THIAGO DOS SANTOS MOREIRA

Integração de mecanismos cardiovasculares e respiratórios na região bulbar

Tese apresentada à Universidade Federal de São Paulo – Escola Paulista de Medicina para obtenção do título de Doutor em Ciências

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A ciência é composta de erros que, por sua vez, são os passos até a verdade

Issac Newton

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Indice

Abreviações

A1, A2, A5, A6: Grupamento noradrenérgico A1, A2, A5, A6.
APC: Área pressora caudal.
bpm: Batimento por minuto.
BDA: Amina dextrana biotinilada.
Böt: Região Bötzinger.
C1: Grupamento adrenérgico C1.
CO_2: Dióxido de carbono.
CVL: Região caudoventrolateral.
CRV: Coluna respiratória ventral.
cVRG: Grupamento respiratório ventro-caudal.
FC: Frequência cardíaca.
GABA: Ácido δ-aminobutírico.
GAD: Glutamina ácido descarboxilase.
Itr: Região inter trigeminal.
KF: Kölliker-Fuse.
mmHg: Milímetro de mercúrio.
mM: Milimolar.
Mo5: Núcleo motor do trigêmio.
nA: Núcleo Ambígus.
NPCV: Neurônios pré-ganglionares cardio-vagais.
NPBL: Núcleo parabraquial lateral.
NTS: Núcleos do trato solitário.
NTScom: Núcleo do trato solitário comissural.
NRL: Núcleo reticular lateral.
NRT: Núcleo retrotrapezóide.
NSPG: Neurônio simpático pré-ganglionar
O_2: Oxigênio.
PAM: Pressão arterial média.
pCO_2: Pressão parcial de CO_2.
Pn: Núcleo pontino.
pO_2: Pressão parcial de O_2.
pre-BötC: Complexo pré-Bötzinger.
RVL: Região rostroventrolateral.
rVRG: Grupamento respiratório ventro-rostral.
Scp: Peduncúlo cerebelar superior.
SNC: Sistema nervoso central.
SO: Oliva superior.
VGLUT: Vesícula para transporte de glutamato.
VlPon: Ponte ventrolateral.
5n: Quinto nervo motor.
7n: Sétimo nervo motor.
7: Núcleo motor do facial.

Resumo

A presente tese de doutorado vai tentar elucidar os mecanismos excitatórios do bulbo relacionados ao controle cardiovascular e respiratório. Para tanto serão discutidos 4 trabalhos recentemente publicados (controle cardiovascular: Moreira e cols., 2005 e 2007; controle cardio-respiratório: Takakura e cols., 2006; Moreira e cols., 2006).

Trabalhos 1 e 2: <u>Relacionado aos mecanismos glutamatérgicos responsáveis pelo controle cardiovascular</u>.

No presente estudo, procuramos investigar os efeitos cardiovasculares promovidos pela inibição da região caudoventrolateral (CVL) do bulbo com a injeção bilateral de muscimol, juntamente com o bloqueio glutamatérgico dos núcleos do trato solitário (NTS) (região postremal ou comissural) com a injeção de ácido quinurênico (antagonista de receptores glutamatérgicos). Foram utilizados ratos Holtzman, anestesiados com uretana e alfa-cloralose e registrou-se a pressão arterial média (PAM), frequência cardíaca (FC) e fluxo sanguíneo para os leitos renal, mesentérico e aórtico. A injeção bilateral de muscimol (agonista GABAérgico do sub-tipo A) (2 mM) na região do CVL ou a injeção de ácido quinurênico (50 mM) no NTS postremal produzem aumento de PAM (186 ± 11 ou 142 ± 6 mmHg, respectivamente, vs. controle: 105 ± 4 mmHg), de FC (407 ± 15 ou 412 ± 18 bpm, respectivamente, vs. controle: 352 ± 12 bpm) e aumento de resistência vascular renal, mesentérica e aórtica. A injeção de ácido quinurênico no NTS comissural (NTScom) não produz alterações nos valores basais da PAM, FC e resistência vascular. Entretanto, em animais com a injeção bilateral de muscimol no CVL, a injeção de ácido quinurênico no NTS (postremal ou comissural) reduziu a PAM e resistência vascular para valores abaixo do valor controle. Do mesmo modo, em ratos com o bloqueio do NTS (postremal ou comissural), a subsequente inibição do CVL produziu também uma redução da PAM e resistência vascular para valores abaixo do valor controle. Em animais com lesão eletrolítica da região do NTScom, a injeção de muscimol na região do CVL

produziu redução da PAM e resistência vascular para valores abaixo do valor controle.

Dinte disso, os resultados sugerem a existência de importantes mecanismos excitatórios no NTS (região postremal e comissural) e no CVL para controle da pressão arterial e resistência vascular.

Trabalhos 3 e 4: <u>Relacionado aos mecanismos bulbares ativados pelos quimiorreceptores centrais no controle cardio-respiratório</u>.

Trabalho 3: Interação entre quimiorreceptores centrais e periféricos.

O núcleo retrotapezóide (NRT) contém neurônios sensíveis ao pH e são característicos como sendo quimiorreceptores centrais. A hipótese desse trabalho é procurar mostrar que a ativação dos quimiorreceptores periféricos promove a ativação dos quimiorreceptores centrais no NRT e que essa ativação é mediada por uma via direta do NTScom para o NRT. Nesse estudo foram utilizados ratos Sprague-Dawley.

Uma densa projeção do NTScom para a região do NRT foi mostrada com o uso de traçadores anterógrados (amina dextrana biotinilada - BDA) injetados por iontoforese na região do NTScom. Na região do NRT, mais de 50% dos terminais que expressam BDA contêm a vesícula de transporte para glutamato (VGLUT2), mas apenas 5% contêm glutamina ácido descarboxilase (GAD67). Em um outro grupo de animais, uma semana após a injeção do traçador retrógrado cólera toxina B (CTB) no NRT, os animais foram expostos a períodos de hipóxia por 3 horas. A presença da proteína Fos serviu para identificar os neurônios que eram ativados pela hipóxia. Neurônios imunorreativos para Fos e CTB foram encontrados na região do NTScom. A grande maioria dos neurônios que eram imunorreativos para Fos e CTB, expressavam VGLUT2 mRNA, enquanto uma minoria expressou GAD67 mRNA.

Na segunda parte do estudo foram utilizados animais anestesiados com uretana e alfa-cloralose e vagotomizados. A injeção bilateral de muscimol no NRT promoveu a eliminação do nervo frênico durante uma situação controle, durante a

estimulação dos quimiorreceptores centrais (hipercapnia – 10% de CO_2) e durante a estimulação dos quimiorreceptores periféricos (injeção endovenosa de cianeto de sódio ou hipóxia (10-15% de O_2).

A atividade dos neurônios quimiossensíveis do NRT foram registrados em animais intactos ou vagotomizados para possíveis comparações. Todos os neurônios quimiossensíveis do NRT foram ativados pelos quimiorreceptores periféricos. A ativação dos quimiorreceptores periféricos não promoveu a ativação dos neurônios do NRT em animais com desnervação dos quimiorreceptores.

A injeção bilateral de muscimol na região do CVL promoveu a eliminação do nervo frênico e a modulação respiratória nos neurônios do NRT. A injeção de muscimol não alterou o limiar e a sensibilidade dos neurônios do NRT durante uma situação de hipercapnia e também durante a ativação dos quimiorreceptores periféricos.

Portanto, podemos concluir nesse estudo que os neurônios do NRT respondem às variações de CO_2 devido a presença de 2 mecanismos: sua quimiossensibilidade intrínseca e por meio da ativação dos quimiorreptores periféricos (via glutamatérgica direta do NTScom para o NRT).

Trabalho 4: Quimiorreceptores centrais e atividade simpática.

O próximo estudo procurou explorar como elevações na pCO_2 pode aumentar a atividade simpática. Foram registrados a atividade simpática eferente do nervo esplâncnico, atividade do nervo frênico e atividade dos neurônios vasomotores da região rostroventrolateral (RVL) do bulbo. Os experimentos foram realizados em ratos Sprague-Dawley, anestesiados com halotana e completamente desnervados. Hipercapnia (variação do CO_2 expirado de 5 para 10%) promoveu aumento da atividade simpática (97 ± 6%) e da atividade dos neurônios do RVL (67 ± 4%). A injeção bilateral de ácido quinurênico no RVL ou no NRT eliminou ou reduziu a atividade do nervo frênico, respectivamente, mas não alterou a atividade simpática eferente produzida pela hipercapnia. A injeção bilateral de ácido quinurênico ou muscimol na região do CVL eliminou a atividade do nervo frênico e promoveu um aumento do efeito estimulatório do CO_2 na

atividade simpática. A injeção de muscimol na região comissural do NTS não alterou as respostas estimulatórias do CO_2 sobre a atividade simpática e atividade do nervo frênico. Como esperado, a injeção de ácido quinurênico no RVL ou a injeção de muscimol no NTS comissural, em ratos intactos, bloqueou os efeitos cardio-respiratórios da estimulação dos quimiorreceptores periféricos com a injeção endovenosa de cianeto de sódio.

Em conclusão, a hipercapnia aumentou a atividade simpática por ativação dos neurônios bulbo-espinais do RVL. Os neurônios excitatórios do RVL podem ser sensíveis a variações de CO_2 e/ou receber projeções excitatórias dos quimiorreceptores centrais da região do NRT. Durante uma situação de hipercapnia, o total de aumento da atividade simpática produzido pela ativação dos quimiorreceptores centrais parece estar reduzido. Esse efeito é, portanto, mediado pela ativação de neurônios barossensíveis da região do CVL ou, então, pela inibição dos quimiorreceptores centrais do NRT.

Abstract

The present thesis will try to elucidate important excitatory mechanisms in the brainstem related to cardio-respiratory control. We will discuss 4 recently published papers (cardiovascular control: Moreira e cols., 2005 e 2007; cardio-respiratory control: Takakura e cols., 2006; Moreira e cols., 2006).

Papers 1 and 2: <u>Glutamatergic mechanisms related to cardiovascular control</u>.

In the present study, we investigated the effects of inhibition of the caudal ventrolateral medulla (CVLM) with the GABA(A) agonist muscimol combined with the blockade of glutamatergic mechanism in the nucleus of the solitary tract (NTS) with kynurenic acid (kyn) on mean arterial pressure (MAP), heart rate (HR), and regional vascular resistances. In male Holtzman rats anesthetized intravenously with urethane/chloralose, bilateral injections of muscimol (120 pmol) into the CVLM or bilateral injections of kyn (2.7 nmol) into the NTS alone increased MAP to 186 ± 11 and to 142 ± 6 mmHg, respectively, vs. control: 105 ± 4 mmHg; HR to 407 ± 15 and to 412 ± 18 beats per minute (bpm), respectively, vs. control: 352 ± 12 bpm; and renal, mesenteric and hindquarter vascular resistances. However, in rats with the CVLM bilaterally blocked by muscimol, additional injections of kyn into the NTS reduced MAP to 88 ± 5 mmHg and mesenteric and hindquarter vascular resistances below control baseline levels. Moreover, in rats with the glutamatergic mechanisms of the NTS blocked by bilateral injections of kyn, additional injections of muscimol into the CVLM also reduced MAP to 92 ± 2 mmHg and mesenteric and hindquarter vascular resistances below control baseline levels. Simultaneous blockade of NTS and CVLM did not modify the increase in HR but also abolished the increase in renal vascular resistance produced by each treatment alone.

The results suggest that important pressor mechanisms arise from the NTS and CVLM to control vascular resistance and arterial pressure under the conditions of the present study.

Papers 3 and 4: <u>Central chemoreceptors brainstem mechanisms related to cardio-respiratory control</u>.

Paper 3: Interaction between central and peripheral chemoreceptors.

The rat retrotrapezoid nucleus (RTN) contains pH-sensitive neurons that are putative central chemoreceptors. Here, we examined whether these neurons respond to peripheral chemoreceptor stimulation and whether the input is direct from the solitary tract nucleus (NTS) or indirect via the respiratory network. A dense neuronal projection from commissural NTS (commNTS) to RTN was revealed using the anterograde tracer biotinylated dextran amine (BDA). Within RTN, 51% of BDA-labelled axonal varicosities contained detectable levels of vesicular glutamate transporter-2 (VGLUT2) but only 5% contained glutamic acid decarboxylase-67 (GAD67). Awake rats were exposed to hypoxia (n = 6) or normoxia (n = 5) 1 week after injection of the retrograde tracer cholera toxin B (CTB) into RTN. Hypoxia-activated neurons were identified by the presence of Fos-immunoreactive nuclei. CommNTS neurons immunoreactive for both Fos and CTB were found only in hypoxia-treated rats. VGLUT2 mRNA was detected in 92 ± 13% of these neurons whereas only 12 ± 9% contained GAD67 mRNA.

In urethane-chloralose-anaesthetized rats, bilateral inhibition of the RTN with muscimol eliminated the phrenic nerve discharge (PND) at rest, during hyperoxic hypercapnia (10% CO_2), and during peripheral chemoreceptor stimulation (hypoxia and/or i.v. sodium cyanide, NaCN). RTN CO_2-activated neurons were recorded extracellularly in anaesthetized intact or vagotomized rats. These neurons were strongly activated by hypoxia (10-15% O_2; 30 s) or by NaCN. Hypoxia and NaCN were ineffective in rats with carotid chemoreceptor denervation. Bilateral injection of muscimol into the ventral respiratory column 1.5 mm caudal to RTN eliminated PND and the respiratory modulation of RTN neurons. Muscimol did not change the threshold and sensitivity of RTN neurons to hyperoxic hypercapnia nor their activation by peripheral chemoreceptor stimulation.

In conclusion, RTN neurons respond to brain pCO_2 presumably via their intrinsic chemosensitivity and to carotid chemoreceptor activation via a direct

glutamatergic pathway from commNTS that bypasses the respiratory network. RTN neurons probably contribute a portion of the chemical drive to breathe.

Paper 4: Central chemoreceptors and sympathetic vasomotor outflow.

The present study explores how elevations in brain pCO_2 increase the sympathetic nerve discharge (SND). SND, phrenic nerve discharge (PND) and putative sympathoexcitatory vasomotor neurons of the rostral ventrolateral medulla (RVLM) were recorded in anaesthetized sino-aortic denervated and vagotomized rats. Hypercapnia (end-expiratory CO_2 from 5% to 10%) increased SND (97 ± 6%) and the activity of RVLM neurons (67 ± 4%). Injection of kynurenic acid (Kyn, ionotropic glutamate receptor antagonist) into RVLM or the retrotrapezoid nucleus (RTN) eliminated or reduced PND, respectively, but did not change the effect of CO_2 on SND. Bilateral injection of Kyn or muscimol into the rostral ventral respiratory group (rVRG-pre-Botzinger region, also called CVLM) eliminated PND while increasing the stimulatory effect of CO_2 on SND. Muscimol injection into commissural part of the solitary tract nucleus (commNTS) had no effect on PND or SND activation by CO_2. As expected, injection of Kyn into RVLM or muscimol into commNTS virtually blocked the effect of carotid body stimulation on SND in rats with intact carotid sinus nerves.

In conclusion, CO_2 increases SND by activating RVLM sympathoexcitatory neurons. The relevant central chemoreceptors are probably located within or close to RVLM and not in the NTS or in the rVRG-pre-Botzinger/CVLM region. RVLM sympathoexcitatory neurons may be intrinsically pH-sensitive and/or receive excitatory synaptic inputs from RTN chemoreceptors. Activation of the central respiratory network reduces the overall sympathetic response to CO_2, presumably by activating barosensitive CVLM neurons and inhibiting RTN chemoreceptors.

Introdução

O sistema nervoso neurovegetativo é, tradicionalmente, descrito como um sistema motor que promove o controle das funções viscerais que são críticas para o controle da homeostasia. Esse sistema consiste em uma coleção de aferências e eferências que permite a conexão entre o sistema nervoso central (SNC) e os orgãos viscerais (Blessing, 1997; Loewy e Spyer, 1990). O sistema nervoso neurovegetativo possui dois grandes ramos eferentes que são classificados como sistema nervoso simpático e o parassimpático, os quais permitem o controle de várias funções fisiológicas. Esse controle, na maioria dos casos, é antagônico nas suas funções. Um exemplo clássico é a inervação para o coração. A estimulação simpática causa taquicardia, enquanto que a estimulação parassimpática promove bradicardia (Dampney, 1994; Guyenet, 2006).

Da mesma maneira, o controle neural do sistema cardiovascular é regulado pelo sistema nevoso neurovegetativo (Blessing, 1987; Loewy e Spyer, 1990). A regulação neural do sistema cardiovascular envolve a ativação de diferentes grupos de sensores periféricos (barorreceptores, receptores cardiopulmonares e quimiorreceptores, entre outros), os quais projetam suas aferências para estruturas do SNC via nervos vago e glossofaríngeo. O processamento destas informações aferentes no SNC irá resultar na modulação das vias autonômicas eferentes, controlando as variáveis cardiovasculares no sentido de manter a homeostase nas diversas situações comportamentais em que os mamíferos são submetidos. No sistema cardiovascular, as variáveis reguladas pelo sistema nervoso neurovegetativo são a freqüência cardíaca, o volume sistólico (força de contração) e a resistência periférica, as quais são determinantes da pressão arterial.

O envolvimento do SNC na manutenção da homeostase do sistema cardiovascular é conhecido desde o século XIX. Os estudos clássicos realizados por Claude Bernard, na França, e publicados em 1863 (apud Gebber, 1990), já mostravam que a transecção da medula espinal cervical em qualquer nível produzia pronunciada queda da PA. No entanto, foram os estudos de Dittmar, em

1873 (apud Gebber, 1990), que primeiro demonstraram a participação do bulbo na manutenção da pressão arterial, sobretudo as porções ventrolaterais bulbares. Nesses estudos, foi observado que a destruição da região ventral do bulbo produzia queda da pressão arterial, o mesmo não acontecia se a destruição fosse realizada na região dorsal. Disso surgiu a hipótese de que a região ventral do bulbo exerceria fundamental importância na manutenção dos níveis considerados normais de pressão arterial, e foi introduzido na época o conceito de tônus vasomotor simpático.

Posteriormente, Bayliss lançou a hipótese de que existiria um centro vasomotor, localizado na região ventral do bulbo e que seria constituído por áreas tanto pressoras como depressoras (Bayliss, 1901).

Mais tarde, o aparecimento da técnica de estereotaxia associada à eletrofisiologia (estimulação elétrica) possibilitou o mapeamento de todo o tronco cerebral e foram identificadas regiões pressoras e depressoras situadas em toda a formação reticular bulbar, que se estendiam desde a região dorsal até a ventral do bulbo (Wang & Ranson, 1939; Alexander, 1946). A idéia de um centro vasomotor simpático com localização delimitada, como proposto por Dittmar passou então a ser desacreditada. Entretanto, os dados obtidos utilizando-se da técnica de estimulação elétrica, que reconhecidamente interfere não só sobre os corpos celulares dos neurônios, como também sobre fibras de passagem, não permitia delimitar com exatidão a localização das regiões envolvidas no controle cardiovascular.

