multiple preparations.

EPSPs and EPSCs were analyzed using Spike2 version 5.19 (Cambridge Electronic Design) and homemade scripts. The area under the curve was measured from the end of the stimulation artifact to the end of the EPSPs.

Data in the text are given as means ± SD unless specified otherwise. Data in A3 Figure 5 are given as mean ± SEM. Statistical analyses were carried out using Origin (OriginLab) or SigmaStat version 3.5 (Systat). A Student t test was used to compare the means of two groups. The Wilcoxon and Mann–Whitney tests were used when the compared distributions did not respect the assumptions of normality of distributions or equality of variance. Distributions were considered statistically significant when the value of P was lower than 0.05.

Histology and axonal tracing experiments

Details of the histological procedures were published elsewhere (36, 41) and are provided in SI Materials and Methods. Electrophysiological and anatomical figures were designed using CorelDraw 4 software (Corel). The projections of all of the intracellularly labeled cells were examined in details from serial sections. One MLR neuron was reconstructed in 3D for illustrative purposes by using Neurolucida (MBF Neuroscience; A3 Figure 3). In the 3D model, the contours of the brainstem; MRRN; posterior rhombencephalic reticular nucleus; V, VII, IX, and X motor nuclei; and pTRG were first outlined on each cross-section.

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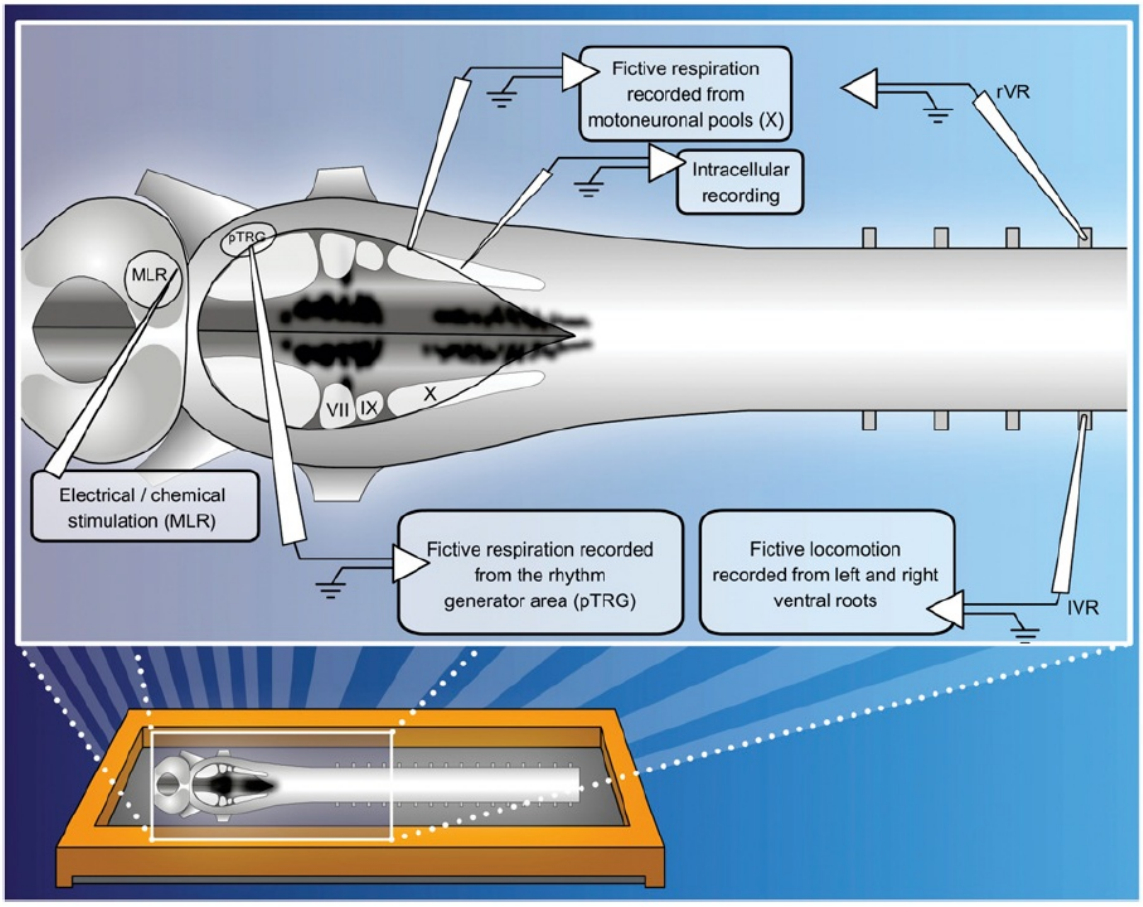
Supporting Information

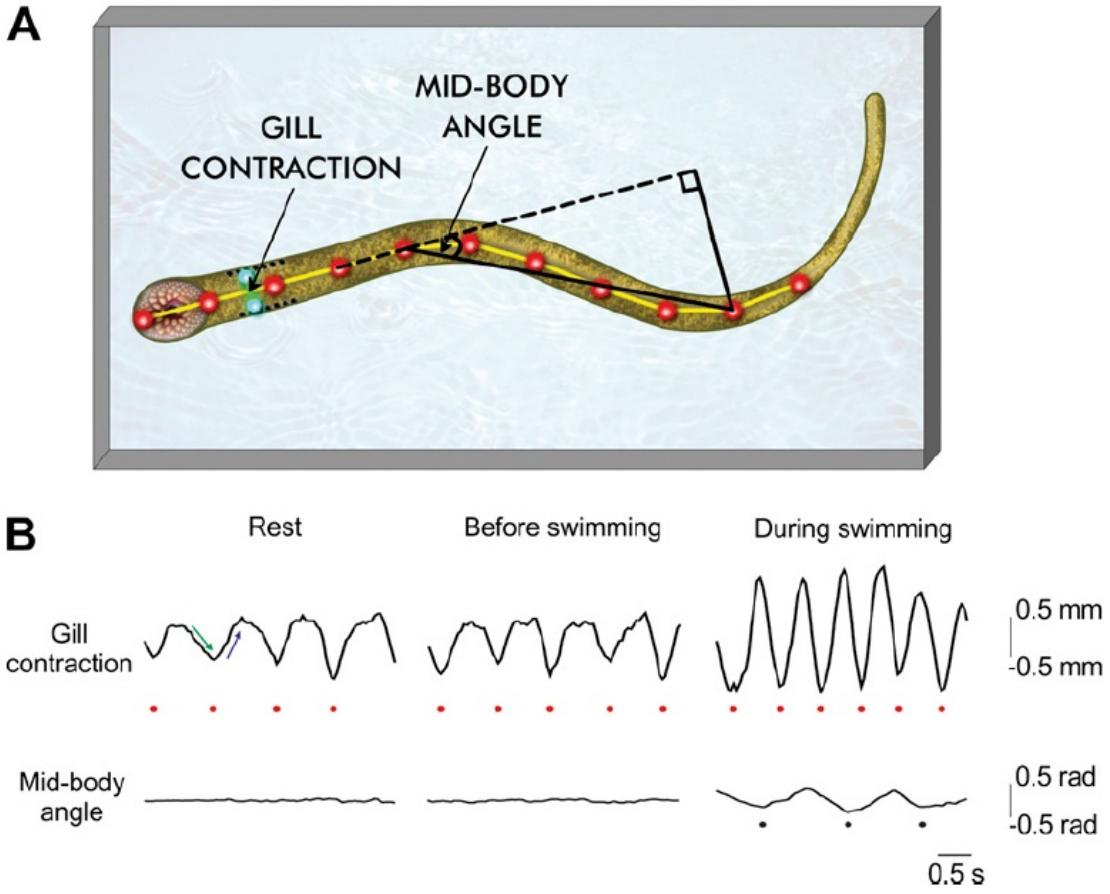
In vitro changes in respiratory activity

An *in vitro* preparation consisting of the isolated brainstem and a portion of the spinal cord was used to examine the role of central connections in the absence of sensory inputs (A3 Supplementary Figure 1). Extracellular discharges were recorded from motoneurons in the glossopharyngeal motor nucleus (IX) or the rostral vagal motor nucleus (X) to monitor respiratory activity (at rest, 0.89 ± 0.30 Hz frequency; 54.5 ± 7.8 ms duration; A3 Figure 1B, B' and A3 Supplementary Figure 3). The mesencephalic locomotor region (MLR) was pharmacologically activated by locally injecting 2.5 mM D-glutamate (n = 48 injections, n = 7 preparations) and fictive locomotion, consisting of bilaterally alternating ventral root discharges, was elicited (A3 Supplementary Figure 3A, Center). At the beginning of the fictive locomotor bout, a period of large and sustained tonic bursts replaced the short phasic bursts of respiratory activity. After this, respiration returned to a stable rhythmic pattern, but with a 91.4 ± 58.6% increase in frequency (0.89 ± 0.30 Hz in control to 1.57 ± 0.39 Hz; one-tailed paired t test; $P < 0.001$; n = 48) and a 58.2 ± 49.4% increase in area of the bursts (100 ± 14.4% in control to 157.8 ± 52.7%; one-tailed paired t test; $P < 0.001$; n = 48). The effects on the frequency lasted significantly longer than those on the burst area (154.6 ± 72.3 vs. 64.2 ± 35.3 s; two-tailed paired t test, $P < 0.001$; n = 48). In 23 cases, the changes in respiratory activity preceded locomotion, and in 25 cases, the respiratory changes followed the onset of locomotion. The delays ranged from −5.7 s to 1.6 s, with negative values indicating respiratory effects preceding locomotion. In a subset of four experiments, the paratrigeminal respiratory group (pTRG) was recorded in addition to the respiratory

motoneurons, and similar respiratory changes were observed (A3 Supplementary Figure 3A).



A3 Supplementary Figure 1: **Schematic representation of the experimental setup.** Experiments were performed using chemical (D-glutamate 2.5 mM) or electrical stimulation (tungsten microelectrode) of the MLR. The respiratory activity was recorded using glass suction electrodes placed over the glossopharyngeal (IX) or the rostral vagal (X) motor nuclei. In some experiments, the pTRG was recorded in combination with respiratory motoneurons to monitor respiration. Locomotor activity was recorded by using glass suction electrodes placed on ventral roots on the right and left sides of the spinal cord (RVR and LVR, respectively). In some experiments, intracellular recordings of respiratory motoneurons in the IX or the X motor nucleus were carried out by using sharp microelectrodes. Injection micropipettes were placed in the pTRG on both sides to pressure-eject a mixture of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and (2R)-amino-5-phosphonovaleric acid (AP5). VII, facial motor nucleus.

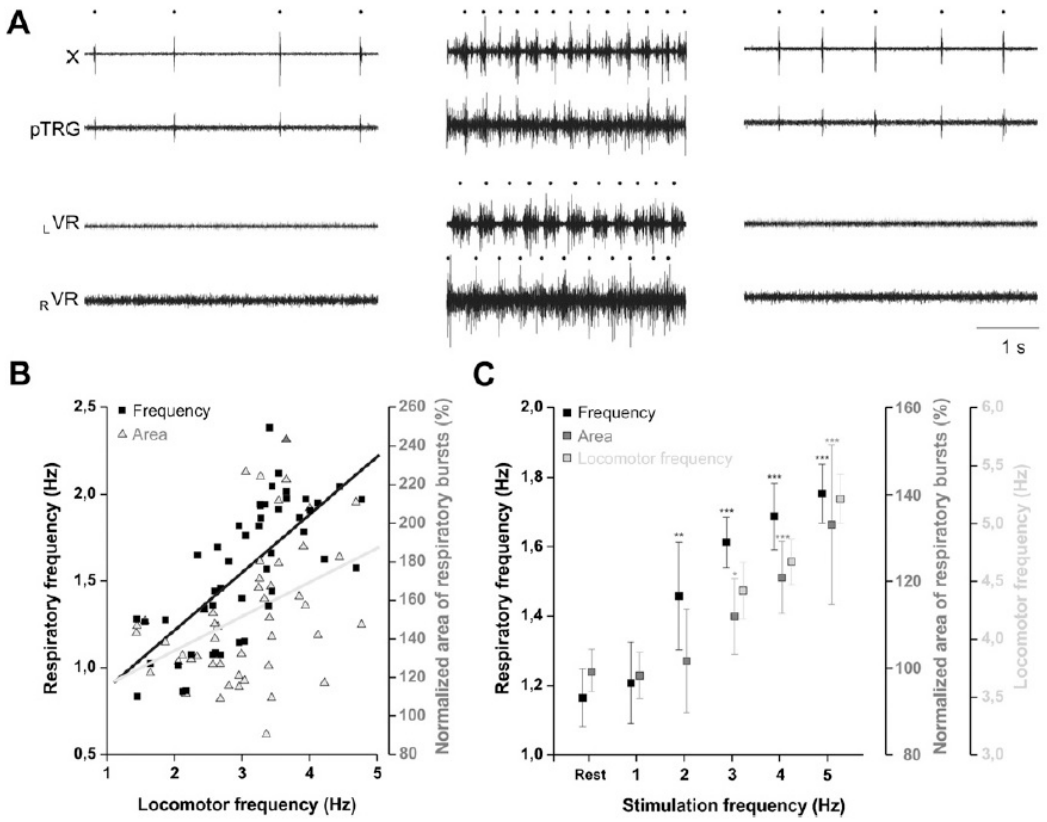


A3 Supplementary Figure 2: ***In vivo*** **respiratory increases associated with locomotion.** (A) Schematic representation of the movement analysis procedure for respiratory and locomotor activities in freely moving adult lampreys. (B) Video tracking of respiratory and swimming movements at rest (Left), 6 s before swimming (Center), and during swimming (Right). Statistical analyses are provided in A3 Figure 1. Red dots indicate peaks of expiration, black dots indicate locomotor cycles, green arrow indicates expiration, and blue arrow indicates inspiration.