Os estudos de Feldberg & Guertzenstein (1972) e Guertzenstein (1973) foram os primeiros a demonstrar a existência da atividade tônica de neurônios localizados na superfície ventral do bulbo rostral de gatos sobre a manutenção da pressão arterial. Inicialmente, eles demonstraram que a administração intracerebroventricular de pentobarbital sódico produzia queda da pressão arterial; a mesma dose administrada endovenosamente ou quando o aqueduto cerebral que liga o terceiro ao quarto ventrículo era obstruído, a hipotensão desaparecia, sugerindo que o pentobarbital atuava na região bulbar (Feldberg & Guertzenstein,

1972). Os dois autores utilizaram-se de drogas que agem, preferencialmente, em corpos celulares, como o ácido gama-aminobutírico (GABA) e glicina que, ao serem aplicados topicamente por meio de anéis plásticos sobre a região estudada (superfície ventral bulbar rostral), produziam acentuada queda da pressão arterial. Foi também demonstrado que a aplicação unilateral de glicina causava uma queda muito maior, atingindo níveis considerados espinais agudos, ou seja, níveis de pressão arterial por volta de 60 mmHg (Guertzenstein & Silver, 1974). Com tais experimentos, foi possível localizar mais precisamente a região bulbar ventral responsável pela manutenção da pressão arterial. Novamente, a idéia de um centro vasomotor delimitado voltou a ser considerada.

A partir das publicações de Guertzenstein e colaboradores (1973, 1974, 1977 e 1984), diferentes grupos de pesquisa começaram a enfocar a participação do bulbo ventrolateral no controle cardiovascular, na tentativa de melhor compreender o papel desta região, não somente na manutenção do tônus vasomotor simpático, mas também no que diz respeito ao seu papel no controle reflexo cardiovascular e ajustes cardiovasculares específicos (Dampney, 1994; Guyente, 2006). Até então, ou seja, em meados de 1970, os núcleos do trato solitário (NTS) localizados na região dorsal do bulbo eram conhecidos como o maior centro integrador cardiovascular, particularmente por receber as informações dos aferentes barorreceptores. Outra região considerada importante era a coluna intermédio-lateral (IML) da medula espinal, pois era sabido que esta região contém os neurônios efetores simpáticos pré-ganglionares. No entanto, o centro responsável pela conexão NTS-IML, ou seja, o centro vasomotor tinha localização indefinida (Chalmers e Pilowsky, 1991). Dessa forma, sobretudo os estudos de Guertzenstein e colaboradores foram fundamentais no sentido de delimitar um centro vasomotor simpático integrador localizado no bulbo ventral rostral e de fundamental importância para manutenção da homeostase cardiovascular.

Atualmente sabe-se que o bulbo participa no controle cardiovascular, gerando sinais que são continuamente modulados pelas informações aferentes cardiovasculares (Spyer, 1990). Os nervos depressor aórtico e do seio carotídeo,

cujas aferências têm acesso ao SNC através dos nervos vago e glossofaríngeo, respectivamente, são responsáveis pela condução das informações aferentes dos baro- e quimiorreceptores para o SNC, mais precisamente no bulbo, como parte integrante na regulação cardiovascular. Os corpos celulares destas fibras vagais e glossofaríngeas estão localizados nos gânglios nodoso e petroso, respectivamente, tendo suas terminações no NTS (Palkovitz e Zarborsky, 1977). A primeira sinapse ou o sítio de terminação destas fibras aferentes no NTS já foi investigado anatômica (Torvik, 1956; Cottle, 1964; Palkovits e Zarborsky, 1977) e eletrofisiologicamente (Fussey e cols., 1967; Jordan e Spyer, 1977; Jordan e Spyer, 1978), bem como a expressão da proteína Fos em neurônios do NTS, após a estimulação dos baro- e quimiorreceptores (Erickson e Millhorn, 1991; Weston e cols., 2003; Takakura e cols., 2006) em várias espécies.

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O NTS é constituído por grupos heterogêneos de neurônios, que estão dispostos dorsalmente no bulbo e se estendem de forma rostrocaudal, como uma coluna bilateral, desde o nível do pólo caudal do núcleo motor do nervo facial até o óbex, onde as duas colunas se unem para formar uma única estrutura na linha média, que continua caudalmente até a parte caudal da decussação piramidal (Ciriello e cols, 1994).

Em relação ao seu aspecto antero-posterior, o NTS pode ser dividido em 3 porções, dada a sua proximidade com a área postrema (Cottle, 1964; Loewy, 1990): NTS rostral; NTS intermediário e NTS comissural.

O NTS intermediário e comissural estão diretamente envolvidos no controle cardiovascular e respiratório, pois todas as projeções aferentes vagais e glossofaríngeas, que conduzem informações cardio-respiratórias, fazem sua primeira sinapse nestas duas porções do NTS.

Sabe-se que a porção intermediária do NTS constitui o principal sítio de entrada das aferências dos barorreceptores, utilizando o L-glutamato como

principal neurotransmissor (Talman e cols., 1980; Weston e cols., 2003). Já a porção comissural do NTS constitui o principal sítio no SNC para o qual se projetam as aferências dos quimiorreceptores carotídeos (Chitravanshi e cols., 1993; Finley e Katz, 1992; Ciriello e cols., 1994). Curiosamente, a injeção de L-glutamato, no NTS intermediário, em animais não anestesiados promoveu resposta pressora (Machado e Bonagamba, 1992; Colombari e cols., 1994), diferentemente da resposta hipotensora produzida pela estimulação dos barorreceptores (Talman e cols., 1980; Ohta e Talman, 1994; Machado e Bonagamba, 1992). Como a resposta produzida pela injeção de L-glutamato no NTS intermediário é semelhante à resposta produzida pela ativação dos quimiorreceptores, uma possibilidade seria que o efeito da injeção de L-glutamato no NTS mimetizasse a ativação do quimiorreflexo (Haibara e cols., 1995). Em animais normotensos, a lesão do NTS comissural bloqueou a resposta pressora do L-glutamato injetado no NTS intermediário e a resposta pressora produzida pela estimulação dos quimiorreceptores periféricos com KCN (Colombari e cols., 1996). Esses resultados levaram à sugestão de que o NTS comissural possui importante participação na ativação do sistema nervoso simpático.

A partir do NTS, as informações provenientes dos receptores periféricos podem ser distribuídas para diversas áreas do SNC, dentre as quais destacam-se as áreas localizadas na região ventral do bulbo que controlam o tônus simpático sobre o sistema cardiovascular.

O sistema nervoso simpático permite o controle a curto e longo prazo da pressão arterial. O nível de atividade do sistema nervoso simpático é controlado por uma rede de neurônios localizados na medula espinal, na região rostroventrolateral (RVL) do bulbo, na região caudoventrolateral (CVL) do bulbo, nos NTS, bem como também em regiões hipotalâmicas (Dampney, 1994; Guyenet, 2006).

O sistema nervoso simpático eferente parece ser, primeiramente, regulado pela região RVL (Dampney e cols., 2002; Guyenet, 2006). Essa região do bulbo contém as células adrenérgicas C1 que, por definição, constitui uma das regiões

do SNC que sintetizam adrenalina (Hokfelt e cols., 1974). A partir do início dos anos 80, a porção da região ventrolateral do bulbo que se relacionava com os neurônios C1, foi definida como sendo o principal centro de regulação da pressão arterial (Brown e Guyenet, 1985; Ross e cols., 1985; Guyenet, 1990; Blessing, 1997; Schreihofer e Guyenet, 1997; Dampney e cols., 2002; Guyenet, 2006). Os neurônios do RVL, responsáveis pelo controle da pressão arterial e atividade simpática, projetam-se para os neurônios pré-ganglionares simpáticos na medula espinal (Guyenet, 2006). Esses neurônios do RVL liberam L-glutamato como neurotransmissor, mas também sintetizam vários outros neurotransmissores, incluindo por exemplo a adrenalina. Os neurônios que sintetizam a adrenalina correspondem a 70% do total de neurônios da região do RVL e são classificados como as células C1 (Ross e cols., 1985; Schreihofer e Guyenet, 1997; Schreihofer e cols., 2000). Entretanto, nem todos os neurônios C1 estão sobre o controle do barorreflexo; um exemplo clássico de neurônios C1 que não são controlados pelo barorreflexo consistem os neurônios que controlam a secreção de adrenalina das células cromafins (Cao e Morrison, 2001; Ritter e cols., 2001). Vale ressaltar ainda que alguns dos neurônios C1 do RVL também projetam-se para a região hipotalâmica (Verbernne e cols., 1999). Esses neurônios são diferentes daqueles que se projetam para a medula espinal, mas possuem características eletrofisiológicas e neuroquímicas muito semelhantes (Verbernne e cols., 1999). Muitos desses neurônios contribuem na modulação excitatória do barorreflexo para áreas hipotalâmicas (núcleo paraventricular e núcleo preóptico mediano) que estão envolvidas no balanço hidroeletrolítico (Verberne e cols., 1999).

Sabe-se que o sistema nervoso simpático sofre oscilações em sua atividade (aumentando e diminuindo) durante a manutenção da pressão arterial (Gebber, 1990; Guyenet 1990; Dampney, 1994; Guyenet, 2006). Essas oscilações são determinadas em grande escala pela atividade dos neurônios bulbo-espinais e excitatórios do RVL (Gebber, 1990; Guyenet, 2006). Apesar do controle da atividade simpática envolver a participação de várias regiões do SNC, em situações de anestesia, os neurônios do RVL aparecem como os principais reguladores da atividade simpática eferente (Dampney, 1994; Guyenet, 2006). Na

maioria das condições anestésicas, o neurotransmissor L-glutamato possui apenas uma pequena parcela de contribuição na atividade basal desses neurônios. Nessas condições, a injeção do antagonista de receptores glutamatérgicos, ácido quinurênico, na região do RVL não produz alterações na pressão arterial ou na atividade simpática (Guyenet e cols., 1987; Ito e Sved, 1997; Horiuchi e cols., 2004). Por outro lado, a transmissão glutamatérgica apresenta-se muito mais significante em algumas situações experimentais. O bloqueio glutamatérgico na região RVL produz alterações significantes na pressão arterial e na atividade simpática eferente em algumas situações peculiares como, por exemplo, em animais que sofreram desidratação extracelular, em animais que apresentam anormalidade nos gases sanguíneos (altos níveis de CO_2 e baixo níveis de O_2), quando reflexos excitatórios são ativados (quimiorreflexo) ou em situações de hipertensão (Guyenet e Stornetta, 2004; Sun, 1996; Brooks e cols., 2004; Guyenet , 2000; Ito e cols., 2000; Ito e cols., 2001). Esses resultados levam a conclusão de que a região do RVL possui um balanço entre os mecanismos excitatórios e inibitórios e que esse balanço estaria alterado em algumas situações experimentais.

Em resumo, a atividade dos neurônios do RVL aparece dependente do neurotransmissor L-glutamato em proporções que variam de acordo com as circunstâncias fisiológicas. Além do L-glutamato, existe uma lista de neurotransmissores que regulam a atividade dos neurônios do RVL, como por exemplo ácido γ-aminobutírico (GABA), acetilcolina, serotonina, oxitocina, angiotensina (Ang), substância P, vasopressina, orexina e também as catecolaminas (Guyenet e Stornetta, 2004).

A área RVL recebe informações de diferentes regiões do bulbo e da ponte. Algumas dessas informações são via região CVL, envolvendo a participação de neurônios GABAérgicos e a via do barorrefexo (Schreihofer e Guyenet 2003). O restante das informações recebidas pelo RVL originam-se da área pressora caudal (APC), de regiões mediais do bulbo (depressoras), de vários subnúcleos do NTS, da área depressora gigantocelular, da região A5 e do núcleo parabraquial lateral (Guyenet e cols., 1987; Koshiya e Guyenet, 1996a; Colombari e cols., 1996;

Blessing, 1997; Aicher e cols., 1999; Campos e McAllen, 1999; Moreira e cols., 2005). Essas regiões bulbares estão, provavelmente, envolvidas também em vários reflexos simpáticos somáticos e viscerais (reflexo pressor do exercício, reflexo nociceptor, reflexo cardiopulmonar e quimiorreflexo) (Campos e cols., 1994; Horiuchi e Dampney, 2002; Verberne e cols., 1999; Barman e cols., 2000; Guyenet, 2000; Colombari e cols., 2001).

A área RVL está sob forte influência de dois grandes reflexos cardio-respiratórios, o barorreflexo e o quimiorreflexo. O mecanismo neural relacionado ao baroreflexo, inclui os neurônios GABAérgicos da região CVL, que recebem informações excitatórias do NTS, e se projetam para os neurônios pré-simpáticos da região RVL (Chan e Sawchenko, 1998; Gordon e Sved, 2002). Os interneurônios GABAérgicos do CVL promovem uma intensa e contínua inibição sobre os neurônios pré-simpáticos do RVL (Schreihofer e Guyenet, 2002). Essa inibição pode também ser independente da via clássica do barorreflexo (Cravo e cols., 1991). Muitos dos neurônios do CVL possuem uma atividade basal mesmo sem a presença das aferências vagais e possuem, ainda, outras influências além do barorreflexo (Cravo e cols., 1991; Schreihofer e Guyenet, 2002). As vias neurais independentes do barorreflexo são, ainda, amplamente inexploradas levando-se em conta sua importância no controle a longo prazo da pressão arterial. A literatura sugere que o barorreflexo possui uma pequena influência no controle a longo prazo da pressão arterial (Osborn e cols., 2005). Esse ponto foi, originalmente, descrito por Cowley, mostrando que a completa remoção dos barorreceptores produzia apenas mudanças transientes na pressão arterial (24 hs) em cachorros acordados (Cowley, 1992).

Em paralelo ao mecanismo do baroreflexo, as aferências dos quimiorreceptores periféricos fazem sua primeira sinapse na região comissural do NTS (NTScom) (Blessing e cols., 1999; Takakura e cols., 2006). Muitos dos neurônios do NTScom que recebem informações dos quimiorreceptores periféricos projetam-se para a região ventrolateral do bulbo, mais especificamente, para região do RVL (neurônios C1) (Aicher e cols., 1996; Colombari e cols., 1996; Koshiya e Guyenet, 1996a; Paton e cols., 2001). A existência de um mecanismo

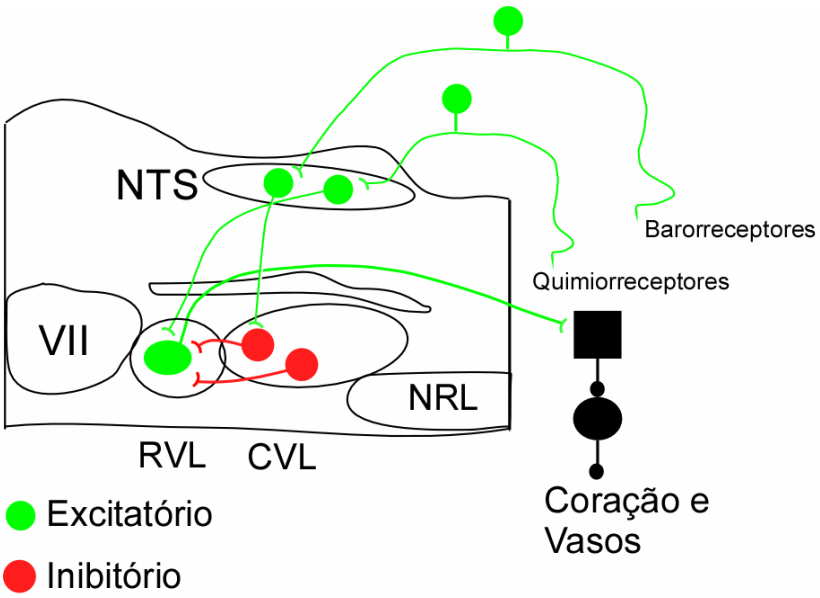
excitatório no NTScom foi demonstrado, primeiramente, em experimentos com animais anestesiados em que observou-se aumento de pressão arterial produzido pelo neurotrasmissor L-glutamato injetado no NTScom, após a inibição da região do CVL com muscimol (agonista GABA-A) (Urbanski e Sapru, 1988). Esse aumento de pressão arterial, produzido pelo L-glutamato no NTScom, foi abolido após o bloqueio prévio de receptores glutamatérgicos na região do RVL (Willette e cols., 1987, Urbanski e Sapru, 1988). Mais uma prova da existência de neurônios excitatórios do NTScom que se projetam para a região RVL foi realizada em animais com lesões eletrolíticas do NTScom (Colombari e cols., 1996). Nesses experimentos, a lesão do NTScom foi capaz de abolir a resposta pressora produzida pela estimulação dos quimiorreceptores periféricos, sem alterações no baroreflexo (Colombari e cols., 1996; Sato e cols., 1999). Evidências eletrofisiológicas também comprovaram que neurônios quimiossensíveis da porção comissural do NTS eram ativados antidromicamente pelo RVL, sugerindo que os neurônios do NTScom projetam-se para o RVL (Koshiya e Guyenet, 1996a). Estudos da literatura indicam que não apenas os neurônios do NTScom, mas também os neurônios do NTS postremal possam estar enviando projeções diretas para o RVL (Koshiya e Guyenet, 1996a; Moreira e cols., 2005, 2007).

Vale lembrar também que além das projeções inibitórias, estudos recentes têm sugerido que a região do CVL pode também enviar projeções excitatórias para a região do RVL (Ito e Sved, 1997; Natarajan e Morrison, 2000; Moreira e cols., 2005; 2007).

Embora os mecanismos bulbares referentes ao baro e quimiorreflexo estejam estabelecidos (Guyenet, 2006), as projeções excitatórias para os neurônios pré-simpáticos do RVL ainda não estão muito bem esclarecidas. O aumento da atividade simpática e da pressão arterial produzido pela inibição do CVL é sugerido pela desinibição da região do RVL. Entretanto, além das projeções inibitórias do CVL, a região RVL pode receber informações excitatórias da região do CVL e também do NTS (região adjacente à área postrema e comissural) (Moreira e cols., 2005; 2007). Contrariando a literatura, estudos de Ito e Sved (1997) mostraram que o bloqueio dos receptores glutamatérgicos na

região do RVL, associado com a inibição bilateral da região do CVL produziu redução da pressão arterial para valores similares ao bloqueio ganglionar com hexametônio (Ito e Sved, 1997). Esse trabalho sugere que a região do RVL recebe importantes informações excitatórias. Sabe-se que o bloqueio dos receptores glutamatérgicos, com ácido quinurênico, não altera os valores basais da pressão arterial, indicando que esse mecanismo excitatório parece não estar ativado em condições basais. (Ito e Sved, 1997). De acordo com Ito e Sved, em condições basais, os mecanismos excitatórios para o RVL é contrabalanceado pela atuação dos mecanismos inibitórios do CVL. Quando esse mecanismo inibitório é bloqueado (injeção de muscimol na região do CVL), o mecanismo excitatório é liberado e a pressão arterial e atividade simpática aumentam (Ito e Sved, 1997).

A situação experimental de inibição da região do CVL parece promover uma super-excitação dos neurônios do RVL e também do NTS. Diante disso, estudos referentes a essa tese de doutorado demonstraram (Trabalho 1 e 2; Moreira e cols., 2005; 2007) que o bloqueio de receptores glutamatérgicos na região do NTS (região adjacente à área postrema - postremal) ou comissural, simultaneamente com a inibição da região do CVL produziram redução da pressão arterial e da resistência vascular para valores abaixo dos valores controles, sugerindo que importantes mecanismos excitatórios existem no NTS e no CVL para controle e manutenção da pressão arterial e resistência vascular (Moreira e cols., 2005; 2007). Portanto, a primeira parte dessa tese de doutorado visa investigar a importância de mecanismos excitatórios na região bulbar, em especial no NTS e na região do CVL para controle das variáveis cardiovasculares.



O esquema acima mostra uma representação de um corte sagital do encéfalo de rato. Esse esquema procura mostrar as possíveis interconexões entre NTS, região CVL e a região RVL discutidas nessa introdução. Neurônios do NTS enviam projeções excitatórias para a região CVL e RVL. Os neurônios da região CVL (CVL inibitório, verde) enviam projeções (baro dependentes e baro idependente) para a região RVL, promovendo a principal fonte de inibição dos neurônios dessa área.

Role of pressor mechanisms from the NTS and CVLM in control of arterial pressure

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Role of pressor mechanisms from the NTS and CVLM in control of arterial pressure

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Moreira, Thiago Santos, Monica Akemi Sato, Ana Carolina Thomaz Takakura, José Vanderlei Menani, and Eduardo Colombari. Role of pressor mechanisms from the NTS and CVLM in control of arterial pressure. *Am J Physiol Regul Integr Comp Physiol* 289: R1416–R1425, 2005. First published July 28, 2005; doi:10.1152/ajpregu.00053.2005.—In the present study, we investigated the effects of inhibition of the caudal ventrolateral medulla (CVLM) with the $GABA_A$ agonist muscimol combined with the blockade of glutamatergic mechanism in the nucleus of the solitary tract (NTS) with kynurenic acid (kyn) on mean arterial pressure (MAP), heart rate (HR), and regional vascular resistances. In male Holtzman rats anesthetized intravenously with urethane/chloralose, bilateral injections of muscimol (120 pmol) into the CVLM or bilateral injections of kyn (2.7 nmol) into the NTS alone increased MAP to 186 ± 11 and to 142 ± 6 mmHg, respectively, vs. control: 105 ± 4 mmHg; HR to 407 ± 15 and to 412 ± 18 beats per minute (bpm), respectively, vs. control: 352 ± 12 bpm; and renal, mesenteric and hindquarter vascular resistances. However, in rats with the CVLM bilaterally blocked by muscimol, additional injections of kyn into the NTS reduced MAP to 88 ± 5 mmHg and mesenteric and hindquarter vascular resistances below control baseline levels. Moreover, in rats with the glutamatergic mechanisms of the NTS blocked by bilateral injections of kyn, additional injections of muscimol into the CVLM also reduced MAP to 92 ± 2 mmHg and mesenteric and hindquarter vascular resistances below control baseline levels. Simultaneous blockade of NTS and CVLM did not modify the increase in HR but also abolished the increase in renal vascular resistance produced by each treatment alone. The results suggest that important pressor mechanisms arise from the NTS and CVLM to control vascular resistance and arterial pressure under the conditions of the present study.

ventrolateral medulla; γ; γ-aminobutyric acid; L-glutamate; muscimol; sympathetic system

THE MEDULLARY CIRCUIT related to cardiovascular control involves the nucleus of the solitary tract (NTS), nucleus ambiguous, and ventrolateral medulla (rostral and caudal) (8, 15, 17). The NTS is the site of the first synapse of the viscerosensory afferents in the brain stem, including those related to cardiovascular baroreceptor and chemoreceptor afferents. The neurotransmitter released by these afferents in the NTS is suggested to be L-glutamate (8, 15, 16, 32, 48). From the NTS, the baroreceptor afferent signals project to the caudal ventrolateral medulla (CVLM) (45, 47). Through GABAergic mechanisms, the CVLM inhibits neurons in the rostral ventrolateral medulla (RVLM) that innervate the preganglionic sympathetic neurons involved in controlling the heart and vascular beds (5, 15, 28, 29, 36, 37). Disinhibition of the RVLM via deactivation of CVLM by electrolytic lesions or injections of the $GABA_A$ receptor agonist muscimol, or the blockade of GABAergic transmission in the RVLM, results in sustained sympathoexcitation and increase in arterial pressure and heart rate (4, 14, 46). Similar to the deactivation of the CVLM, the blockade of the NTS with injections of muscimol or the ionotropic glutamatergic antagonist kynurenic acid (kyn) also produces sympathoexcitation and increases arterial pressure and heart rate (18).

Parallel to the inhibitory mechanisms, the RVLM also receives important excitatory projections (17, 20, 24, 26). Anatomical and immunohistochemical studies have shown that the NTS sends monosynaptic connections to the RVLM (13, 21, 35, 39, 41) and these projections from the NTS to the RVLM may convey peripheral chemoreceptor signals (30, 31, 49). The existence of pressor mechanisms in the NTS is supported by the increase in arterial pressure produced by L-glutamate injections into the NTS in awake rats (9, 33). Although L-glutamate injected into the NTS in anesthetized rats usually reduces arterial pressure, similar to baroreflex activation, L-glutamate into the NTS induces pressor responses in anesthetized rats after the inhibition of the CVLM with muscimol (49). This pressor response to L-glutamate into the NTS in anesthetized rats is abolished by the blockade of excitatory amino acid (EAA) receptors in the RVLM, which suggests the existence of a pressor pathway from the NTS to the RVLM (50, 54). Besides the inhibitory projection, there are some studies that suggest that the CVLM by direct or indirect projections may activate RVLM neurons (26, 38). In spite of some controversies (25), Ito and Sved (26) have shown that the blockade of the EAA receptors by bilateral injections of kyn into the RVLM combined with the inhibition of the CVLM with bilateral injections of muscimol reduced arterial pressure to a level similar to that produced by complete autonomic blockade, suggesting that the RVLM receives important tonic excitatory drive. However, bilateral injections of kyn alone into the RVLM in rats do not significantly change resting arterial pressure, which indicate that these excitatory mechanisms are not active in resting conditions. According to Ito and Sved (26), under resting conditions the excitatory drive to the RVLM is counterbalanced by the activation of the CVLM

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inhibitory mechanisms that, in turn, is also activated by signals under control of the RVLM excitatory drive. When the inhibitory mechanism is blocked by injections of muscimol into the CVLM, the excitatory mechanisms that reach the RVLM are released and sympathetic activity and arterial pressure increase (26).

Therefore, according to the work of Ito and Sved (26), the deactivation of the inhibitory influences that reach the RVLM by blocking the CVLM with muscimol or the EAA receptors with kyn into NTS increases sympathetic activity, arterial pressure, and heart rate. Additional studies have suggested that the RVLM also receives excitatory projections from the NTS and from the CVLM. Considering that the relative importance of the excitatory and inhibitory signals from the CVLM and NTS to the RVLM for cardiovascular regulation is still not completely clear, in the present study, we investigated the effects produced by the inhibition of the CVLM with muscimol combined with the blockade of EAA receptors in the NTS with kyn on mean arterial pressure (MAP), heart rate (HR) and renal, mesenteric and hindquarter vascular resistances. To better compare the relative importance of the mechanisms present in each area for cardiovascular control, two sequences of treatments were tested in different rats: in one group of rats the first treatment was muscimol into the CVLM, and the second treatment, performed 10 min later, was kyn into the NTS; in another group of rats, the first treatment was kyn into the NTS followed 10 min later by muscimol into the CVLM.

MATERIALS AND METHODS

Surgical procedures. All experiments were performed in accordance with the Brazilian National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes and were approved by the Animal Experimentation Ethics Committee of the Federal University of São Paulo, School of Medicine.

Male Holtzman rats weighing 300–350 g were used. One day before the experiment, the rats were anesthetized with ketamine (80 mg/kg body wt) combined with xylazine (7 mg/kg body wt), and the femoral artery and vein were cannulated for arterial pressure measurement and drug administration, respectively. The arterial and venous catheters (PE-10 connected to PE-50) were tunneled subcutaneously and fixed on the back of the rat with suture thread. On the next day, immediately before the experiments, the animals were anesthetized with urethane (1.0 g/kg iv) combined with α-chloralose (60 mg/kg iv). A midline laparotomy was performed and miniature pulsed Doppler flow probes were placed around the renal artery, superior mesenteric artery, and lower abdominal aorta for measurement of renal, mesenteric, and hindquarter blood flows, respectively. The probes were fixed to the surrounding tissues with suture thread, and the animals were immediately placed in a stereotaxic apparatus in a prone position with the incisor bar at 11 mm below the intra-aural line. A partial occipital craniotomy was performed to expose the dorsal surface of the caudal brain stem.

Arterial pressure, heart rate and regional blood flow recordings. The catheter inserted into the femoral artery was connected to a P23 Db pressure transducer (Statham Gould) coupled to a preamplifier (model ETH-200 Bridge Bio Amplifier, CB Sciences) connected to the Powerlab computer recording system (model Powerlab 16SP, ADInstruments) for measurement of pulsatile arterial pressure, MAP, and HR. The flow probes were connected to a Doppler flowmeter (Dept. of Bioengineering, University of Iowa, Iowa City, IA) also coupled to the Powerlab computer recording system. Details of the Doppler flow recording technique, including the reliability of the method for estimation of flow velocity, have been described previously by Haywood and colleagues (22). Relative renal, mesenteric, and hindquarter vascular resistance changes were calculated as the ratio of MAP and Doppler shifts. Data from animals in which the probes moved during the experiment were not considered for analysis.

The rectal temperature was maintained at 37°C with a thermostatically controlled heating pad. During the surgical procedure or recording period, if the animals were responsive to noxious toe pinch, a supplementary dose of urethane (0.1 g/kg) combined with α-chloralose (20 mg/kg) was administered intravenously.

Central injections. Injections (50 nl, delivered over 5 s) of muscimol (120 pmol) or saline into the CVLM and kyn (2.7 nmol) or vehicle into the intermediate regions of the NTS were performed using the same single-barrel glass pipette (20 μm tip diameter) coupled to a pressure injection apparatus (PicoSpritzer II). An injection was first performed in one side; the pipette was withdrawn from the brain and the contralateral injection was made; thus the two injections were made ~1 min apart. The volume of each injection was estimated from the displacement of the fluid meniscus in the pipette using a calibrated reticule. Injections into the CVLM were made 0.5 mm rostral to the calamus scriptorius, 1.8 mm lateral to midline and

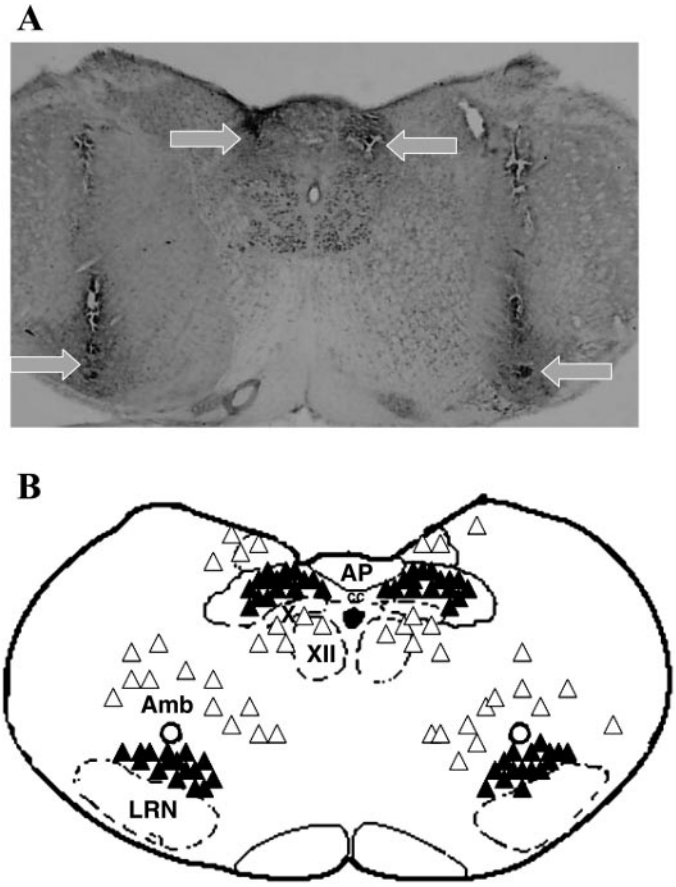

Fig. 1. *A*: photomicrograph of a coronal section of the brain stem showing the typical bilateral injections into the intermediate region of the nucleus of the solitary tract (NTS) and into the caudal ventrolateral medulla (CVLM). Arrows indicate the center of the injections. *B*: diagrammatic composite of a transverse section of the rat medulla showing the sites where muscimol and kyn were injected approximately at the level 13.7 mm caudal to bregma according to Paxinos and Watson (40). Solid triangles indicate the centers of injection sites of kyn into the NTS ($n = 16$) or muscimol into the CVLM ($n = 16$), and open triangles indicate the centers of injection sites into the neighboring regions of the NTS ($n = 9$) and CVLM ($n = 12$). Amb, nucleus ambiguus; AP, area postrema; cc, central canal; LRN, lateral reticular nucleus; X, dorsal motor vagus nucleus; XII, hypoglossal nucleus.

Table 1. *Baseline MAP and HR in the different groups of rats that received injections of saline or muscimol into the caudal ventrolateral medulla and vehicle or kynurenic acid into the nucleus of the solitary tract*

Treatments	Baseline MAP, mmHg	Baseline HR, bpm
sal + veh	105±6	352±12
musc + veh	99±2	343±10
sal + kyn	102±3	368±7
musc + kyn	103±3	340±9
veh + sal	101±2	334±16
kyn + sal	105±6	326±7
veh + musc	103±5	338±11
kyn + musc	100±4	332±8

The results are presented as means ± SE; $n = 8$ rats for each treatment. sal, saline; veh, vehicle; musc, muscimol; kyn, kynurenic acid; MAP, mean arterial pressure; HR, heart rate.

1.9 to 2.2 mm below the dorsal surface of the brain stem. Injections into the NTS were made 0.5 mm rostral to the calamus scriptorius, 0.5 mm lateral to midline, and 0.5 mm below the dorsal surface of the brain stem.

Drugs. Muscimol and kyn were purchased from Sigma Chemical. Muscimol was dissolved in isotonic saline. Kyn was initially dissolved in 100 mM sodium bicarbonate (in a volume that corresponded to 10% of the final volume) and then diluted with isotonic saline until reaching the final volume. The pH of kynurenic acid solution was around 7.4.

Histology. At the end of the experiments, a 2% solution of Evans blue was injected into the CVLM and NTS (50 nl) using the same pipette that was previously used for drug injection. Saline followed by 10% buffered formalin was perfused through the heart. The brains were removed, fixed in 10% formalin for at least 2 days, frozen, cut coronally into 50-μm sections and stained with Giemsa. The sections were analyzed by light microscopy to confirm the injections bilaterally into the CVLM and NTS.

Statistical analysis. Data are expressed as means ± SE. Statistical analysis of baseline MAP, HR, and changes in vascular resistances were performed using two-way ANOVA followed by the Student-Newman-Keuls post hoc test. Significance level was set at $P < 0.05$.

Experimental Protocols1

Effects of kynurenic acid into the NTS on MAP, HR, and regional vascular resistances in rats pretreated with muscimol into the CVLM. Blood flows, MAP and HR were continuously recorded during 60 min and were analyzed at every 10 min starting the recording 10 min after the connection of the arterial line to the pressure transducer. Control (baseline) values were recorded for 10 min and were analyzed immediately before bilateral injections of muscimol or saline into the CVLM (first treatment). These values were used as reference to calculate the changes produced by the treatments. Ten minutes after muscimol or saline into the CVLM, kyn or vehicle was bilaterally injected into the NTS and the cardiovascular responses were evaluated during the next 40 min.

Four groups of animals ($n = 8$ in each group) were used to investigate the cardiovascular effects of the combination of injections of muscimol or saline into the CVLM followed by injections of kyn or vehicle into the NTS. In each rat, only one of the following combinations was tested: *1*) saline into the CVLM followed by vehicle into the NTS (control); *2*) saline into the CVLM followed by kyn into the NTS; *3*) muscimol into the CVLM followed by vehicle into the NTS; and *4*) muscimol into the CVLM followed by kyn into the NTS.

Effects of muscimol into the CVLM on MAP, HR, and regional vascular resistances in rats pretreated with kynurenic acid into the NTS. The protocol used was similar to that described above (item 1), except that the first treatment was kyn or vehicle into the NTS and the second treatment was muscimol or saline into the CVLM.

Four groups of animals ($n = 8$ each group) were also used, and in each rat only one of the following combinations was tested: *1*) Vehicle into the NTS followed by saline into the CVLM (control); *2*) Kyn

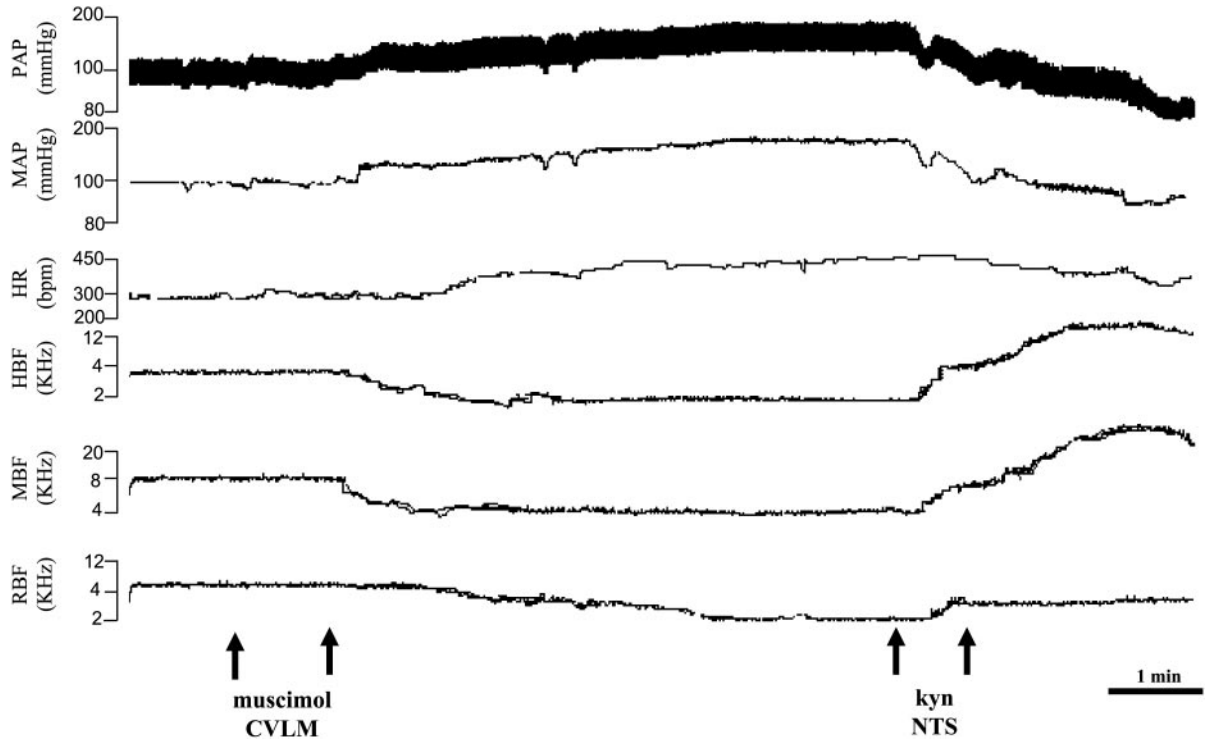

Fig. 2. Typical recording showing changes in pulsatile arterial pressure (PAP), mean arterial pressure (MAP), heart rate (HR), renal (RBF), mesenteric (MBF) and hindquarter (HBF) blood flows produced by bilateral injections of muscimol (120 pmol/50 nl) injected into the CVLM followed by bilateral injections of kynurenic acid (kyn, 2.7 nmol/50 nl) into the NTS. Arrows indicated the moment of the injections.

into the NTS followed by saline into the CVLM; *3*) Vehicle into the NTS followed by muscimol into the CVLM; and *4*) Kyn into the NTS followed by muscimol into the CVLM.