The intensity of the respiratory changes was correlated to the locomotor frequency (frequency, strong correlation; $r = 0.72$; linear fit; $P < 0.0001$; $n = 48$; area, weak correlation; $r = 0.41$; linear fit; $P < 0.01$; $n = 48$; A3 Supplementary Figure 3B). In five additional experiments, the MLR was electrically stimulated ($n = 163$) at progressively increasing intensities (1–10 μA; 3 Hz; 2-ms pulse duration; 10 s) or

frequencies (1–5 Hz; 5 μA; 2-ms pulse duration; 10 s; A3 Supplementary Figure 3C). As previously shown in semi-intact lampreys (1), the locomotor frequency increased linearly with the stimulation intensity (average correlation coefficient $r = 0.61 \pm 0.13$; linear fits; $P < 0.05$ for each preparation). In addition, the respiratory effects were correlated with the intensity of the MLR stimulation (average correlation coefficient for frequency $r = 0.65 \pm 0.17$; linear fits; $P < 0.05$ for each preparation; area, $r = 0.71 \pm 0.14$; linear fits; $P < 0.01$ for each preparation). These results show that, even in the absence of sensory feedback, the MLR induces respiratory effects in lampreys as previously seen in mammals (2–4).

We found that respiratory changes can occur in the absence of fictive locomotor activity from the ventral roots when the MLR was stimulated at weaker intensities (2 Hz; A3 Supplementary Figure 3C). This suggests that the pathways increasing breathing can operate without actual movement. We also reproduced these results with local D-glutamate injections in the MLR (A3 Figure 1D). At such low stimulation strengths (20–86% of the intensity needed to induce locomotion), the respiratory frequency increased by $38.8 \pm 24.8\%$ (0.86 ± 0.15 Hz in control, 1.17 ± 0.20 Hz after stimulation; one-tailed paired t test, $P < 0.001$; $n = 40$) and the burst area by $11.0 \pm 15.7\%$ ($100.0 \pm 6.3\%$ in control, $110.9 \pm 16.9\%$ after stimulation; Wilcoxon signed-rank test, $P < 0.001$; $n = 40$).



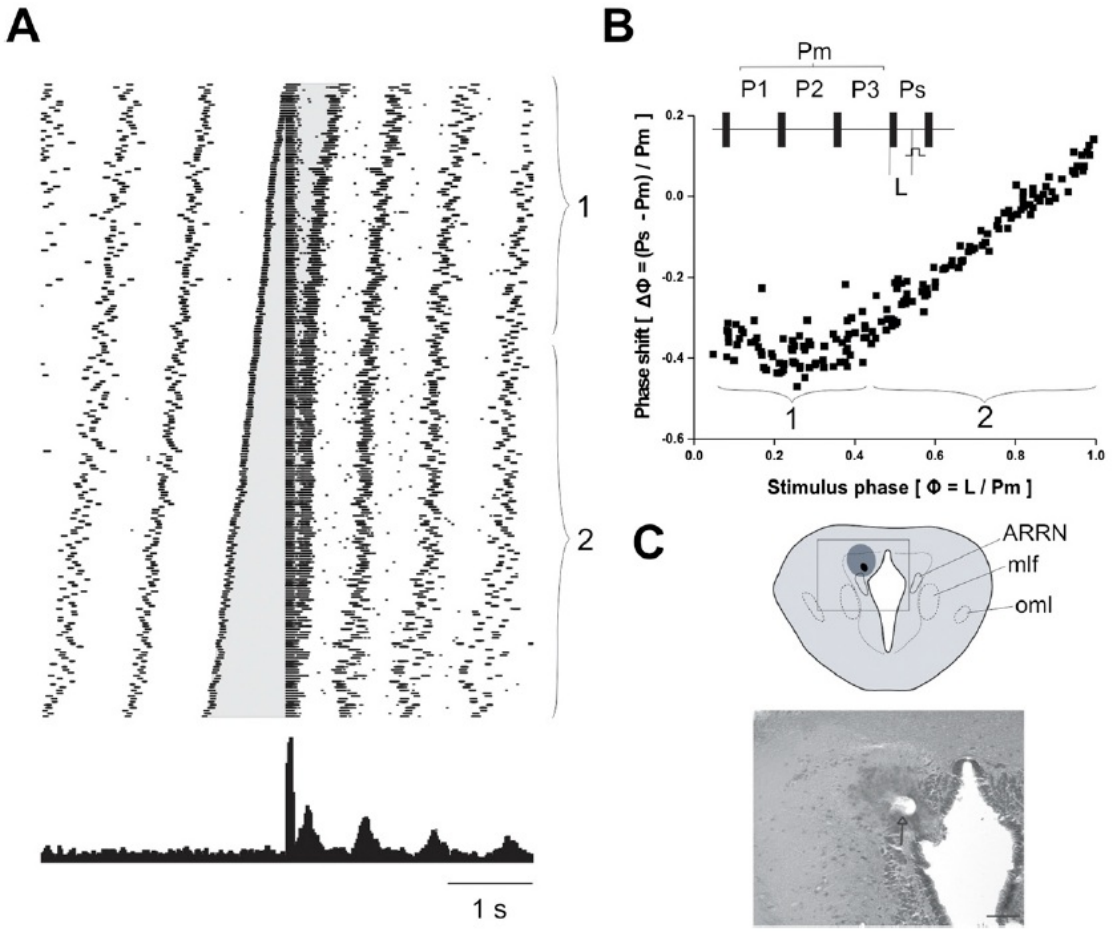
A3 Supplementary Figure 3: **Changes in respiratory activity associated with locomotion in an *in vitro* brainstem–spinal cord preparation.** (A) Respiratory changes following D-glutamate (D-GLU, 2.5 mM) injection in the MLR. Neurographic recordings show the extracellular activity of the respiratory motoneurons (X), the pTRG, and left and right ventral roots of the spinal cord (LVR and RVR, respectively) before (Left), during (Center), and after (Right) fictive locomotion induced by chemical activation of the MLR. Dots indicate respiratory and locomotor bursts. (B) Graph illustrating the respiratory frequency and the normalized area of respiratory bursts vs. the locomotor frequency. Data were pooled from seven preparations (48 injections). (C) Graph illustrating the respiratory frequency, the normalized area of respiratory bursts, and the locomotor frequency vs. the MLR stimulation frequency. The data are illustrated for one preparation (*P < 0.05, **P < 0.01, and ***P < 0.001).

Resets of respiratory rhythm following MLR stimulation

To examine the physiological connections between the MLR and the respiratory central pattern generator, the former was stimulated with short trains of stimuli (100 Hz, n = 3 stimuli). We then determined whether there was a reset of the respiratory rhythm (n = 7 preparations, n = 2,053). When the stimulation was applied in the late phase of the respiratory cycle, a reset of the respiratory rhythm occurred immediately after the stimulus (A3 Supplementary Figure 4A and B, bracket 2), providing strong support for the presence of connections between the MLR and the respiratory central pattern generator. When the stimulation was delivered at the beginning of the cycle, the resetting effect occurred with a delay (A3 Supplementary Figure 4A and B, bracket 1). The respiratory frequency of the three respiratory bursts preceding the stimulation was significantly lower (1.06 ± 0.06 Hz) than that of the three respiratory bursts following the stimulation (1.25 ± 0.18 Hz; two-tailed t test, $P < 0.001$; n = 6,159 for both groups). Electrolytic lesions made at the end of these experiments confirmed that the stimulation site was located in the dorsal part of the MLR (A3 Supplementary Figure 4C).

Retrograde labeling of population of MLR cells projecting to respiratory nuclei

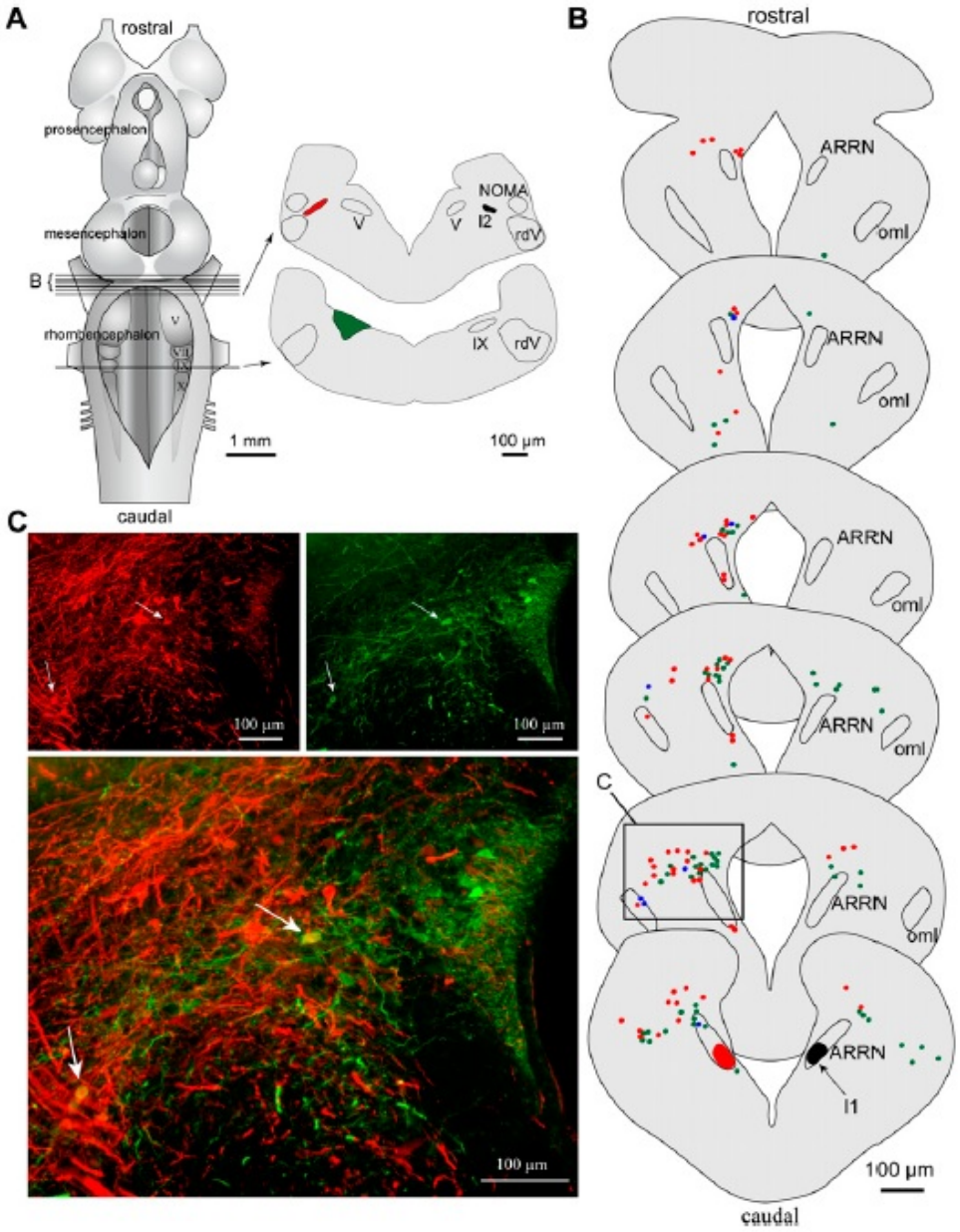
Anatomical experiments were carried out by using retrograde tracers to reveal the population of MLR cells projecting to the respiratory nuclei. The cells presented in the main article were all labeled by using injections in the motor nuclei, but injections were also performed in the pTRG. Injections were made either in the pTRG on one side (n = 6 preparations), in the respiratory motoneuronal pools (n = 9 preparations), or in both the



A3 Supplementary Figure 4: **Resetting of the respiratory rhythm by short trains of electrical stimulation of the MLR.** (A) Raster representation of extracellular activity recorded from the vagal motor nucleus aligned on the onset of MLR stimulation (vertical line, middle). Stimulation (trains of three stimuli at 100 Hz) was performed at different phases of the respiratory cycle (bracket 1, early during the cycle; bracket 2, late in the cycle). The gray area shows the respiratory cycle during which the stimulation is delivered. The histogram below shows the summation of the raster points (bin width, 20 ms). (B) Plot of the phase shift vs. stimulus phase. Note that a linear relationship between phase shift and stimulus phase is only present for Φ values greater than 0.4. (C) A typical MLR stimulation site identified by an electrolytic lesion. Top: Schematic illustration of a transverse section of the brainstem at the level of the isthmus. Bottom: Photomicrograph illustrating the electrolytic lesion, which is located in the MLR. (Scale bar: 50 μm.) ARRN, anterior rhombencephalic reticular nucleus; mlf, medial longitudinal fasciculus; oml, lateral octavomesencephalic tract (***$P < 0.001$).