Baroreflex test in rats treated with muscimol into the CVLM and kynurenic acid into the NTS. In 6 rats that received muscimol into the CVLM followed by kyn into the NTS, and in 5 rats treated with injections of kyn into the NTS followed by injections of muscimol into the CVLM, the baroreflex was tested by intravenous injection of a pressor dose of phenylephrine (5 μg/kg body wt) and a depressor dose of sodium nitroprusside (30 μg/kg body wt). The injections of phenylephrine and sodium nitroprusside were performed from 4 to 8 min before the first central injection (control) and from 10 to 20 min after the second central injection.

Effects of kynurenic acid and muscimol injected in sites outside the NTS and CVLM on MAP, HR, and regional vascular resistances. To confirm the specificity of injection sites for the effects of muscimol and kynurenic acid on MAP, HR, and regional vascular resistances, results from rats in which the injections did not reach the NTS or the CVLM bilaterally (misplaced injections) were also analyzed and presented in the RESULTS section.

RESULTS

Histological analysis. Figure 1*A* is a photomicrograph showing the typical sites of the bilateral injections into the intermediate region of the NTS and into the CVLM in one rat representative of the rats used in the present study. Figure 1*B* is a diagrammatic composite showing the injection sites into the medulla in rats that received injections of muscimol and kyn. According to Paxinos and Watson (40), these coronal sections are located ~13.7 mm caudal to bregma. The injection sites in the NTS shown in Fig. 1 are the same that previous studies have already shown to produce depressor responses to L-glutamate injections and pressor responses to bilateral injections of kyn (18, 32). Sympathoinhibitory neurons have been described in the CVLM in sites similar to those shown in Fig. 1 (1, 3, 28, 34), and previous studies have showed that bilateral injections of muscimol in these sites induced pressor responses (25, 26, 49).

Changes in MAP, HR, and regional vascular resistances induced by kynurenic acid into the NTS in rats pretreated with muscimol into the CVLM. The baseline levels of MAP and HR were similar in all four experimental groups tested (Table 1).

Bilateral injections of muscimol (120 pmol/50 nl) into the CVLM followed by vehicle into the NTS resulted in sustained (for at least 30 min) hypertension (186 ± 11 mmHg vs. saline: 105 ± 4 mmHg), tachycardia [407 ± 15 beats per minute (bpm) vs. saline: 352 ± 12 bpm] and increase in renal (364 ± 38% vs. saline: 6 ± 12%), mesenteric (389 ± 53% vs. saline: 8 ± 4%) and hindquarter vascular resistances (403 ± 48% vs. saline: 7 ± 9%), whereas renal (−123 ± 51% vs. saline: 5 ± 5%), mesenteric (−184 ± 46% vs. saline: 11 ± 13%) and hindquarter (−196 ± 47% vs. saline: 8 ± 10%) blood flows were reduced (Figs. 2 and 3).

Similar to muscimol into the CVLM, bilateral injections of kyn (2.7 nmol/50 nl) into the NTS preceded by saline into the CVLM also increased HR to 397 ± 14 bpm. Although the other responses were less intense than those produced by muscimol into the CVLM, bilateral injections of kyn into the NTS also increased MAP to 144 ± 5 mmHg and renal, mesenteric, and hindquarter vascular resistances, while the blood flows in the same beds were reduced for at least 30 min (Figs. 2 and 3). However, in rats pretreated with muscimol into the CVLM, bilateral injections of kyn (2.7 nmol/50 nl) into the NTS immediately reduced MAP to 88 ± 5 mmHg and mesenteric (−28 ± 19%) and hindquarter vascular resistances (−31 ± 11%) below control preinjection baseline levels and abolished the changes in renal vascular resistance but did not modify the effects of muscimol on HR (Figs. 2-4). Mesenteric (33 ± 12%) and hindquarter (52 ± 16%) blood flows increased to above control preinjection baseline levels, and renal blood flow was restored to control baseline level by the combination of muscimol into the CVLM and kyn into the NTS (Figs. 3 and 4). No significant changes in breathing occurred after injections of kyn into the NTS or muscimol into the CVLM.

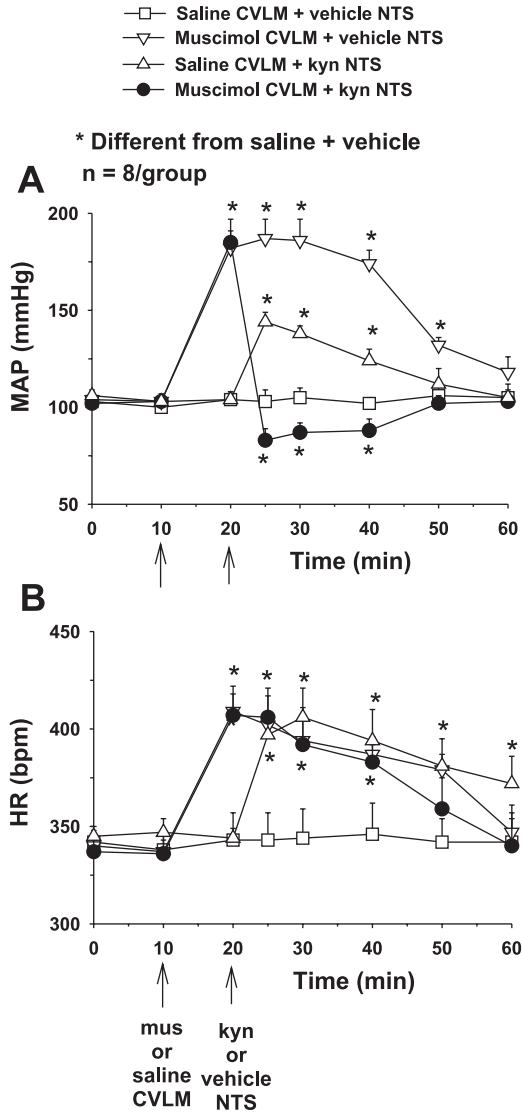

Fig. 3. A: mean arterial pressure (MAP) and (*B*) heart rate (HR) in rats treated with bilateral injections of muscimol (mus; 120 pmol/50 nl) or saline into the CVLM followed by bilateral injections of kynurenic acid (kyn; 2.7 nmol/50 nl) or vehicle into the NTS. The results are represented as means ± SE. *n* = number of rats. ANOVA showed significant differences among the treatments for MAP [$F(3, 165) = 32.10$, $P < 0.01$] and HR [$F(3, 165) = 43.51$, $P < 0.01$].

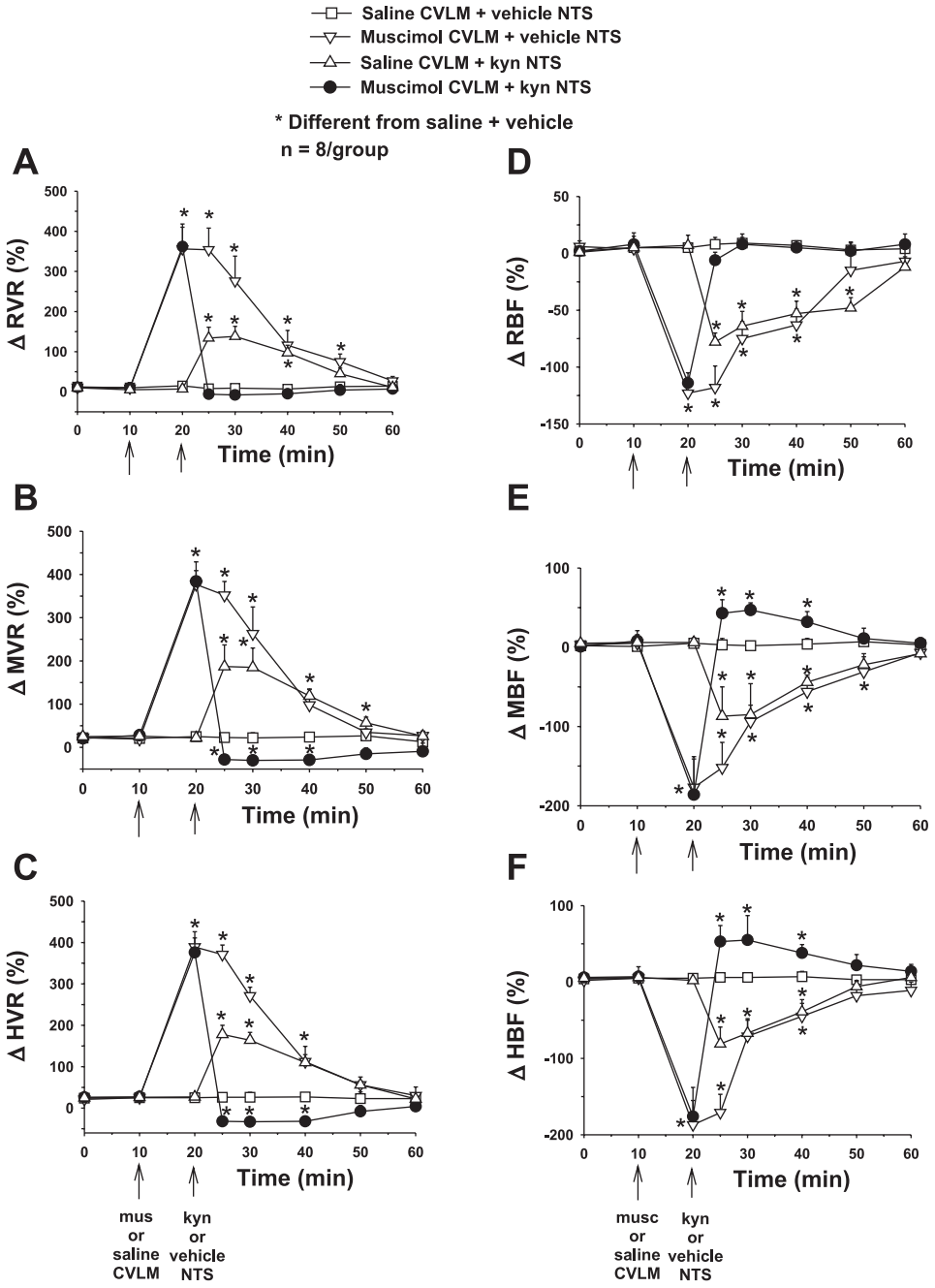

Fig. 4. Changes in (*A*) renal (RVR), (*B*) mesenteric (MVR) and (*C*) hindquarter (HVR) vascular resistances and in (*D*) RBF, (*E*) MBF, and (F) HBF produced by bilateral injections of mus (120 pmol/50 nl) or saline into the CVLM followed by bilateral injections of kyn (2.7 nmol/50 nl) or vehicle into the NTS. The results are represented as means ± SE. n = number of rats. ANOVA showed significant differences among the treatments for renal [$F(3, 165) = 43.19$, $P < 0.01$], mesenteric [$F(3, 165) = 29.34$, $P < 0.01$], and hindquarter [$F(3, 165) = 25.24$, $P < 0.01$] vascular resistances and for renal [$F(3, 165) = 188.22$, $P < 0.01$], mesenteric [$F(3, 165) = 179.74$, $P < 0.01$], and hindquarter [$F(3, 165) = 171.40$, $P < 0.01$] blood flows.

Changes in MAP, HR, and regional vascular resistances induced by muscimol into the CVLM in rats pretreated with kynurenic acid into the NTS. The baseline levels of MAP and HR were similar in all four experimental groups tested (Table 1).

Bilateral injections of kyn (2.7 nmol/50 nl) into the NTS followed by saline into the CVLM produced sustained (for at least 30 min) increases in MAP (142 ± 6 mmHg vs. vehicle: 101 ± 2 mmHg), HR (412 ± 18 bpm vs. vehicle: 334 ± 16 bpm) and renal (138 ± 13% vs. saline: 8 ± 9%), mesenteric (168 ± 26% vs. saline: 11 ± 7%) and hindquarter vascular resistances (154 ± 34% vs. saline: 6 ± 5%), while renal (−81 ± 19% vs. saline: −5 ± 15%), mesenteric (−94 ± 21% vs. saline: 4 ± 6%), and hindquarter (−72 ± 22% vs. saline: 9 ± 14%) blood flows were reduced (Figs. 5 and 6).

Bilateral injections of muscimol (120 pmol/50 nl) into the CVLM preceded by vehicle into the NTS also increased MAP to 183 ± 7 mmHg, HR to 443 ± 11 bpm, and renal, mesenteric and hindquarter vascular resistances, while the blood flows in the same beds were reduced for at least 30 min (Figs. 5 and 6). However, bilateral injections of muscimol (120 pmol/50 nl) into the CVLM in rats pretreated with kyn (2.7 nmol/50 nl) into the NTS immediately reduced MAP to 92 ± 2 mmHg and mesenteric (−24 ± 11%) and hindquarter (−26 ± 9%) vascular resistances below control baseline levels and abolished the changes in renal vascular resistance but not the changes in

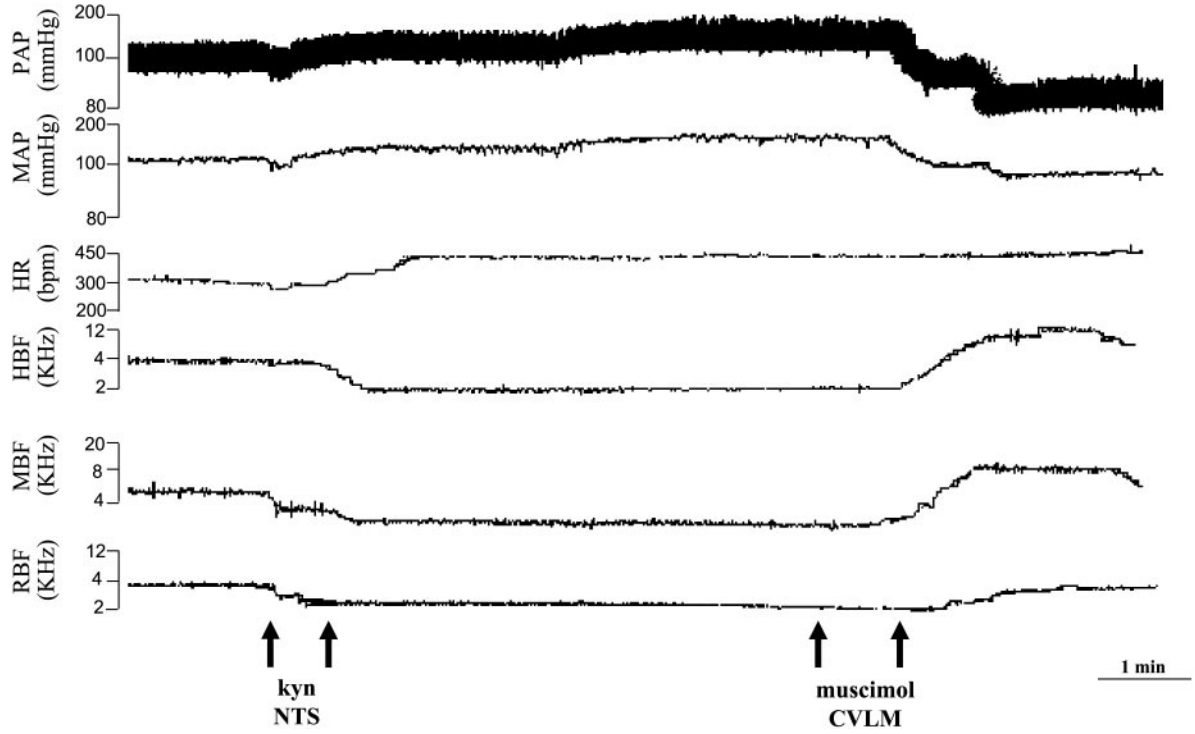

Fig. 5. Typical recording showing changes in PAP, MAP, HR, RBF, MBF, and HBF blood flows produced by bilateral injections of kyn (2.7 nmol/50 nl) into the NTS followed by bilateral injections of mus (120 pmol/50 nl) into the CVLM. Arrows indicated the moment of the injections.

HR (Figs. 5-7). Mesenteric (51 ± 14%) and hindquarter (55 ± 7%) blood flows increased above control preinjection baseline levels, and renal blood flow was restored to control baseline level by the combination of kyn into the NTS and muscimol into the CVLM (Figs. 6 and 7).

Baroreflex test in rats treated with muscimol into the CVLM and kynurenic acid into the NTS. The combination of bilateral injections of muscimol into the CVLM and kyn into the NTS abolished the reflex bradycardia (−1 ± 2 bpm vs. control: −54 ± 6 bpm; n = 11) produced by an intravenous injection of phenylephrine (5 μg/kg body wt) and also abolished the reflex tachycardia (3 ± 4 bpm vs. control: 66 ± 7 bpm) to intravenous injection of sodium nitroprusside (30 μg/kg body wt). The pressor response to intravenous phenylephrine (36 ± 5 mmHg vs. control: 44 ± 9 mmHg) and the hypotension to intravenous sodium nitroprusside (−35 ± 3 mmHg vs. control: −37 ± 5 mmHg) were not modified by the simultaneous blockade of the CVLM with muscimol and the NTS with kyn.

Effects of muscimol and kynurenic acid injected outside the CVLM and NTS on MAP, HR, and regional vascular resistances. In the first series of experiments, bilateral injections of muscimol or kyn outside the CVLM or NTS, respectively (Fig. 1), produced no significant changes in the baseline MAP (8 ± 3 mmHg vs. vehicle: 9 ± 6 mmHg; n = 12 and 8, respectively), HR (13 ± 11 bpm vs. vehicle: 7 ± 7 bpm) or renal (11 ± 6% vs. vehicle: 9 ± 15%), mesenteric (7 ± 5% vs. vehicle: 12 ± 17%) and hindquarter (6 ± 8% vs. vehicle: 6 ± 11%) vascular resistances. Injections of kyn outside the NTS after muscimol into the CVLM produced no additional changes in MAP (184 ± 11 mmHg vs. vehicle: 188 ± 8 mmHg; n = 12 and 8, respectively), HR (409 ± 11 bpm vs. vehicle: 413 ± 7 bpm) or renal (345 ± 26% vs. vehicle: 363 ± 32%), mesenteric (352 ± 35% vs. vehicle: 366 ± 13%) and hindquarter (374 ± 51% vs. vehicle: 358 ± 33%) vascular resistances.

In a second series of experiments, the bilateral injections of kyn or muscimol outside the NTS or CVLM, respectively, produced no significant changes in the baseline MAP (4 ± 6 mmHg vs. vehicle: −2 ± 11 mmHg; n = 9 and 8, respectively), HR (7 ± 9 bpm vs. vehicle: 13 ± 6 bpm) or renal (21 ± 16% vs. vehicle: 23 ± 11%), mesenteric (12 ± 5% vs. vehicle: 11 ± 11%) and hindquarter (9 ± 12% vs. vehicle: 8 ± 9%) vascular resistances. Injections of muscimol outside the CVLM after kyn into the NTS produced no additional changes in MAP (144 ± 9 mmHg vs. vehicle: 138 ± 15 mmHg; n = 9 and 8, respectively), HR (417 ± 9 bpm vs. vehicle: 423 ± 16 bpm) or renal (142 ± 36% vs. vehicle: 153 ± 41%), mesenteric (162 ± 25% vs. vehicle: 161 ± 13%) and hindquarter (159 ± 32% vs. vehicle: 158 ± 19%) vascular resistances.

DISCUSSION

As previously demonstrated, the blockade of the glutamatergic receptors in the NTS by injections of kyn or the inhibition of the CVLM with muscimol increases MAP and HR (15, 18, 26, 53). The novelty in the present study is that the blockade of the glutamatergic receptors of the NTS simultaneously with the inhibition of the CVLM reduces MAP and mesenteric and hindquarter vascular resistances below control preinjection baseline levels. Misplaced injections of muscimol or kyn outside the CVLM or NTS produced no significant changes in the baseline vascular resistances, MAP, and HR. Therefore, the present results suggest that important pressor mechanisms arising from the NTS and the CVLM are involved

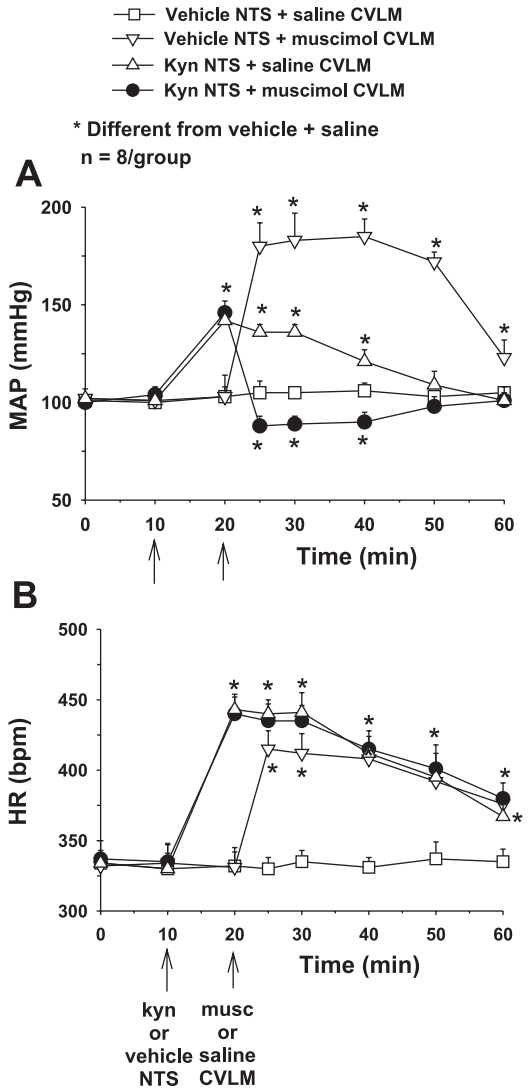

Fig. 6. *A*: MAP and (*B*) HR in rats treated with bilateral injections of kyn (2.7 nmol/50 nl) or vehicle into the NTS followed by bilateral injection of mus (120 pmol/50 nl) or saline into the CVLM. The results are represented as means ± SE. n = number of rats. ANOVA showed significant differences among the treatments for MAP [$F(3, 165) = 26.15$, $P < 0.01$] and HR [$F(3, 165) = 19.73$, $P < 0.01$].

in the control of vascular resistance and arterial pressure in the conditions used in the present study.

Important connections among the NTS, CVLM, and RVLM for cardiovascular regulation have been proposed (11, 30). Chemoreceptors probably activate an excitatory pathway that connects the NTS to sympathetic premotor neurons in the RVLM either directly (10, 31, 49) or through brain stem pathways that involve neurons also in the A_5 region (19). Baroreceptor afferents provide excitatory inputs to second-order neurons in the NTS that project to the CVLM and activate inhibitory projections from CVLM to the RVLM (11, 16). Glutamate is suggested to be the neurotransmitter released by baroreceptor and chemoreceptor afferent fibers in the NTS (8, 15, 16, 32, 48). Blocking the baroreflex with injections of kyn into the NTS, the CVLM inhibitory mechanism is deactivated reducing the inhibition of the RVLM which in turn increases sympathetic activity, vascular resistance, MAP, and HR. Injections of muscimol into the CVLM also remove the inhibition of the RVLM producing effects qualitatively similar to kyn into the NTS (4, 12, 53). Although baroreceptor signals that arise through NTS are important to inhibit the RVLM (2, 12, 18, 28), it is necessary to consider that part of the inhibition that the RVLM receives from the CVLM is not baroreceptor dependent (12), which may explain why vascular resistance and MAP increases are almost double after CVLM blockade with muscimol than after only the blockade of the baroreflex influences with kyn into the NTS.

Although the treatment with muscimol into the CVLM or kyn into the NTS independently disinhibits the RVLM and increases sympathetic activity, vascular resistance, MAP, and HR, when both treatments are combined simultaneously, MAP and mesenteric and hindquarter vascular resistances fall below control preinjection levels without changing the HR increase. These results suggest that the increases in MAP and regional vascular resistances produced by the inhibition of the CVLM alone depend on pressor mechanisms arising from the NTS. Moreover, the effects on MAP and vascular resistances produced by the blockade of the glutamatergic mechanisms of the NTS alone depend on pressor mechanisms that arise from the CVLM. Excitatory mechanisms arising from different central areas are important to activate RVLM neurons in resting conditions or hypertensive states (26, 27, 43). Although the blockade of EAA receptors in the RVLM produces no significant effects on baseline arterial pressure in rats (18, 26), it reduces arterial pressure and sympathetic nerve activity in rabbits (23). Ito and Sved (26) also showed that arterial pressure was reduced below control resting levels by the blockade of EAA receptors with kyn in the RVLM combined with the blockade of the CVLM with muscimol, which suggests that the increase in arterial pressure produced by the blockade of the CVLM is dependent on RVLM excitation produced by EAA release. Similar to Ito and Sved (26), the present results also support the importance of facilitatory mechanisms to the RVLM for the pressor response, resulting from muscimol into the CVLM, with the difference that the present results suggest that the facilitatory projection to the RVLM arises and is activated by glutamatergic mechanisms in the NTS.

The existence of pressor mechanisms in the NTS is suggested by the pressor responses usually produced by L-glutamate injections into the NTS in awake rats or by the antihypertensive effects of commissural NTS lesions in spontaneously hypertensive rats (9, 33, 42, 44). Although L-glutamate injected into the NTS in anesthetized rats usually induces depressor responses, the same injection into the NTS after the blockade of the CVLM with muscimol produces pressor responses in anesthetized rats (49), which suggest that L-glutamate injections into the NTS may also activate pressor mechanisms in anesthetized rats. However, in anesthetized rats the pressor mechanism activated by L-glutamate injections into the NTS is completely masked by the larger depressor responses dependent on CVLM. Anatomical and imunohistochemical studies have suggested the existence of a direct excitatory projection from the NTS to the RVLM (49, 50), which may convey peripheral chemoreceptor signals (30, 31). Although kyn injections into the NTS might block chemoreflex pathways (6, 7, 51), it is necessary to consider that chemoreceptors are usually silent in the absence of a proper stimulus. Therefore,

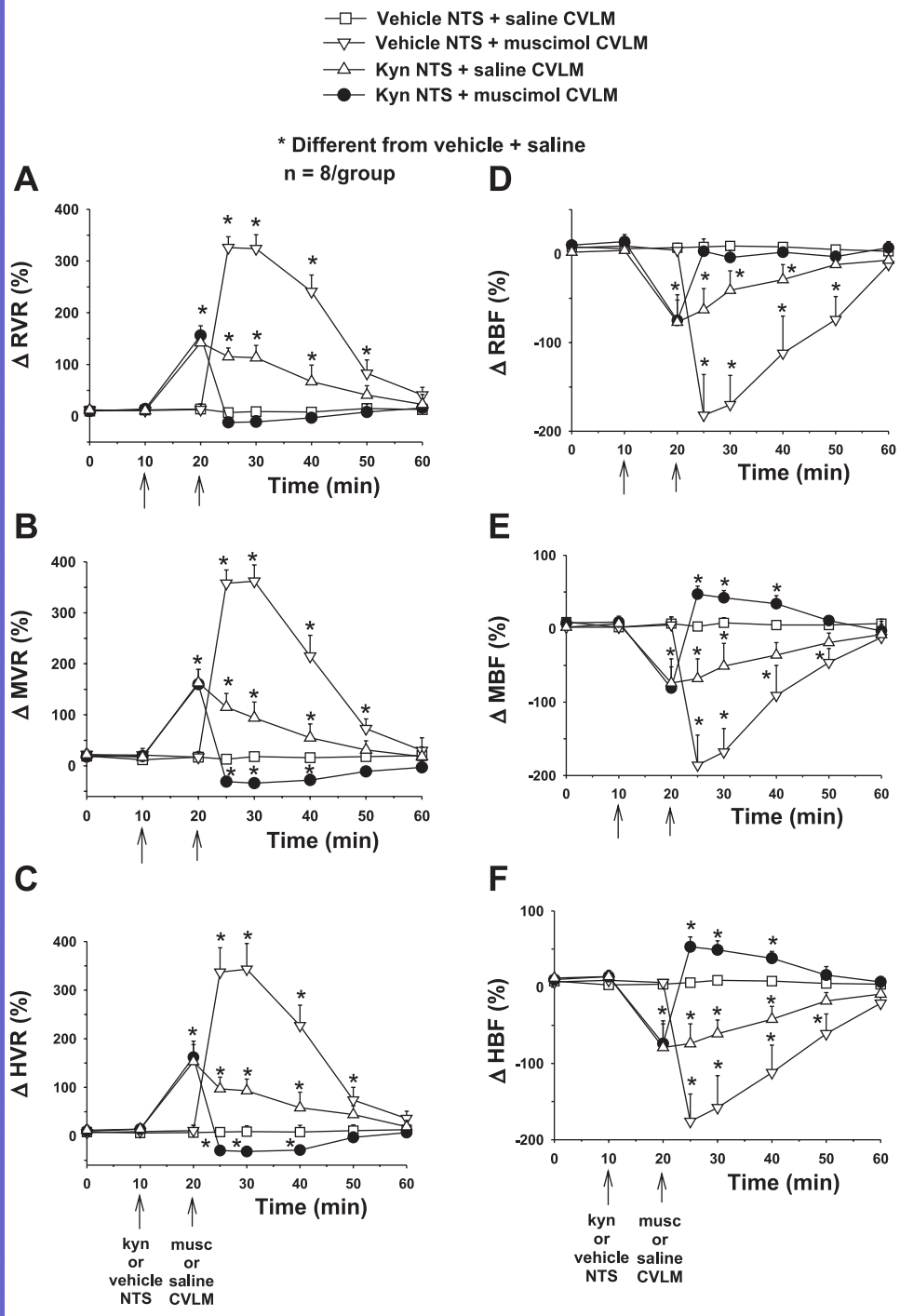

Fig. 7. Changes in (*A*) RVR, (*B*) MVR, and (*C*) HVR vascular resistances and in (*D*) RBF, (*E*) MBF, and (*F*) HBF blood flows produced by bilateral injections of kyn (2.7 nmol/50 nl) or vehicle into the NTS followed by bilateral injection of mus (120 pmol/50 nl) or saline into the CVLM. The results are represented as means ± SE; n = number of rats. ANOVA showed significant differences among the treatments for renal [$F(3, 165) = 43.63$, $P < 0.01$], mesenteric [$F(3, 165) = 28.51$, $P < 0.01$], and hindquarter [$F(3, 165) = 32.44$, $P < 0.01$] vascular resistances and renal [$F(3, 165) = 145.87$, $P < 0.01$], mesenteric [$F(3, 165) = 256.82$, $P < 0.01$], and hindquarter [$F(3, 165) = 241.62$, $P < 0.01$] blood flows.

during the blockade of the CVLM with muscimol, a mechanism probably not related to the chemoreflex seems to activate EAA receptors in the NTS causing sympathetic activation and pressor responses. A previous study suggested that aortic depressor nerve activity can stimulate pressor mechanisms and increase arterial pressure when the CVLM was blocked by muscimol (52). Although the involvement of mechanisms related to the aortic depressor nerve should be considered, the present results do not illuminate the mechanism that activates NTS glutamatergic pressor pathways after muscimol injections into the CVLM. However, it is clear that MAP and vascular resistance are strongly dependent on NTS glutamatergic mechanisms after the blockade of the CVLM. On the other hand, the pressor response produced by kyn injections into the NTS is abolished by muscimol injections into the CVLM, suggesting that excitatory mechanisms from the CVLM blocked by muscimol injections are involved in the pressor response to kyn injections into the NTS. Injections of kyn into the RVLM abolish the pressor response to muscimol injections into the NTS (26), which suggests that the excitatory projection to the RVLM arises from other sources in addition to the NTS. Similar to previous studies (26, 38), the present results showing

that the pressor response to kyn injections into the NTS is abolished by muscimol injections into the CVLM also suggest that the CVLM by direct or indirect projections may activate RVLM neurons. Other studies have already suggested that nonglutamatergic excitatory projections arise from the CVLM to the RVLM (26) and that the cardiovascular effects of the caudal pressor area (CPA) activation also depend on an excitatory projection from the CVLM to the RVLM (38).

It is important to note that independent of the sequence of the CVLM and NTS blockades, HR increased after the first blockade, and this increase was not affected by the second blockade. Therefore, the fact that dual blockade produced different effects on MAP and HR suggests that different brainstem mechanisms are activated in these responses and may also involve changes in parasympathetic activity for the tachycardic responses. It is also important to consider that the combination of muscimol into the CVLM and kyn into the NTS reduced mesenteric and hindquarter vascular resistances below control baseline levels, while renal vascular resistance was maintained at the baseline level, which suggests that the hypotension after the combination of the two treatments depends on vasodilation of specific vascular beds. Although NTS glutamatergic and CVLM excitatory mechanisms are important for the increase in renal, mesenteric and hindquarter vascular resistances after the blockade of each area individually, these excitatory mechanisms apparently are not important to maintain baseline renal vascular resistance when both areas are blocked simultaneously. This differs from hindquarter and mesenteric vascular resistances that are dependent on NTS and CVLM excitatory mechanisms to maintain resting levels.

In conclusion, the present results suggest that the pressor responses produced by the blockade of the inhibitory mechanism to the RVLM are strongly dependent on the excitatory projections that arise from the CVLM and from the NTS. In addition, these excitatory pressor mechanisms are also important to maintain baseline arterial pressure and vascular resistance because the blockade of both mechanisms simultaneously reduced MAP and vascular resistance to below control resting levels

Perspectives

One important mechanism still not completely understood is how RVLM neurons are activated to maintain tonic baseline activity of sympathetic nervous system. Besides the importance of the inhibitory mechanisms to control RVLM and sympathetic activity, recent studies have suggested that excitatory projections to the RVLM seem to play an important role in cardiovascular regulation. The present results suggest that excitatory mechanisms that arise from the NTS and CVLM are important to activate RVLM and the sympathetic system. In addition, they suggest that a balance between NTS and CVLM excitatory and inhibitory mechanisms seems to be essential to maintain baseline arterial pressure. Studies using different methodologies are necessary to show more details and to establish the role of the excitatory mechanisms proposed in the present study in the control of sympathetic activity and arterial pressure. An important mechanism still not understood is how the excitatory projections from CVLM and NTS to RVLM are activated under physiological conditions. Other questions for further investigation include whether the pathway relaying excitatory signals from the NTS to RVLM is also involved in chemoreceptor signaling and whether baroreceptor signaling is involved in the cardiovascular responses evoked from NTS when the CVLM is blocked by muscimol.

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REFERENCES

1. **Agarwal SK, Gelsema AJ, and Calaresu FR.** Inhibition of rostral VLM by baroreceptor activation is relayed through caudal VLM. *Am J Physiol Regul Integr Comp Physiol* 258: R1271–R1278, 1990.
2. **Agarwal S and Calaresu F.** Monosynaptic connection from caudal to rostral ventrolateral medulla in the baroreceptor reflex pathway. *Brain Res* 555: 70–74, 1991.
3. **Badoer E, McKinley MJ, Oldfield BJ, and McAllen RM.** Localization of barosensitive neurons in the caudal ventrolateral medulla which project to the rostral ventrolateral medulla. *Brain Res* 657: 258–268, 1994.
4. **Blessing WW.** Depressor neurons in rabbit caudal medulla act via GABA receptors in rostral medulla. *Am J Physiol Heart Circ Physiol* 254: H686–H692, 1988.
5. **Brown DL and Guyenet PG.** Electrophysiological study of cardiovascular neurons in the rostral ventrolateral medulla in rats. *Circ Res* 56: 359–369, 1985.
6. **Chitravanshi VC, Kachroo A, and Sapru HN.** A midline area in the nucleus commissuralis of NTS mediates the phrenic nerve responses to carotid chemoreceptor stimulation. *Brain Res* 662: 127–133, 1994.
7. **Chitravanshi VC and Sapru HN.** Chemoreceptor-sensitive neurons in commissural subnucleus of nucleus tractus solitarius of the rat. *Am J Physiol Regul Integr Comp Physiol* 268: R851–R858, 1995.
8. **Ciriello J, Hochstenbach S, and Roder S.** Central projections of baroreceptor and chemoreceptor afferent fibers in the rat. In: *Nucleus of the Solitary Tract*, edited by Barraco IRA. Boca Raton, FL: CRC, p. 35–50, 1994.
9. **Colombari E, Bonagamba LG, and Machado BH.** Mechanisms of pressor and bradycardic responses to L-glutamate microinjected into the NTS of conscious rats. *Am J Physiol Regul Integr Comp Physiol* 266: R730–R738, 1994.
10. **Colombari E, Menani JV, and Talman W.** Commissural NTS contribute to pressor responses to glutamate injected into the medial NTS of awake rats. *Am J Physiol Regul Integr Comp Physiol* 270: R1220–R1225, 1996.
11. **Colombari E, Sato MA, Cravo SL, Bergamaschi CT, Campos RR Jr, and Lopes OU.** Role of the medulla oblongata in hypertension. *Hypertension* 38: 549–554, 2001.
12. **Cravo SL, Morrison SF, and Reis DJ.** Differentiation of two cardiovascular regions within caudal ventrolateral medulla. *Am J Physiol Regul Integr Comp Physiol* 261: R985–R994, 1991.
13. **Dampney RA, Czachurski J, Dembowsky K, Goodchild K, and Seller H.** Afferent connections and spinal projections of the pressor region in the rostral ventrolateral medulla of the cat. *J Auton Nerv Syst* 20: 73–86, 1987.
14. **Dampney RA, Blessing WW, and Tan E.** Origin of tonic GABAergic inputs to vasopressor neurons in the subretrofacial nucleus of the rabbit. *J Auton Nerv Syst* 24: 227–339, 1988.
15. **Dampney RA.** Functional organization of central pathways regulating the cardiovascular system. *Physiol Rev* 74: 323–364, 1994.
16. **Gordon FJ and Sved AF.** Neurotransmitters in central cardiovascular regulation: glutamate and GABA. *Clin Exp Pharmacol Physiol* 29: 522–524, 2002.
17. **Guyenet PG.** Role of the ventral medulla oblongata in blood pressure regulation. *In Central Regulation of Autonomic Functions*. A. D. Loewy and K. M. Spyer, Eds. Oxford University Press: New York, p. 145–167, 1990.
18. **Guyenet PG, Filtz TM, and Donaldson SR.** Role of excitatory amino acids in rat vagal and sympathetic baroreflexes. *Brain Res* 407: 272–284, 1987.