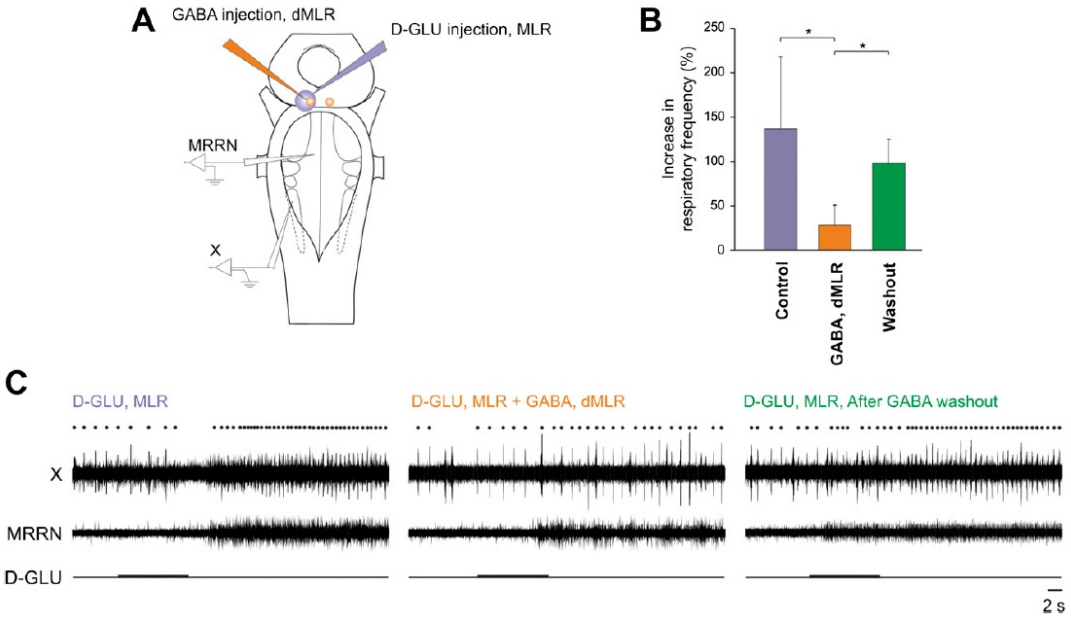
pTRG and the motoneuronal pools on the same side (n = 9 preparations; A3 Supplementary Figure 5). Both motoneuronal pool and pTRG injections yielded retrogradely labeled cells in the ipsilateral and contralateral MLR (A3 Supplementary Figure 5B and C). The largest proportion of the labeled cells were found medially and dorsally within the isthmic periventricular cell layer, sometimes intermingled with the large neurons of the anterior rhombencephalic reticular nucleus. Very few cells were labeled more ventrolaterally in the isthmic tegmentum. Overall, numerous labeled cells were located in the area where electrolytic lesions were made as the MLR was physiologically identified (A3 Supplementary Figure 4C). In double-labeling experiments, there were cells in the area containing both tracers (A3 Supplementary Figure 5). The labeled cells in the MLR were counted in five preparations in which the pTRG and the motoneuronal pools were injected with different tracers. The counts were made every second section to avoid counting neurons twice. Therefore, the counts represent approximately half the population. Injections in the motoneuronal pools labeled on average 76 ± 35 neurons in the ipsilateral MLR and 16 ± 9 neurons in the contralateral MLR. Injections in the pTRG labeled 49 ± 41 neurons in the ipsilateral MLR and 35 ± 31 neurons in the contralateral MLR. Counts were also made in the nearby reticular nucleus, the anterior rhombencephalic reticular nucleus. No cells were labeled from the motoneuronal injections. pTRG injections labeled on average 8 ± 1 neurons in the ipsilateral MLR and 6 ± 4 contralaterally. On average, 6 ± 3 neurons were found double-labeled by the pTRG and motoneuronal pool injections when located on the same side, and 3 ± 1 neurons were double-labeled when the two injections were located on opposite sides. The experiments into which single MLR cells were injected with an intracellular

dye revealed, on the contrary, that all the labeled MLR cells projected to the pTRGs and respiratory motoneurons on both sides (A3 Figure 3). The tracer injections in the pTRG could have destroyed some fibers that are descending to the motoneuronal pools or the injections may also miss some of the axonal collaterals (A3 Supplementary Figure 5). The neurons projecting to the pTRG and the respiratory motoneurons might thus be systematically underrepresented in the tracing experiments compared with single-cell injection experiments.



A3 Supplementary Figure 5: **Distribution of neurons in the MLR projecting to the pTRG and/or to respiratory motoneurons.** (A) Schematic dorsal view of the lamprey brain indicating the levels of the transverse sections illustrated in B. Right: Schematized transverse sections at the levels of the tracer injections in the pTRG (red) and the respiratory motoneurons (green). (B) Transverse sections in the MLR showing retrogradely labeled neurons from the two injection sites. A section was skipped between each illustrated one to avoid including some neurons twice. Each dot represents a labeled neuron. Double-labeled neurons are represented by blue dots. (C) Photomicrographs of the boxed area in B with neurons labeled from the pTRG (red) and from the vagal motor nucleus (green). The arrows indicate double-labeled neurons, which appear in yellow on

the superimposed images. ARRN, anterior rhombencephalic reticular nucleus; NOMA, anterior octavomotor nucleus; oml, lateral octavomesencephalic tract; rdV, descending trigeminal root; V, trigeminal nucleus; VII, facial motor nucleus; IX, glossopharyngeal motor nucleus; X, vagal motor nucleus. I1 and I2 are two large reticulospinal cells used as anatomical landmarks.