19. **Guyenet PG.** Neural structures that mediate sympathoexcitation during hypoxia. *Respir Physiol* 121: 147–162, 2000.
20. **Granata AR.** Modulatory inputs on sympathetic neurons in the rostral ventrolateral medulla in the rat. *Cell Mol Neurobiol* 23: 665–80, 2003.
21. **Hancock MB.** Evidence for direct projections from the nucleus of the solitary tract onto medullary adrenaline cells. *J Comp Neurol* 276: 460–468, 1988.
22. **Haywood JR, Shaffer A, Fastenow C, Fink GD, and Brody MJ.** Regional blood flow measurement with pulsed Doppler flowmeter in conscious rat. *Am J Physiol Heart Circ Physiol* 241: H273–H278, 1981.
23. **Horiuchi J and Dampney RA.** Evidence for tonic disinhibition of RVLM sympathoexcitatory neurons from the caudal pressor area. *Auton Neurosci* 99: 102–110, 2002.
24. **Horiuchi J, McAllen RM, Allen AM, Killinger S, Fontes MA, Dampney RA.** Descending vasomotor pathways from the dorsomedial hypothalamic nucleus: role of medullary raphe and RVLM. *Am J Physiol Regul Integr Comp Physiol* 287: R824–R832, 2004.
25. **Horiuchi J, Killinger S, and Dampney RA.** Contribution to sympathetic vasomotor tone of tonic glutamatergic inputs to neurons in the RVLM. *Am J Physiol Regul Integr Comp Physiol* 287: R1335–R1343, 2004.
26. **Ito S and Sved AF.** Tonic glutamate-mediated control of rostral ventrolateral medulla and sympathetic vasomotor tone. *Am J Physiol Regul Integr Comp Physiol* 273: R487–R494, 1997.
27. **Ito S, Komatsu K, Tsukamoto K, and Sved AF.** Excitatory amino acids in the rostral ventrolateral medulla support blood pressure in spontaneously hypertensive rats. *Hypertension* 35: 413–417, 2000.
28. **Jeske I, Morrison SF, Cravo SL, and Reis DJ.** Identification of baroreceptor reflex interneurons in the caudal ventrolateral medulla. *Am J Physiol Regul Integr Comp Physiol* 264: R169–R178, 1993.
29. **Jeske I, Reis DJ, and Milner TA.** Neurons in the barosensory area of the caudal ventrolateral medulla project monosynaptically on to sympathoexcitatory bulbospinal neurons in the rostral ventrolateral medulla. *Neuroscience* 65: 343–353, 1995.
30. **Koshiya N, Huangfu D, and Guyenet PG.** Ventrolateral medulla and sympathetic chemoreflex in the rat. *Brain Res* 609:174–184, 1993.
31. **Koshiya N and Guyenet PG.** NTS neurons with carotid chemoreceptor inputs arborize in the rostral ventrolateral medulla. *Am J Physiol Regul Integr Comp Physiol* 270: R1273–R1278, 1996.
32. **Leone C and Gordon FJ.** Is L-glutamate a neurotransmitter of baroreceptor information in the nucleus of the tractus solitarius? *J Pharmacol Exp Ther* 250: 953–962, 1989.
33. **Machado BH and Bonagamba LG.** Microinjection of L-glutamate into the nucleus tractus solitarii increases arterial pressure in conscious rats. *Brain Res* 576: 131–138, 1992.
34. **Minson JB, Llewellyn-Smith IJ, Chalmers JP, Pilowsky PM, and Arnolda LF.** c-fos identifies GABA-synthesizing barosensitive neurons in caudal ventrolateral medulla. *Neuroreport* 8: 3015–3021, 1997.
35. **Morilak DA, Somogyi P, McIlhinney RA, and Chalmers J.** An enkephalin-containing pathway from nucleus tractus solitarius to the pressor area of the rostral ventrolateral medulla of the rabbit. *Neuroscience* 31: 187–194, 1989.
36. **Morrison SF and Reis DJ.** Reticulospinal vasomotor neurons in the RVL mediate the somatosympathetic reflex. *Am J Physiol Regul Integr Comp Physiol* 256: R1084–R1097, 1989.
37. **Morrison SF, Callaway J, Milner TA, and Reis DJ.** Rostral ventrolateral medulla: a source of the glutamatergic innervation of the sympathetic intermediolateral nucleus. *Brain Res* 562: 126–135, 1991.
38. **Natarajan M and Morrison SF.** Sympathoexcitatory CVLM neurons mediate responses to caudal pressor area stimulation. *Am J Physiol Regul Integr Comp Physiol* 279: R364–R374, 2000.
39. **Otake K, Ezure K, Lipski J, and Wong She RB.** Projections from the commissural subnucleus of the nucleus of the solitary tract: an anterograde tracing study in the cat. *J Comp Neurol* 324: 365–378, 1992.
40. **Paxinos G and Watson C.** *The Rat Brain in Stereotaxic Coordinates* (4th ed.). New York: Academic, 1988.
41. **Ross CA, Ruggiero DA, and Reis DJ.** Projections from the nucleus tractus solitarii to the rostral ventrolateral medulla. *J Comp Neurol* 242: 511–534, 1985.
42. **Sato MA, Menani JV, Lopes OU, and Colombari E.** Commissural NTS lesions and cardiovascular responses in aortic baroreceptor-denervated rats. *Hypertension* 34: 739–743, 1999.
43. **Sato MA, Colombari E, and Morrison SF.** Inhibition of neurons in commissural nucleus of solitary tract reduces sympathetic nerve activity in SHR. *Am J Physiol Heart Circ Physiol* 282: H1679–H1684, 2002.
44. **Sato MA, Schoorlemmer GH, Menani JV, Lopes OU, Colombari E.** Recovery of high blood pressure after chronic lesions of the commissural NTS in SHR. *Hypertension* 42: 713–718, 2003.
45. **Schreihofer AM and Guyenet PG.** The baroreflex and beyond: control of sympathetic vasomotor tone by GABAergic neurons in the ventrolateral medulla. *Clin Exp Pharmacol Physiol* 29: 514–521, 2002.
46. **Sun MK and Guyenet PG.** GABA-mediated baroreceptor inhibition of reticulospinal neurons. *Am J Physiol Regul Integr Comp Physiol* 249: R672–R680, 1985.
47. **Sved AF, Ito S, and Madden CJ.** Baroreflex dependent and independent roles of the caudal ventrolateral medulla in cardiovascular regulation. *Brain Res Bull* 51: 129–133, 2000.
48. **Talman WT, Perrone MH, and Reis DJ.** Evidence for L-glutamate as the neurotransmitter of baroreceptor afferent nerve fibers. *Science* 209: 813–815, 1980.
49. **Urbanski RW and Sapru HN.** Evidence for a sympathoexcitatory pathway from the nucleus tractus solitarii to the ventrolateral medullary pressor area. *J Auton Nerv Syst* 23: 161–174, 1988.
50. **Urbanski RW and Sapru HN.** Putative neurotransmitters involved in medullary cardiovascular regulation. *J Auton Nerv Syst* 25: 181–193, 1988.
51. **Vardhan A, Kachroo A, and Sapru HN.** Excitatory amino acid receptors in commissural nucleus of the NTS mediate carotid chemoreceptor responses. *Am J Physiol Regul Integr Comp Physiol* 264: R41–R50, 1993.
52. **Willette RN, Punnen S, Krieger AJ, and Sapru HN.** Interdependence of rostral and caudal ventrolateral medullary areas in the control of blood pressure. *Brain Res* 321: 169–174, 1984.
53. **Willette RN, Punnen-Grandy S, Krieger AJ, and Sapru HN.** Differential regulation of regional vascular resistance by the rostral and caudal ventrolateral medulla in the rat. *J Auton Nerv Syst* 18: 143–151, 1987.
54. **Zagon A and Spyer KM.** Stimulation of aortic nerve evokes three different response patterns in neurons of rostral VLM of the rat. *Am J Physiol Regul Integr Comp Physiol* 271: R1720–R1728, 1996.

Glutamatergic mechanisms in the commissural nucleus of the solitary tract are important for pressor response produced by CVLM inhibition

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ABSTRACT

In the present study we investigated the role of commissural nucleus of the solitary tract (commNTS) on the excitatory responses produced by CVLM deactivation. For that, we evaluated the effects on mean arterial pressure (MAP), heart rate (HR) and renal, mesenteric and hindquarter vascular resistances produced by either electrolytic lesion or blockade of glutamatergic mechanisms in the commNTS with kynurenic acid (kyn) combined with the deactivation of the caudal ventrolateral medulla (CVLM) mechanisms by injections of the $GABA_A$ agonist muscimol or kyn. Male Holtzman rats anesthetized with urethane/chloralose, iv, were used. Bilateral injections of muscimol (2 mM – 50 nL) into the CVLM alone increased MAP (181 ± 6 mmHg, vs. control: 104 ± 3 mmHg), HR (418 ± 16 bpm, vs. control: 322 ± 12 bpm) and vascular resistances. However, the combination of muscimol into the CVLM with acute commNTS lesions or kyn into the commNTS reduced MAP to 67 ± 5 and 75 ± 3 mmHg, respectively HR to 279 ± 8 and 303 ± 7 bpm, respectively and vascular resistances below to control baseline levels. Bilateral injections of kyn (50 mM – 50 nL) into the CVLM alone also increased MAP, HR and vascular resistances, while the combination of kyn into the CVLM with kyn into the NTS also reduced MAP (81 ± 7 mmHg), HR (306 ± 11 bpm) and vascular resistances below to control baseline levels. The results suggest that important glutamatergic excitatory mechanisms are activated in the commNTS and CVLM to control arterial pressure, HR and vascular resistance under the conditions of the present experiments.

INTRODUCTION

The nucleus of the solitary tract (NTS) is the first synaptic integrative site for viscerosensory afferents in the brainstem including those related to respiratory and cardiovascular control and among them arterial baro and chemoreflexes (Ciriello et al., 1994; Dampney, 1994; Guyenet, 2006).

The central mechanisms related to baroreceptor reflexes includes GABAergic neurons in the caudal ventrolateral medulla (CVLM) that receive an excitatory projection from the NTS and in turn project to presympathetic neurons in the rostral ventrolateral medulla (RVLM) (Chan and Sawchenko, 1998; Gordon and Sved, 2002). Based upon numerous studies using anatomical, pharmacological and physiological approaches, baroreceptor-mediated decreases in sympathetic activity and blood pressure are believed to depend on activation of GABAergic CVLM neurons that project to presympathetic RVLM neurons. In parallel with the baroreceptor mechanisms, chemoreceptor afferents primarily innervate commissural NTS (commNTS) (Blessing et al., 1999). Most of the commNTS neurons that are activated by carotid body stimulation are not respiratory modulated and therefore are probably not part of the respiratory pattern generator (Koshiya and Guyenet, 1996; Paton et al., 2001). Many of these carotid body-responsive neurons project to the ventrolateral medulla where one of their targets has long been assumed to be the arterial pressure-regulating C1 neurons (Aicher et al., 1996; Koshiya and Guyenet, 1996; Paton et al., 2001). The existence of pressor mechanisms in the commNTS is supported by the increase in arterial pressure produced by L-glutamate injections into the NTS in anesthetized rats after the inhibition of the CVLM with muscimol (Urbanski and Sapru, 1988). This pressor response induced by L-glutamate into the commNTS in anesthetized rats is abolished by the blockade of excitatory amino acid (EAA) receptors in the RVLM

which suggests the existence of a pressor pathway from the commNTS to the RVLM, (Willette et al., 1987, Urbanski and Sapru, 1988). Electrolytic lesions in commNTS abolish the pressor response produced by stimulation of peripheral chemoreceptor with potassium cyanide without significant changes in baroreflex activity (Colombari et al., 1996; Sato et al., 2001).

Besides the inhibitory projection, studies have suggested that CVLM also sends excitatory projections to the RVLM (Ito and Sved, 1997; Natarajan and Morrison, 2000; Moreira et al., 2005). In accordance with this suggestion, a previous study from our laboratory (Moreira et al., 2005) demonstrated that the blockade of the EAA receptors with bilateral injection of kyn into the NTS (postremal region) combined with the inhibition of the CVLM with muscimol reduced arterial pressure and vascular resistance to a level below the baseline levels, suggesting that important pressor mechanisms arising from the NTS and CVLM are involved in the control of cardiovascular functions.

Although the medullary circuitry connecting baroreceptors and chemoreceptor inputs to sympathetic vasomotor outflow is well established (Guyenet, 2006), the excitatory inputs that activate presympathetic neurons in the RVLM are not completely understood. The increase in sympathetic activity and arterial pressure produced by the inhibition of the CVLM is suggested to be due to the disinhibition of the RVLM. However, besides inhibitory inputs from the CVLM, the RVLM may also receive excitatory projections from the CVLM and from the NTS (postremal region) that are important to control sympathetic activity and arterial pressure (Moreira et al., 2005). Therefore, the aim of the present study was to investigate the importance of excitatory mechanisms that arise from commNTS and CVLM for cardiovascular regulation. In addition, the possible activation by glutamatergic mechanisms of these excitatory pathways in the commNTS

and CVLM were also investigated. We also hypothesize that the postremal (Moreira et al., 2005) and the commissural (present results) region of the NTS could contribute for excitatory influences to RVLM neurons after blockade of the CVLM. For this, we tested: 1) the effects of electrolytic lesions of the commNTS on cardiovascular responses induced by bilateral inhibition of the CVLM with muscimol and, 2) the cardiovascular responses produced by bilateral deactivation of the CVLM mechanisms with muscimol or blockade of the EAA with kynurenic acid (kyn) combined with the blockade of EAA receptors within the commNTS with kyn. Some of these data have been published in abstract and presented at the 2006 Experimental Biology meeting.

RESULTS

Histological analysis

As shown in Figure 1, lesions of the commNTS were located on the midline above the central canal and extended from the level of the obex to ~1 mm caudal to the obex. Lesions destroyed the commNTS keeping intact the area postrema and lateral regions of the NTS. Other tissues like the ventromedial portions of the gracile nucleus and medial portions of the dorsal motor nucleus of vagus that lay adjacent to the lesioned area, presented only minimal damage. The size of the lesions was similar to that of previous studies (Colombari et al., 1996; Sato et al., 1999; 2001; 2003).

Figure 2A and 2B are photomicrographs showing the typical sites of the injections into the commissural region of the NTS and into the CVLM, respectively, in one rat representative of the rats used in the present study. Figure 2C and 2D are diagrammatic composites showing the injection sites in rats that received injections of kyn into the commNTS and muscimol into the CVLM. According to Paxinos and Watson (Paxinos and

Watson, 1988), Figure 2C represent slices of brain located approximately 14.08 mm and 14.3 mm caudal to bregma level and 2D represent slices of brain located approximately 13.3 mm and 13.6 mm caudal to bregma level.

The injection of dye extended 210 ± 13 μm on each side of the injection center and therefore muscimol, which probably diffuses more than the dye has likely reached the respiratory neurons located in the pre-Bötzinger complex (dorsal to the CVLM cardiovascular neurons). To exclude eventual effects related to respiratory responses, animals that presented respiratory changes were not analyzed.

Effects of bilateral injections of muscimol into the CVLM on MAP, HR and regional vascular resistances in rats with commNTS lesions.

Acute (30 min) commNTS-lesions did not affect baseline MAP (103 ± 5 mmHg, vs. sham lesions: 106 ± 3 mmHg) and HR (320 ± 9 bpm, vs. sham lesions: 324 ± 14 bpm).

Bilateral injections of the $GABA_A$ agonist muscimol (2 mM) into the CVLM in sham lesioned rats produced an intense increase in MAP (181 ± 6 mmHg), tachycardia (418 ± 16 bpm), and increase in renal (312 ± 24%), mesenteric (324 ± 39%) and hindquarter (350 ± 51%) vascular resistance, (Figure 3). However, in acute commNTS-lesioned rats, muscimol into the CVLM reduced MAP (67 ± 5 mmHg), HR (279 ± 8 bpm) and renal (-43 ± 12%), mesenteric (-55 ± 18%) and hindquarter (-58 ± 7%) vascular resistance below control baseline pre-injection level (Figure 3). The commNTS lesion performed in these series of experiments was the same as shown before that was capable to block the effect of peripheral chemoreflex activation and no effect on arterial baroreflex (Colombari et al., 1996; Sato et al., 1999; 2001; 2003).

Changes in MAP, HR and regional vascular resistances induced by kynurenic acid into the commNTS combined with muscimol into the CVLM.

The experiments described in the commNTS lesion experiments suggested that the commNTS neurons have an important role in the sympathoexcitatory effect produced by CVLM-inhibition, but we do not know exactly if this effect is due a destruction of fibers of passage or cell body located into the commNTS. In this next series of experiments, we injected kyn into the commNTS in animals with or without inhibition of the CVLM.

Baseline pre-treatment levels of MAP (100 ± 2 mmHg, $p>0.05$) and HR (325 ± 3 bpm, $p>0.05$) were similar in all experimental groups tested.

Bilateral injections of muscimol (2 mM in 50 nL) alone into the CVLM again induced sustained (at least 30 min of duration) hypertension, tachycardia, increase in renal, mesenteric, and hindquarter vascular resistances, while injections of kyn (50 mM in 50 nL) alone into the commNTS produced no change in MAP, HR and renal, mesenteric and hindquarter vascular resistances (Figure 4 and 5).

Differently from the responses after muscimol alone into the CVLM or kyn alone into the commNTS, the combination of muscimol into the CVLM with kyn into the commNTS immediately reduced MAP, HR and renal, mesenteric and hindquarter vascular resistances to a level below control pre-injection baseline levels independent if the injections of muscimol into the CVLM were performed before (Figure 4) or after kyn into the NTS (Figure 5).

Changes in MAP, HR and regional vascular resistances induced by combining injections of kynurenic acid into the commNTS and into the CVLM.

The next series of experiments we injected kyn into the commNTS in animals with or without blockade of the EAA receptors in the CVLM.

The pre-treatment baseline levels of MAP (108 ± 5 mmHg, $p>0.05$) and HR (324 ± 4 bpm, $p>0.05$) were similar in all experimental groups tested.

Bilateral injections of kyn (50 mM in 50 nL) alone into the CVLM increased MAP, HR and renal, mesenteric and hindquarter vascular resistances, while injections of kyn (50 mM in 50 nL) into the commNTS again produced no change on MAP, HR and renal, mesenteric and hindquarter vascular resistances (Figure 6 and 7).

Differently from the responses after kyn alone into the CVLM or into the commNTS, the combination of kyn into the CVLM with kyn into the commNTS immediately reduced MAP, HR and renal, mesenteric and hindquarter vascular resistances to a level below control pre-injection baseline levels independent if the injections of kyn into the CVLM were performed before (Figure 6) or after kyn into the commNTS (Figure 7).