A3 Supplementary Figure 6: **Effect of a GABA injection in the dorsal part of the MLR on the MLR-induced respiratory increases.** (A) Schematic dorsal view of the *in vitro* brainstem preparation indicating the injection sites for D-glutamate (D-GLU, 2.5 mM), GABA (1 mM), and the recording sites [middle rhombencephalic reticular nucleus (MRRN) and X]. (B) Analysis of the increases in respiratory frequency following D-glutamate injections in the MLR in the control condition, after GABA injection bilaterally in the dorsal part of the MLR, and after washout. D-glutamate injections were performed every 20 min. First, a control injection was made. It was followed by a D-glutamate injection immediately after injecting GABA (<1 min). After 20 min of washout, a third D-glutamate injection was made. These experiments were performed on five preparations. The GABA injection markedly reduced the increase in respiratory frequency induced by the D-glutamate injection (137.0 ± 80.6% in control, 28.7 ± 22.2% after GABA; ANOVA on ranks; n = 5 injections; $P < 0.05$). After washout, the effect recovered (98.0 ± 27.2%; ANOVA on ranks; n = 5 injections; $P < 0.05$ for difference with effect, $P > 0.05$ for difference with control). (C) Raw traces of the MLR-induced respiratory effects (*$P < 0.05$). D-GLU, D-glutamate; X, vagal motor nucleus; dMLR, dorsal part of the MLR. Dots indicate expiratory activity.

Supplementary methods - In vivo video tracking

Experiments were conducted *in vivo* to evaluate the changes in respiratory frequency in relation to locomotion. Markers were used to track gill movements. The animals were anesthetized with tricaine methanesulfonate (MS 222, 100 mg/L). Six white dots (2 mm diameter, n = 3 ventrally, n = 3 dorsally) were tattooed on the skin of the gill region by using tattoo needles and white ink. The contractions of the gills during expiration were visible both ventrally and dorsally, such that respiratory movements could be tracked independently from the angle of the lamprey with respect to the camera. After the marking procedure, the animals were returned to their aquarium to recover from the procedure for at least 24 h. The animals were then transferred to a recording aquarium (98 × 35 cm) filled with 5 cm of water (approximately twice the height of lampreys). This allowed us to record swimming with reduced movements in the vertical plane, thus facilitating video tracking. The animals were allowed to acclimate for 2 to 4 h before data acquisition. The water temperature was kept between 8 °C and 10 °C. A video camera (GZ-HD3U; JVC) equipped with a wide-angle lens (0.42×) was placed below the aquarium (30 frames/s, 1,920 × 1,080 pixels). The recording sessions lasted several hours. The images were corrected for spatial distortion introduced by the wide angle.

Markers were manually pointed on the video images using homemade scripts in Matlab. The distance between any visible markers bilaterally placed on the gills provided a measure of respiratory movements. The low-frequency component of the measured distance signal was composed of various nonrespiratory signals such as movements across the aquarium and tilting. This component was removed by subtracting a moving average filtered version of the signal from the original signal at a frequency that was

sufficiently low to leave any respiratory signal present (smooth function in Matlab; cutoff, 0.5 Hz). A moving average was then applied on the signal to remove high-frequency noise (smooth function in Matlab; cutoff, 6 Hz). Locomotor movements were tracked by using markerless geometrical analysis of the body. The analysis relies on overlaying equidistant points on the body axis starting from the head down to the tail of the animal. The rostral extremity of the head was identified by the experimenter on every video frame. A set of pixels equidistant to the first point were calculated, thus forming a circle. The maximal contrast points along the circumference of that circle were found. These contrast points corresponded to the borders of the body. The mean angle between these two points with respect to the previous body point allowed calculating the position of the next body axis point. This analysis was repeated 10 times, providing a set of position vectors for 11 arbitrary equidistant segments of the body on every video frame. The angle between the point corresponding to segment i (Pi) and another point Pj with respect to a straight line defined by Pi and Pi−1 provided a measure of local bending of the body between segments i and j. The angle signal was filtered using a band-pass filter based on moving averages similar to that for respiration (bandwidth, 0.5–6 Hz).

Resetting of respiratory rhythm

Whether electrical stimulation in the MLR induced phase shifts in the respiratory activity was tested by using an analysis similar to that of Morin and Viala (5). Phase shift was obtained by calculating a prediction of the respiratory period based on the mean period of the three preceding respiratory bursts (Pm; A3 Supplementary Figure 4B). The

shifted period (Ps) was obtained by measuring the period of the cycle modified by the stimulation. Dividing by Pm the difference between Ps and Pm provided a measure of how much the phase was shifted on a −1 to 0 scale (A3 Supplementary Figure 4B, y axis). The phase of the stimulus was obtained by calculating L divided by Pm, where L is the latency between the stimulation and the preceding respiratory burst (A3 Supplementary Figure 4B, x axis).

Histology and Axonal Tracing Experiments

In some preparations, electrolytic lesions were made in the MLR at the end of the electrophysiological experiments to confirm the exact position of the stimulation. The brain tissue was rapidly fixed by immersion in PBS solution (0.1 M, pH 7.4) containing 4% paraformaldehyde (Fisher Scientific) for 24 h at 4 °C. The tissue was then transferred in phosphate buffer (0.1 M, pH 7.4) containing 20% sucrose overnight at 4 °C. The next day, the tissue was quickly frozen at 50 °C in 2-methylbutane (Fisher Scientific) and cut transversally with a cryostat at 25 μm thickness. The transverse sections were mounted on ColorFrost Plus slides (Fisher Scientific), washed in PBS solution, stained with H&E, dehydrated in increasing concentrations of alcohol, cleared in xylene, and mounted with Entellan (EMD Chemicals).

In experiments in which patch-recorded cell projections were traced, the patch electrodes used contained 0.1% biocytin. The preparations were perfused with Ringer solution for 24 h after the experiment to allow biocytin to fill the injected neuron. The preparations were then fixed in paraformaldehyde, transferred in sucrose, and cut as

described earlier. Biocytin was then revealed by using Alexa Fluor 488-conjugated streptavidin (Invitrogen) diluted (1:200) in PBS solution for 60 min at room temperature. The sections were then mounted with Vectashield/DAPI and observed and photographed with an Eclipse 600 microscope equipped with a DXM1200 video camera (Nikon).

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Discussion

Nous avons caractérisé une nouvelle population de neurones localisée dans la région du pons qui génère la respiration chez la lamproie, le pTRG. De plus, nous avons caractérisé une nouvelle population de neurones localisée dans la MLR qui projette directement vers le pTRG et les motoneurones respiratoires. Nous avons par la suite montré que le blocage de la région dorsale de la MLR, où les neurones projetant vers les centres respiratoires sont situés, réduit considérablement l'augmentation de l'activité respiratoire reliée à la locomotion. Ces résultats montrent qu'il existe des connexions directes entre la MLR et les centres respiratoires et qu'une région spécifique de la MLR joue un rôle essentiel dans les changements respiratoires reliés à la locomotion.