Effects of muscimol and kynurenic acid injected outside the CVLM and commNTS on MAP, HR and regional vascular resistances

Bilateral injections of muscimol outside the CVLM or injection of kyn outside the commNTS, respectively produced no significant changes in the baseline MAP, HR or renal, mesenteric and hindquarter vascular resistances. Injections of kyn outside the NTS after muscimol into the CVLM produced no additional changes in MAP (177 ± 13 mmHg, vs. vehicle: 174 ± 6 mmHg, $p > 0.05$), HR (414 ± 13 bpm, vs. vehicle: 406 ± 8 bpm, $p > 0.05$) or renal (315 ± 31%, vs. vehicle: 313 ± 32%, $p > 0.05$), mesenteric (326 ± 28%, vs. vehicle:

319 ± 15%, p > 0.05) and hindquarter (356 ± 43%, vs. vehicle: 349 ± 33%, p > 0.05) vascular resistances.

The injections of kyn outside the commNTS or bilateral injection of muscimol outside the CVLM, respectively produced no significant changes in the baseline MAP, HR or renal, mesenteric and hindquarter vascular resistances. Injections of muscimol outside the CVLM after kyn into the commNTS produced no additional changes in MAP (104 ± 3 mmHg, vs. vehicle: 106 ± 5 mmHg, p > 0.05), HR (326 ± 8 bpm, vs. vehicle: 323 ± 16 bpm, p > 0.05) or renal (14 ± 36%, vs. vehicle: 15 ± 41%, p > 0.05), mesenteric (16 ± 25%, vs. vehicle: 16 ± 13%, p > 0.05) and hindquarter (15 ± 32%, vs. vehicle: 18 ± 19%, p > 0.05) vascular resistances.

Effects of kynurenic acid injected outside the CVLM and commNTS on MAP, HR and regional vascular resistances

Bilateral injections of kyn outside the CVLM or injection of kyn outside the commNTS, respectively, produced no significant changes in the baseline MAP, HR or renal, mesenteric and hindquarter vascular resistances. Injections of kyn outside the NTS after kyn into the CVLM produced no additional changes in MAP (138 ± 6 mmHg, vs. vehicle: 144 ± 7 mmHg, p > 0.05), HR (372 ± 15 bpm, vs. vehicle: 368 ± 8 bpm, p > 0.05) or renal (112 ± 21%, vs. vehicle: 120 ± 32%, p > 0.05), mesenteric (126 ± 18%, vs. vehicle: 129 ± 15%, p > 0.05) and hindquarter (124 ± 19%, vs. vehicle: 128 ± 25%, p > 0.05) vascular resistances.

The injections of kyn outside the commNTS or outside the CVLM, respectively produced no significant changes in the baseline MAP, HR or renal, mesenteric and hindquarter vascular resistances. Injections of kyn outside the CVLM after kyn into the

commNTS produced no additional changes in MAP (117 ± 12 mmHg, vs. vehicle: 108 ± 9 mmHg, $p > 0.05$), HR (326 ± 8 bpm, vs. vehicle: 323 ± 16 bpm, $p > 0.05$) or renal (14 ± 36%, vs. vehicle: 15 ± 41%, $p > 0.05$), mesenteric (16 ± 25%, vs. vehicle: 16 ± 13%, $p > 0.05$) and hindquarter (15 ± 32%, vs. vehicle: 18 ± 19%, $p > 0.05$) vascular resistances.

DISCUSSION

As shown by the present or previous studies (Natarajan and Morrison, 2000; Houriuchi et al., 2004; Schreihofer et al., 2005; Moreira et al., 2005; Moreira et al., 2006), inhibition of CVLM by muscimol or blockade of glutamatergic receptors of CVLM increases MAP, HR and renal, mesenteric and hindquarter vascular resistances. On the other hand, commNTS lesions or blockade of glutamatergic mechanisms in the commNTS produces no change of MAP, HR and vascular resistance. However, deactivation of CVLM mechanisms in rats with lesions of the commNTS or blockade of the glutamatergic receptors of the commNTS reduces MAP, HR and vascular resistances below control baseline levels. Misplaced injections of muscimol or kyn outside the CVLM or commNTS produce no significant changes in the baseline vascular resistances, MAP and HR. Therefore, the present results suggest that excitatory glutamatergic mechanisms arising from the commNTS and the CVLM are capable of maintaining arterial pressure, heart rate and vascular resistance under these experimental conditions.

Relationship with prior study

Besides the important pressor mechanisms arising from the postremal region of the NTS and from the CVLM (Moreira et al., 2005), the novelty in the present study is that the blockade of the glutamatergic receptors of the commNTS simultaneously with the

blockade of the CVLM reduces MAP, HR and vascular resistances below control preinjection baseline levels. Indeed, the present study confirms and extends the results from the previous study showing that excitatory mechanisms also arise from the commNTS and that these mechanisms depend on glutamatergic receptors. In our earliest study related to the postremal region of the NTS and CVLM (Moreira et al., 2005), we did encounter that after glutamatergic blockade of the NTS simultaneously with the CVLM inhibition reduced arterial pressure, mesenteric and hindquarter vascular resistance. However, in the previous study, we conclude that important excitatory mechanisms arising from the NTS (postremal) and CVLM control arterial pressure and vascular resistance. On the other hand, the present study have shown that the blockade of the commissural region of the NTS together with CVLM inhibition produced a much greater reduction in arterial pressure, heart rate and vascular resistance (renal, mesenteric and hindquarter). In view of the present results, we conclude and confirm the literature (Urbanski and Sapru, 1988; Colombari et al., 1996) that important excitatory mechanisms arise especially from commNTS to control and maintain cardiovascular functions.

Possible pathways: relationship with chemoreceptors

The tonic activity of RVLM sympathetic vasomotor neurons results from the combination of excitatory and inhibitory influences (Lipski et al., 1996; Sved et al., 2001; Dampney et al., 2003). The RVLM neurons also receive inputs from different medullary and pontine sources and part of them are GABAergic inputs from the CVLM that in turn are activated by excitatory baroreceptor-related inputs from the NTS (Chan and Sawchenko, 1998; Weston et al., 2003; Guyenet, 2006). Acute blockade of the CVLM neuron activity removes baroreceptor-mediated inhibition of presympathetic RVLM

neurons increasing their basal activity and the sympathetic vasomotor tone leading to increased arterial pressure (Koshiya and Guyenet, 1996; Horiuchi et al., 2004).

The excitatory influence to the RVLM sympathetic vasomotor neurons is related to peripheral chemoreflex and this pathway might become tonically active under the condition of CVLM-inhibition. Alternatively, a mechanism not related to the chemoreflex seems to activate excitatory mechanisms in the commNTS causing sympathetic activation. The stimulation of peripheral chemoreceptors increases sympathetic activity through a pathway that connects the commNTS to sympathetic premotor neurons in the RVLM (Urbaski and Sapru, 1988; Colombari et al., 1996; Koshiya and Guyenet, 1996) or through brainstem pathways that involve neurons located in the ventrolateral pons (Koshiya and Guyenet, 1994). Several studies have shown that commNTS neurons have monosynaptic connections with the RVLM (Ross et al., 1985; Dampney et al., 1987; Hancock, 1988; Morilak et al., 1989; Otake et al., 1992), which, in turn, projects monosynaptically to preganglionic sympathetic neurons (Hancock, 1988; Otake et al., 1992).

The deactivation of CVLM mechanisms increases vascular resistance, HR and MAP, while lesions of the commNTS or blockade of the EAA receptors with kyn into the commNTS do not affect MAP, HR and vascular resistance. However, the deactivation of CVLM mechanisms together with lesions of the commNTS or blockade of the EAA receptors with kyn into the commNTS reduces MAP, HR and vascular resistance to below control levels. These results suggest MAP, HR and vascular resistance increases produced by the inhibition of CVLM depend on facilitatory mechanisms arising from the commNTS. The commNTS may contain other types of neurons that up-regulate sympathetic tone besides those that convey peripheral chemoreceptor inputs as evidenced by: 1) the increase in arterial pressure elicited by microinjection of glutamate into commNTS (Urbaski et al.,

1988) and, 2) inhibition of commNTS neurons block the increase in blood pressure evoked by inhibiting neurons located in the CVLM (Natarajan *and* Morrison, 1998; Moreira et al., 2005).

A sympathoexcitatory pathway from the commNTS to the RVLM is suggested to exist based on anatomical and imunohistochemical studies and these mechanisms are related to peripheral chemoreceptor signals (Koshiya et al., 1993; Koshiya and Guyenet, 1996). The amino-acid L-glutamate is suggested to be the neurotransmitter released by the sympathoexcitatory pathway from commNTS to RVLM and by chemoreceptor afferent fibers in the commNTS (Vardhan et al., 1993; Chitravashi et al., 1994; 1995). Although kyn into the commNTS may block the chemoreflex, it is necessary to consider that chemoreceptors are usually silent in the absence of a proper stimulus and would not activate the pressor pathway from the commNTS to RVLM. But the present results demonstrate that the pressor responses to muscimol or kyn into the CVLM depend on a sympathoexcitatory mechanism that activates EAA receptors in the commNTS and probably involves the projections from commNTS to RVLM. We can not exclude the possibility of a pathway from commNTS to RVLM through the forebrain like PVN or the hypothalamus (Hardy, 2001). Excitation of the commNTS has been shown to increase MAP in both unanesthetized and urethane-anesthetized rats (Colombari et al., 1994; Urbanski et al., 1988). According to the present results, after the inhibition of the CVLM with muscimol, the EAA receptor activation in the commNTS is probably the mechanism that keeps arterial pressure, heart rate and vascular resistance above control levels.

Excitatory mechanisms in the brainstem: NTS and CVLM

Many studies have suggested the participation of excitatory mechanisms arising from different central areas to activated RVLM neurons in resting conditions or hypertensive states (Guyenet et al., 1987; Ito and Sved, 1997; Ito et al., 2000). Ito and Sved (1997) have demonstrated that arterial pressure is reduced below control resting levels by the blockade of EAA receptors with kyn in the RVLM combined with a previous blockade of the CVLM with muscimol. These data suggest that the increase in arterial pressure produced by the blockade of the CVLM is dependent on the RVLM excitation produced by EAA release (Ito and Sved, 1997). Similarly, the present study suggests that the activation of excitatory mechanisms is essential for the pressor response to muscimol into the CVLM, i.e. the sympathetic activation during CVLM inhibition is not only due to reduction of RVLM gabaergic inhibition but also results from the activation of glutamatergic facilitatory mechanisms in the NTS. Therefore, the present results suggest that one important excitatory projection to the RVLM activated during CVLM inhibition arises from the commNTS and utilize L-glutamate as a neurotransmitter in the commNTS.

Besides the blockade of the pressor response to muscimol into the CVLM, kyn into the RVLM also abolished the pressor response to muscimol into the NTS (Ito and Sved, 1997), which suggests that the excitatory projection to the RVLM arises not only from the NTS. Again, similar to previous studies (Ito and Sved, 1997; Natrajan and Morrison, 2000; Moreira et al., 2005), the present results suggest that the CVLM sends excitatory projections to the RVLM that are capable of maintaining arterial pressure, heart rate and vascular resistance. The evidence for this suggestion is the reduction of MAP, HR and vascular resistance to below control baseline levels after the dual blockade of the commNTS and CVLM. The blockade of glutamatergic receptors in the commNTS with kyn alone produces no alteration of MAP, HR and vascular resistance. However if

glutamatergic receptors of the commNTS were already blocked, the additional blockade of CVLM mechanisms with muscimol or kyn results in reduction of MAP, HR and vascular resistance below control levels. These results suggest that the maintenance of MAP, HR and vascular resistance at baseline levels after the blockade of glutamatergic receptors of the commNTS alone depends on excitatory mechanisms that arise from the CVLM and that are activated by L-glutamate in this area.

Neurons located into the caudal pressor area (CPA) may have a role as one of the sources of excitatory drive from the CVLM to the RVLM (Natarajan and Morrison, 2000). The paraventricular nucleus of the hypothalamus also sends a glutamatergic projection to the CVLM (Yang and Coote, 1999; Schreihofer, 2003). Other potential excitatory inputs to the CVLM may arise from respiratory-related pre-Bötzinger neurons, as evidenced by the effects of lesion of pre-Bötzinger neurons reducing the ability of the CVLM to change arterial pressure (Wang et al., 2002). Presympathetic RVLM neurons and sympathetic vasomotor nerves have respiratory-related activity (Haselton and Guyenet, 1989; Moreira et al., 2006) that may be conveyed by respiratory-related excitation of the CVLM. The spinal cord may be also another source of excitation to the CVLM because neurons in laminae I-IV in spinal dorsal horn project directly to the region of the CVLM (Spike et al., 2003).

Conclusion

Overall the present results suggest that the pressor responses produced by the blockade of the inhibitory mechanism to the RVLM are strongly dependent on the sympathoexcitatory projections that arise from the CVLM and from commNTS and are activated by the release of L-glutamate in these areas. In addition, these excitatory mechanisms are also important to maintain baseline arterial pressure and vascular

resistance because the blockade of both mechanisms simultaneously reduced arterial pressure and vascular resistance below control resting levels. However, more studies are still necessary to find the sources of the inputs that tonically activate the neurons in the CVLM and commNTS.

MATERIALS AND METHODS

General procedures. All experiments were performed in accordance with the Brazilian National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes and were approved by the Animal Experimentation Ethics Committee of the Federal University of São Paulo - School of Medicine.

Male Holtzman rats weighing 300-350 g were used. One day before the experiment, the rats were anesthetized with ketamine (80 mg kg^{-1} of body weight) combined with xylazine (7 mg kg^{-1} of body weight) and the femoral artery and vein were cannulated for arterial pressure measurement and drug administration, respectively. The arterial and venous catheters (PE-10 connected to PE-50) were tunneled subcutaneously and fixed on the back of the rat with suture thread. On the next day, immediately before the experiments the animals were anesthetized with urethane (1.0 g kg^{-1}, iv) combined with α-chloralose (60 mg kg^{-1}, iv). A midline laparotomy was performed and miniature pulsed Doppler flow probes were placed around the renal artery, superior mesenteric artery and low abdominal aorta for measurement of renal, mesenteric and hindquarter blood flows, respectively. The probes were fixed to the surrounding tissues with suture thread, and the animals were immediately place in a stereotaxic apparatus in a prone position with the incisor bar at 11

mm below the intra-aural line. A partial occipital craniotomy was perfomed to expose the dorsal surface of the brainstem.

Arterial pressure, heart rate and regional blood flow recordings. The catheter inserted into the femoral artery was connected to a P23 Db pressure transducer (Statham Gould) coupled to a pre-amplifier (model ETH-200 Bridge Bio Amplifier, CB Sciences) connected to the Powerlab computer recording system (model Powerlab 16SP, ADInstruments) for measurement of pulsatile arterial pressure, mean arterial pressure (MAP) and heart rate (HR). The flow probes were connected to a Doppler flowmeter (Dept of Bioengineering, University of Iowa, Iowa City, IA, USA) also coupled to the Powerlab computer recording system. Details of the Doppler flow recording technique, including the reliability of the method for estimation of flow velocity, have been described previously (Haywood et al., 1981). Relative renal, mesenteric and hindquarter vascular resistance changes were calculated as the ratio of MAP and Doppler shifts. Data from animals in which the probes moved during the experiment were not considered for analysis.

The rectal temperature was maintained at 37°C with a thermostatically controlled heating pad. During the surgical procedure or recording period, if the animals were responsive to noxious toe pinch, a supplementary dose of urethane (0.1 g kg^{-1}) combined with α-chloralose (20 mg kg^{-1}) was administered iv.

Eletrolytic commNTS lesions. A partial craniotomy of the occipital bone was performed, and the dorsal surface of the brainstem was exposed. A tungsten electrode was positioned in the commNTS (0.2 to 0.3 mm caudal to the *calamus scriptorius*, 0.5 mm below the dorsal surface of the brain stem and in the midline) and a 2-mA current was passed for 10

seconds, as previously described (Colombari et al., 1996; Sato et al., 1999; 2001; 2003). Sham-lesioned rats were submitted to the same procedures, but no electric current was passed.

Central injections. Injections (50 nL, delivered over 5 s) of muscimol (2 mM) or saline into the CVLM and kynurenic acid (kyn, 50 mM) or vehicle into the commissural subnucleus of the NTS were performed using the same single-barrel glass pipette (20 µm tip diameter) coupled to a pressure injection apparatus (PicoSpritzer II). An injection was first performed in one side, the pipette was withdrawn from the brain and the contralateral injection was made; thus the two injections were made approximately 1 min apart, as the injection was performed bilaterally into the CVLM. The volume of each injection was estimated from the displacement of the fluid meniscus in the pipette using a calibrated reticule. Injections into the CVLM were made 0.5 mm rostral to the *calamus scriptorius*, 1.8 mm lateral to midline and 1.9 to 2.0 mm below the dorsal surface of the brainstem. Injections into the commNTS were made in the midline, 0.2 to 0.3 mm caudal to the *calamus scriptorius* and 0.5 mm below the dorsal surface of the brainstem.

Drugs. Muscimol and kyn were purchased from Sigma Chemical Co., USA. Muscimol was dissolved in isotonic saline. Kyn was initially dissolved in 100 mM sodium bicarbonate (in a volume that corresponded to 10% of the final volume) and then diluted with isotonic saline until reaching the final volume. The pH of kyn solution was around 7.4.

Histology. At the end of the experiments, a 2% solution of Evans blue was injected into the CVLM and commNTS (50 nL) using the same pipette that was previously used for drug

injection. Saline followed by 10% buffered formalin was perfused through the heart. The brains were removed, fixed in 10% formalin for at least 2 days, frozen, cut coronally into 50 μm sections and stained with Giemsa. The sections were analyzed by light microscopy to confirm the sites of injections into the CVLM or into the commNTS and the lesions of commNTS. Section alignment between brains was done relative to a reference section. To align sections around CVLM level, the most rostral section containing an identifiable cluster of facial motor neurons was identified in each brain and assigned the level 11.6 mm caudal to Bregma (Bregma -11.6 mm) according to the atlas of Paxinos and Watson (1998). Levels rostral or caudal to this reference section were determined by adding a distance corresponding to the interval between sections multiplied by the number of intervening sections. In case of lesions or injection sites at commissural NTS level, the reference section used to align all others was the one closest to mid-area postrema level (Bregma -13.8 mm).

Statistical analysis. Statistical analysis was done with Sigma Stat version 3.0 (Jandel Corporation, Point Richmond**,** CA). Data are reported as means ± standard error of the mean. Two-way parametric ANOVA followed by the Newman-Keul multiple comparisons test were used as appropriate. Significance was set at $P<0.05$.

Experimental protocols

1) Effects of bilateral injections of muscimol into the CVLM on MAP, HR and regional vascular resistances in commNTS-lesioned rats.

Blood flows, MAP and HR were continuously recorded during 80 minutes and were analyzed at every 10 minutes, starting the recording 10 minutes after the connections of the

arterial line to the pressure transducer. Control (baseline) values were recorded for 10 minutes and were analyzed immediately before electrolytic commNTS or sham lesion. These values were used as reference to calculate the changes produced by the treatments. Thirty minutes after commNTS or sham lesion, muscimol or saline was bilaterally injected into the CVLM and the cardiovascular responses were evaluated during the next 40 minutes.

Four groups of animals (n = 8 each group) were used to investigate the cardiovascular effects of bilateral injections of muscimol or saline into the CVLM in rats submitted to commNTS or sham lesions. In each rat, only one of the following combinations were tested:

1) Sham lesion + saline into the CVLM (control);

2) Sham lesion + muscimol into the CVLM;

3) CommNTS lesion + saline into the CVLM;

4) CommNTS lesion + muscimol into the CVLM.