Bilatéralité des connexions dans le réseau respiratoire

D'abord, nous avons caractérisé les cellules du pTRG projetant vers les motoneurones respiratoires. En enregistrant ces neurones sur des préparations de tronc cérébral entier, nous avons pu injecter de la biocytine et suivre l'ensemble des projections de ces neurones dans le tronc cérébral. Ces neurones possèdent des arborisations dendritiques qui s'étendent dans la partie ventro-latérale du pons. Nous avons montré que ces neurones projettent vers trois sites distincts : le pTRG contralatéral, la colonne de motoneurones ipsilatérale et la colonne de motoneurones contralatérale. Ces résultats suggèrent que ces neurones pourraient occuper un rôle à la fois dans le contrôle descendant et dans la synchronisation bilatérale du rythme respiratoire. L'un des résultats les plus marquants est qu'une grande proportion des neurones du pTRG qui ont été

enregistrés possédait les trois types de projections. Nous avons aussi montré que ces neurones répondent à une stimulation du pTRG contralatéral, suggérant un fort couplage entre les cellules du générateur des deux côtés du cerveau. Le type de synapses permettant ce couplage bilatéral reste à déterminer. Il reste aussi à déterminer si ces neurones n'occupent qu'un rôle de relai du rythme respiratoire ou s'ils participent eux-mêmes à la genèse du rythme. La technique utilisée pour marquer ces neurones fait en sorte que seuls les neurones projetant vers les motoneurones ont été marqués et enregistrés. À l'avenir, il faudra déterminer si ces neurones constituent le cœur du générateur, ou s'ils sont entourés d'autres neurones qui pourraient être responsables de la genèse du rythme. Il a déjà été montré chez le rat que des cellules respiratoires peuvent posséder des projections descendantes vers les pools de motoneurones ainsi que des projections bilatérales vers les régions du bulbe et du pons impliquées dans la genèse respiratoire (Ezure et al., 2003). Certains des neurones excitateurs participant à la genèse de la locomotion dans la moelle épinière de la lamproie présentent des similarités avec les neurones du pTRG – ils projettent vers le côté controlatéral de la moelle et possèdent aussi des projections descendantes activant les motoneurones ipsilatéraux caudaux (Buchanan et Grillner, 1987; Buchanan et al., 1989). Il serait intéressant de déterminer si les cellules du pTRG possèdent des similarités pharmacologiques avec les cellules du CPG locomoteur. Entre autres, certains neurones impliqués dans la locomotion montrent des oscillations intrinsèques en présence de NMDA, ce qui pourrait contribuer à leurs propriétés rythmogènes (Grillner et Wallén, 1985).

Modulation de la respiration par la MLR

Nous avons montré que des neurones individuels dans la MLR possèdent une arborisation axonale très complexe qui s'étend sur plusieurs noyaux respiratoires du tronc cérébral et bilatéralement. Il était connu depuis les travaux pionniers d'Eldridge et al. (1981) que la stimulation des centres locomoteurs supra-spinaux induisait une augmentation de la respiration. Trois questions fondamentales demeuraient malgré tout. D'abord, les centres locomoteurs influencent-ils la respiration par des connexions directes? Sachant que les centres locomoteurs activent la locomotion par une série de relais qui implique les neurones de commande de la formation réticulée dans le tronc cérébral ainsi que les neurones des CPGs de la moelle épinière (Le Ray et al., 2003; Brocard et Dubuc, 2003; Dubuc, 2009), la possibilité demeurait que certaines des régions locomotrices localisées en aval de la MLR puissent influencer le rythme respiratoire par des projections vers les centres respiratoires. Ensuite, y a-t-il dans les centres locomoteurs des populations de neurones spécifiques pour le contrôle de la respiration et de la locomotion? Finalement, est-il possible d'abolir la contribution des centres locomoteurs aux changements respiratoires sans abolir leurs effets locomoteurs? La préparation de lamproie nous a offert des avantages cruciaux qui nous ont permis de nous attaquer à ces questions. D'abord, les préparations de tronc cérébral et de moelle épinière dans lesquelles les réseaux de neurones qui sous-tendent la respiration et la locomotion sont présents et fonctionnels sont rares. De plus, la possibilité d'enregistrer des neurones par des électrodes de patch dans le tronc cérébral sans couper le tissu nerveux en tranches ou en bloc est aussi exceptionnelle. La technique utilisée pour accéder aux cellules de la MLR ou du pTRG consiste à couper une fine partie du tronc cérébral dorsal à l'aide d'un

vibratome, ce qui permet de préserver intacts le reste des réseaux de neurones importants pour la respiration et la locomotion. Cette technique s'inspire directement de celle utilisée par le Dr. Simon Alford, technique qui a été utilisée pour enregistrer d'autres neurones du réseau locomoteur (Smetana et al., 2010).

Les neurones de la MLR qui ont été identifiés dans notre étude ne possèdent pas de projections vers les centres locomoteurs. De plus, la population de neurones projetant vers les centres respiratoires est localisée plus dorsalement dans la MLR alors que les neurones projetant vers les centres locomoteurs sont localisés plus ventralement. Ces résultats suggèrent que des populations différentes de neurones au sein de la MLR pourraient contrôler spécifiquement la respiration et la locomotion. On savait déjà que le blocage de la transmission glutamatergique dans la MLR abolissait la nage (Derjean et al., 2010). Mes résultats montrent qu'une injection de bloqueurs glutamatergiques restreinte à la partie dorsale de la MLR abolit une majeure partie des changements respiratoires reliés à la locomotion.

Nous avons montré par des enregistrements de type patch qu'une stimulation de la MLR induit des réponses excitatrices glutamatergiques dans les neurones respiratoires du pTRG. Ces réponses ont été bloquées par du CNQX et de l'AP-5. Ces résultats montrent l'existence d'une connexion glutamatergique activant des récepteurs ionotropiques sur les cellules du pTRG. Ils évoquent aussi la possibilité que l'augmentation de la fréquence des bouffées respiratoires lors de l'activation de la MLR puisse être induite par l'activation de récepteurs de type AMPA et NMDA et par la dépolarisation des neurones du pTRG qui s'en suit. Malheureusement, cette hypothèse n'est pas facilement testable. D'abord il faut noter que les récepteurs glutamatergiques

contribuent suffisamment à l'excitabilité du générateur pour être considérés essentiels à la genèse respiratoire de base, du moins en condition normal. Il a été montré chez la lamproie comme chez les mammifères (Bongianni et al., 1999; Shao et Feldman, 2001; Mutolo et al., 2005; Martel et al., 2007) que le blocage des récepteurs glutamatergiques ionotropes arrête la genèse de rythme respiratoire. La transmission glutamatergique ionotrope peut donc être considérée comme essentielle à la genèse du rythme. Par conséquent, il est impossible de bloquer les récepteurs ionotropes pendant la locomotion afin de mesurer la contribution relative des récepteurs glutamatergiques à l'augmentation de la fréquence puisque l'application des bloqueurs abolit instantanément le rythme respiratoire, rendant impossible l'étude de sa modulation.

Il y a cependant des méthodes indirectes qui suggèrent que la modulation du rythme respiratoire par les récepteurs glutamatergiques est du moins plausible. D'abord nous avons montré chez la lamproie qu'une injection localisée d'AMPA ou de NMDA, des agonistes des récepteurs glutamatergiques ionotropes, dans le pTRG est suffisante pour augmenter le rythme respiratoire à des fréquences similaires à celles observées pendant la locomotion (Bongianni et al., 1999; Martel et al., 2007). De plus, des antagonistes des mêmes récepteurs ralentissent ou abolissent le rythme respiratoire (Bongianni et al., 1999; Martel et al., 2007). Il a aussi été montré que le blocage des récepteurs AMPA chez le rat néonatal induit une réduction dose-dépendante de la fréquence respiratoire (Greer et al., 1991; Funk et al., 1993). Ces données suggèrent que les récepteurs glutamatergiques ionotropes peuvent participer à la modulation du rythme respiratoire, mais l'hypothèse selon laquelle ces récepteurs seraient impliqués dans l'augmentation respiratoire induite par l'activation de la MLR reste à déterminer. Parmi

les possibilités alternatives à considérer, il est possible que d'autres types de récepteurs sous-tendent l'effet respiratoire comme les récepteurs glutamatergiques métabotropiques, les récepteurs cholinergiques ou les récepteurs neurokinines de la substance P. L'activation de ces récepteurs augmente le rythme respiratoire chez les mammifères ainsi que chez la lamproie (Mironov et Richter, 2000; Lieske et Ramirez, 2006; Doi et Ramirez, 2008).

Bien que les effets de la MLR sur le système descendant de contrôle locomoteur étaient bien connus, mes travaux ont permis de comprendre les mécanismes neuronaux au sein même de la MLR qui permettent le contrôle de deux comportements distincts, la locomotion et la respiration. Ces travaux s'inscrivent dans une tendance plutôt récente suivie par d'autres laboratoires étudiant la lamproie qui vise à comprendre non seulement le contrôle des comportements au niveau des voies descendantes et spinales, mais à explorer les voies rostrales qui contrôlent ces comportements comme celles du diencéphale et du télencéphale (Derjean et al., 2010; Stephenson-Jones et al., 2011; Stephenson-Jones et al., 2012a; Stephenson-Jones et al., 2012b).

Conclusions et perspectives

Finalement, mes travaux ont permis de caractériser un nouveau groupe de neurones dans le générateur de la respiration chez la lamproie. De plus, nos résultats suggèrent que les changements respiratoires reliés à la locomotion sont induits par une population spécifique de neurones du mésencéphale qui projette vers le générateur de la respiration. Ces résultats constituent un exemple de contrôle parallèle de deux activités

motrices différentes mais reliées l'une à l'autre d'une part par les besoins énergétiques imposés par la locomotion et d'autre part par le rôle primordial de la respiration dans le maintien des réserves d'oxygène du corps.

Certains aspects de la genèse du rythme pourront être explorés plus en détails par des travaux futurs. La préparation que nous avons développée permet les enregistrements du type patch des neurones du pTRG sur des cerveaux intacts, il sera donc possible d'étudier à la fois les propriétés intrinsèques de ces neurones ainsi que le rôle des connexions avec d'autres groupes de neurones dans la genèse du rythme respiratoire. Entre autres, les connexions bilatérales excitatrices entre les deux côtés du cerveau au niveau du pTRG pourraient contribuer au maintien du rythme. De plus, d'autres régions comme le générateur du rythme respiratoire lent, localisé caudalement dans le tronc cérébral, pourraient influencer le générateur. Des expériences pourraient être développées permettant de neutraliser l'activité de ces régions et d'évaluer l'impact de cette inactivation sur le rythme respiratoire et sur les activités de neurones du pTRG. Par exemple, nous avons montré que des bouffées respiratoires à basse fréquence sont interlacées avec le rythme respiratoire normal et arrêtent celui-ci lorsqu'elles s'activent (Martel et al., 2007). Celles-ci avaient aussi été observées précédemment (Rovainen, 1977; Thompson, 1985). Il serait intéressant de déterminer si l'arrêt du rythme respiratoire normal lors de ces grosses bouffées est dû à une inhibition du générateur respiratoire ou plutôt à une surexcitation de celui-ci par des connexions excitatrices.

Plusieurs questions pourront être explorées quant au contrôle des populations de neurones de la MLR. D'abord, il serait intéressant de connaître le rôle du tubercule postérieur dans l'activation des cellules de la MLR. L'une des questions importantes sera

de savoir si les populations de neurones reliés aux centres locomoteurs et respiratoires peuvent être contrôlées indépendamment par les voies rostrales ou si ces voies projettent plutôt vers les deux populations de manière semblable. Ces travaux pourraient nous permettre de comprendre les mécanismes par lesquels la respiration peut être modifiée avant le début de la locomotion. Le rôle des voies supérieures, incluant celui des ganglions de la base, dans le contrôle des voies descendantes pourrait reposer en partie sur le relai de la MLR, et le rôle de cette voie dans la genèse de comportements dynamiques dans des contextes naturels tels que lors de la reproduction, de la migration, et de la prédation reste à explorer (Derjean et al., 2010; Stephenson-Jones et al., 2011; Stephenson-Jones et al., 2012a; Stephenson-Jones et al., 2012b). Il faudra aussi déterminer les neurotransmetteurs impliqués dans l'augmentation du rythme. Des bloqueurs des récepteurs cholinergiques, par exemple, pourraient être appliqués pour évaluer la contribution de ces récepteurs à l'augmentation du rythme respiratoire.

Finalement, le modèle de lamproie nous a permis une étude approfondie de cellules impliquées dans la genèse et la modulation du rythme respiratoire. L'un des avantages de la préparation de lamproie réside dans le fait que des neurones peuvent être enregistrés en configuration patch alors qu'une grande partie des réseaux neuronaux demeurent intacts et fonctionnels. Cela permettra une étude des liens qui pourraient exister entre les propriétés intrinsèques des neurones respiratoires et les connexions présentes au sein du générateur et avec d'autres régions du tronc cérébral. Les recherches effectuées tant chez la lamproie que chez d'autres vertébrés comme les poissons, amphibiens et mammifères nous renseigneront sur comment les réseaux de neurones du

tronc cérébral génèrent la respiration et pourraient aussi nous permettre de comprendre les modifications qu'ont subies ces réseaux au cours de l'évolution.

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