2) Effects of kynurenic acid into the commNTS combined with muscimol into the CVLM on MAP, HR and regional vascular resistances

Muscimol or saline was injected into the CVLM before and after kyn or vehicle into the commNTS.

Blood flows, MAP and HR were continuously recorded during 60 minutes and were analyzed at every 10 minutes, starting the recording 10 minutes after the connections of the arterial line to the pressure transducer. Control (baseline) values were recorded for 10 minutes and were analyzed immediately before the first treatment. These values were used as reference to calculate the changes produced by the treatments. The second treatment was

performed 10 minutes later and the cardiovascular responses were evaluated during the next 40 minutes.

To test the first sequence of drug combinations, 4 groups of animals (n = 7 each group) were used and in each rats only one of the following combinations were tested:

1) Saline into the CVLM + vehicle into the commNTS (control);

2) Saline into the CVLM + kyn into the commNTS;

3) Muscimol into the CVLM + vehicle into the commNTS;

4) Muscimol into the CVLM + kyn into the commNTS.

To test the second sequence of drug combinations, 4 groups of animals (n = 7 each group) were also used and in each rats only one of the following combinations were tested:

1) Vehicle into the commNTS + saline into the CVLM (control);

2) Kyn into the commNTS + saline into the CVLM;

3) Vehicle into the commNTS + muscimol into the CVLM;

4) Kyn into the commNTS + muscimol into the CVLM.

3) Changes in MAP, HR and regional vascular resistances induced by combining injections of kynurenic acid into the commNTS and into the CVLM.

The experimental design was similar to that described on item 2 above, except that kyn was injected into the CVLM, instead of muscimol.

Four groups of animals (n = 8 each group) were used to test the cardiovascular effects produced by the combination of kyn or vehicle into the CVLM followed by kyn or vehicle into the commNTS. Another 4 groups of animals (n = 7 each group) were used to investigate the cardiovascular effects produced by the combination of kyn into the commNTS followed by kyn into the CVLM.

4) Effects of muscimol and kynurenic acid injected in sites outside the CVLM and commNTS on MAP, HR and regional vascular resistances.

To confirm the specificity of injection sites for the effects of muscimol and kyn on MAP, HR and regional vascular resistances, results from rats in which the injections did not reach the CVLM or the commNTS (misplaced injections) were also analyzed and presented in the Results section.

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REFERENCES

1) Aicher SA, Saravay RH, Cravo S, Jeske I, Morrison SF, Reis DJ, & Milner TA (1996). Monosynaptic projections from the nucleus tractus solitarii to C1 adrenergic neurons in the rostral ventrolateral medulla: Comparison with input from the caudal ventrolateral medulla. *J Comp Neurol* **373**, 62-75.

2) Blessing WW, Yu YH, & Nalivaiko E (1999). Medullary projections of rabbit carotid sinus nerve. *Brain Res* **816**, 405-410.

3) Chan RKW &Sawchenko PE (1998). Organization and transmitter specificity of medullary neurons activated by sustained hypertension: Implications for understanding baroreceptor reflex circuitry. *J Neurosci* **18**: 371-387.

4) Chitravanshi VC, Kachroo A & Sapru HN (1994). A midline area in the nucleus commissuralis of NTS mediates the phrenic nerve responses to carotid chemoreceptor stimulation. *Brain Res.* **662 (1-2)**: 127-133.

5) Chitravanshi VC & Sapru HN (1995). Chemoreceptor-sensitive neurons in commissural subnucleus of nucleus tractus solitarius of the rat. *Am. J. Physiol. (Reg. Integr. Comp. Physiol)* **268**: R851-R858.

6) Ciriello J, Hochstenbach S & Roder S (1994). Central projections of baroreceptor and chemoreceptor afferent fibers in the rat. In: *Nucleus of the Solitary Tract*, edited by Barraco IRA. Boca Raton, FL: CRC, p. 35-50.

7) Colombari E, Bonagamba LG & Machado BH (1994). Mechanisms of pressor and bradycardic responses to L-glutamate microinjected into the NTS of conscious rats. *Am. J. Physiol. (Reg. Integr. Comp. Physiol)* **266**: R730-R738.

8) Colombari E, Menani JV & Talman W (1996). Commissural NTS contribute to pressor responses to glutamate injected into the medial NTS of awake rats. *Am. J. Physiol. (Reg. Integr. Comp. Physiol.* **270**: R1220-R1225.

9) Dampney RA, Czachurski J, Dembowsky K, Goodchild K & Seller H (1987). Afferent connections and spinal projections of the pressor region in the rostral ventrolateral medulla of the cat. *J. Auton. Nerv. Syst.* **20**: 73-86.

10) Dampney RA (1994). Functional organization of central pathways regulating the cardiovascular system. *Physio.l Rev.* **74(2)**: 323-364.

11) Gordon FJ & Sved AF (2002). Neurotransmitters in central cardiovascular regulation: glutamate and GABA. *Clin. Exp. Pharmacol. Physiol.* **29(5-6)**: 522-524.

12) Dampney RAL, Horiuchi J, Tagawa T, Fontes MAP, Potts PD, and Polson JW (2003). Medullary and supramedullary mechanisms regulating sympathetic vasomotor tone. Acta Physiol Scand **177**: 209–218.

13) Guyenet PG, Filtz TM & Donaldson SR (1987). Role of excitatory amino acids in rat vagal and sympathetic baroreflexes. *Brain Res.* **407(2)**: 272-284.

14) Guyenet PG (2006). The sympathetic control of blood pressure. *Nat Rev Neurosci* **7(5)**: 335-346.

15) Hancock MB (1988). Evidence for direct projections from the nucleus of the solitary tract onto medullary adrenaline cells. *J. Comp. Neurol.* **276**: 460-468.

16) Hardy SG. (2001). Hypothalamic projections to cardiovascular centers of the medulla. *Brain Res.* **894(2)**: 233-240.

17) Haselton JR & Guyenet PG (1989). Central respiratory modulation of medullary sympathoexcitatory neurons in rat. *Am. J. Physiol. (Regul. Integr. Comp. Physiol.)* **256**: R739–R750.

18) Haywood JR, Shaffer A, Fastenow C, Fink GD & Brody MJ (1981). Regional blood flow measurement with pulsed Doppler flowmeter in conscious rat. *Am. J. Physiol. (Heart Circ. Physiol.)* **241**: H273-H278.

19) Horiuchi J, Killinger S & Dampney RA (2004). Contribution to sympathetic vasomotor tone of tonic glutamatergic inputs to neurons in the RVLM. *Am J Physiol (Reg. Integr. Comp. Physiol.)* **287(6)**: R1335-R1343.

20) Ito S & Sved AF (1997). Tonic glutamate-mediated control of rostral ventrolateral medulla and sympathetic vasomotor tone. *Am. J. Physiol. (Reg. Integr. Comp. Physiol.)* **273 (2)**: R487-R494.

21) Ito S, Komatsu K, Tsukamoto K & Sved AF (2000). Excitatory amino acids in the rostral ventrolateral medulla support blood pressure in spontaneously hypertensive rats. *Hypertension* **35 (1)**: 413-417.

22) Koshiya N, Huangfu D & Guyenet PG (1993). Ventrolateral medulla and sympathetic chemoreflex in the rat. *Brain Res.* **609 (1-2)**:174-184.

23) Koshiya N & Guyenet PG (1994). Role of the pons in the carotid sympathetic chemoreflex. *Am. J. Physiol. (Reg. Integr. Comp. Physiol.)* **267 (2)**: R508-518.

24) Koshiya N & Guyenet PG (1996). NTS neurons with carotid chemoreceptor inputs arborize in the rostral ventrolateral medulla. *Am. J. Physiol. (Reg. Integr. Comp. Physiol.)* **270**: R1273-R1278.

25) Lipski J, Kanjhan R, Kruszewska B, and Rong W (1996). Properties of presympathetic neurons in the rostral ventrolateral medulla in the rat: an intracellular study "in vivo." J Physiol **490**: 729–744.

26) Moreira TS, Sato MA, Takakura AC, Menani JV & Colombari E (2005). Role of pressor mechanisms from the NTS and CVLM in contro of arterial pressure. *Am. J. Physiol. (Reg. Integr. Comp. Physiol.)* **289**: R1416-R1425.

27) Moreira TS, Takakura AC, Colombari E & Guyenet PG (2006). Central chemoreflex and sympathetic vasomotor outflow. *J .Physiol.* in press.

28) Morilak DA, Somogyi P, McIlhinney RA & Chalmers, J (1989). An enkephalin-containing pathway from nucleus tractus solitarius to the pressor area of the rostral ventrolateral medulla of the rabbit. *Neuroscience* **31**: 187-194.

29) Natarajan M & Morrison SF (2000). Sympathoexcitatory CVLM neurons mediate responses to caudal pressor area stimulation. *Am. J. Physiol. (Reg. Integr. Comp. Physiol.)* **279**: R364-R374.

30) Natarajan, M & Morrison SF (1998). Tonic sympathoinhibition from the caudal ventrolateral medulla. Soc Neurosci Abstr **24**: 371.

31) Otake K, Ezure K, Lipski J & Wong She RB (1992). Projections from the commissural subnucleus of the nucleus of the solitary tract: an anterograde tracing study in the cat. *J. Comp. Neurol.* **324**: 365-378.

32) Paton JF, Deuchars J, Li YW, & Kasparov S (2001). Properties of solitary tract neurones responding to peripheral arterial chemoreceptors. *Neurosci* **105**, 231-248.

33) Paxinos G & Watson C (1988). The rat brain stereotaxic coordinate (4th ed.). New York: Academic press.

34) Ross CA, Ruggiero DA & Reis DJ (1985). Projections from the nucleus tractus solitarii to the rostral ventrolateral medulla. *J. Comp. Neurol.* **242**: 511-534.

35) Sato MA, Menani JV, Lopes OU & Colombari, E (1999). Commissural NTS lesions and cardiovascular responses in aortic baroreceptor-denervated rats. *Hypertension* **34 (2)**: 739-743.

36) Sato MA, Menani JV, Lopes OU & Colombari E (2001). Lesions of the commissural nucleus of the solitary tract reduce arterial pressure in spontaneously hypertensive rats. *Hypertension* **38**: 560–564.

37) Sato MA, Schoorlemmer GH, Menani JV, Lopes OU & Colombari E (2003). Recovery of high blood pressure after chronic lesions of the commissural NTS in SHR. *Hypertension* **42(4)**: 713-718.

38) Schreihofer AM (2003). Glutamatergic projections to the caudal ventrolateral medulla (CVLM) in the rat. *FASEB J* **17**: 578.8,.

39) Schreihofer AM, Ito S & Sved AF. (2005). Brain stem control of arterial pressure in chronic arterial baroreceptor-denervated rats. Am. J. Physiol. (Regul. Integr. Comp. Physiol.) **289(6)**: R1746-R1755.

40) Spike RC, Puskar Z, Andrew D & Todd AJ (2003). A quantitative and morphological study of projection neurons in lamina I of the rat lumbar cord. *Eur. J. Neurosci.* **18**: 2433–2448.

41) Sved AF, Ito S, Madden CJ, Stocker SD & Yajima Y (2001). Excitatory inputs to the RVLM in the context of the baroreceptor reflex. Ann NY Acad Sci **940**: 247–258.

42) Urbanski RW & Sapru HN (1988). Evidence for a sympathoexcitatory pathway from the nucleus tractus solitarii to the ventrolateral medullary pressor area. *J. Auton. Nerv. Syst.* **23**: 161-174.

43) Vardhan A, Kachroo A & Sapru HN (1993). Excitatory amino acid receptors in commissural nucleus of the NTS mediate carotid chemoreceptor responses. *Am. J. Physiol. (Reg. Integr. Comp. Physiol.)* **264 (1)**: R41-R50.

44) Wang H, Germanson TP & Guyenet PG (2002). Depressor and tachypneic responses to chemical stimulation of the ventral respiratory group are reduced by ablation of neurokinin-1 receptor-expressing neurons. *J. Neurosci.* **22**: 3755–3764.

45) Weston M, Wang H, Stornetta RL, Sevigny CP & Guyenet PG (2003). Fos expression by glutamatergic neurons of the solitary tract nucleus after phenylephrine-induced hypertension in rats. *J Comp Neurol* **460**: 525-541.

46) Willette RN, Punnen-Grandy S, Krieger AJ & Sapru HN (1987). Differential regulation of regional vascular resistance by the rostral and caudal ventrolateral medulla in the rat. *J. Auton. Nerv. Syst.* **18(2)**: 143-151.

47) Yang Z & Coote JH (1999). The influence of the paraventricular nucleus on baroreceptor dependent caudal ventrolateral medullary neurons of the rat. *Pflügers Arch.* **438**:47–52.

FIGURE LEGENDS

Figure 1: Location of commissural nucleus of the solitary tract (commNTS) lesion.

Photomicrograph of a coronal section of the brainstem showing the typical commNTS lesion (arrow). AP, area postrema; py, pyramidal tract**;** Sp5, spinal tract of trigeminal nerve; XII, hypoglossal nucleus.

Figure 2: Location of commissural nucleus of the solitary tract (commNTS) and caudal ventrolateral medulla (CVLM) injections.

Photomicrographs of a coronal sections of the brainstem showing the typical injections (arrow) into the (A) commissural region of the NTS and (B) CVLM and diagrammatic composite of a transverse sections of the rat medulla showing the sites where kyn and muscimol were injected approximately at the level (C) 14.08 mm - 14.3 mm, respectively and (D) 13.3 mm - 13.6 mm, respectively, caudal to bregma according to Paxinos and Watson, 1988. Filled circles indicate the centers of injection sites of kyn into the commNTS (n = 29) or muscimol (n = 22) or kyn (n = 15) into the CVLM, and open triangles indicate the centers of injection sites into the neighboring regions of the commNTS (n = 5) and CVLM (n = 6). Amb, nucleus ambiguous; cc, central canal; Gr, gracile nucleus; IO, inferior olive; LRt, Lateral reticular nucleus; NTS, nucleus of the solitary tract; for other abbreviations see Figure 1. Scale = 1 mm.

Figure 3: Cardiovascular effects produced by bilateral injections of muscimol into the CVLM in commNTS-lesioned rats.

(A) typical recording showing changes in pulsatile arterial pressure (PAP), mean arterial pressure (MAP), heart rate (HR), renal (RBF), mesenteric (MBF) and hindquarter (HBF) blood flows produced by bilateral injections of muscimol (mus, 2 mM) into the CVLM after commNTS lesion. Arrows indicated the moment of the lesions and the injections. (B) Mean arterial pressure (MAP), (C) heart rate (HR) and changes in (D) renal (RVR), (E) mesenteric (MVR) and (F) hindquarter (HVR) vascular resistances produced by bilateral injections of mus (2 mM) or saline into the CVLM before and after commNTS lesions. The results are represented as means ± SEM. ANOVA showed significant differences among the treatments for MAP [$F(3, 99) = 12.22$, $p<0.01$], HR [$F(3, 99) = 62.51$, $p<0.01$], renal [$F(3, 99) = 33.13$, $p<0.01$], mesenteric, [$F(3, 99) = 47.18$, $p<0.01$] and hindquarter [$F(3, 99) = 29.17$, $p<0.01$] vascular resistances. N = 8/group. *Different from sham commNTS + saline CVLM.

Figure 4: Cardiovascular effects produced by bilateral injections of muscimol into the CVLM combined with kynurenic acid into the commNTS.

(A) typical recording showing changes in pulsatile arterial pressure (PAP), mean arterial pressure (MAP), heart rate (HR), renal (RBF), mesenteric (MBF) and hindquarter (HBF) blood flows produced by bilateral injections of muscimol (mus, 2 mM) injected into the CVLM followed by injections of kynurenic acid (kyn, 50 mM) into the commNTS. Arrows indicated the moment of the injections. (B) Mean arterial pressure (MAP), (C) heart rate (HR) and changes in (D) renal (RVR), (E) mesenteric (MVR) and (F) hindquarter (HVR) vascular resistances produced by bilateral injections of mus (2 mM) or saline into the CVLM followed by injections of kyn (50 mM) or vehicle into the commNTS. The results are represented as means ± SEM. ANOVA showed significant differences among the

treatments for MAP [$F(3, 113) = 22.04$, $p<0.01$], HR [$F(3, 113) = 113.74$, $p<0.01$], renal [$F(3, 115) = 62.66$, $p<0.01$], mesenteric, [$F(3, 113) = 52.13$, $p<0.01$] and hindquarter [$F(3, 113) = 55.69$, $p<0.01$] vascular resistances. N = 7/group. *Different from saline CVLM + vehicle commNTS.

Figure 5: Cardiovascular effects produced by injection of kynurenic acid into the commNTS combined with muscimol into the CVLM.

(A) typical recording showing changes in pulsatile arterial pressure (PAP), mean arterial pressure (MAP), heart rate (HR), renal (RBF), mesenteric (MBF) and hindquarter (HBF) blood flows produced by injections of kynurenic acid (kyn, 50 mM) into the commNTS followed by bilateral injections of muscimol (mus, 2 mM) into the CVLM. Arrows indicated the moment of the injections. (B) Mean arterial pressure (MAP), (C) heart rate (HR) and changes in (D) renal (RVR), (E) mesenteric (MVR) and (F) hindquarter (HVR) vascular resistances produced by injections of kyn (50 mM) or vehicle into the commNTS followed by bilateral injection of mus (2 mM) or saline into the CVLM. The results are represented as means ± SEM. ANOVA showed significant differences among the treatments for MAP [$F(3, 113) = 15.84$, $p<0.01$], HR [$F(3, 113) = 29.37$, $p<0.01$], renal [$F(3, 113) = 95.67$, $p<0.01$], mesenteric [$F(3, 113) = 129.26$, $p<0.01$] and hindquarter [$F(3, 113) = 56.92$, $p<0.01$] vascular resistances. N = 7/group. *Different from vehicle commNTS + saline CVLM.

Figure 6: Cardiovascular effects produced by bilateral injections of kynurenic acid into the CVLM combined with kynurenic acid into the commNTS.

(A) typical recording showing changes in pulsatile arterial pressure (PAP), mean arterial pressure (MAP), heart rate (HR), renal (RBF), mesenteric (MBF) and hindquarter (HBF) blood flows produced by bilateral injections of kynurenic acid (kyn, 50 mM) injected into the CVLM followed by injections of kyn (50 mM) into the commNTS. Arrows indicated the moment of the injections. (B) Mean arterial pressure (MAP), (C) heart rate (HR) and changes in (D) renal (RVR), (E) mesenteric (MVR) and (F) hindquarter (HVR) vascular resistances produced by bilateral injections of kyn (50 mM) or vehicle into the CVLM followed by injections of kyn (50 mM) or vehicle into the commNTS. The results are represented as means ± SEM. ANOVA showed significant differences among the treatments for MAP [$F(3, 99) = 112.06$, $p<0.01$], HR [$F(3, 99) = 87.47$, $p<0.01$], renal [$F(3, 99) = 125.66$, $p<0.01$], mesenteric, [$F(3, 99) = 49.33$, $p<0.01$] and hindquarter [$F(3, 99) = 113.77$, $p<0.01$] vascular resistances. N = 8/group. *Different from vehicle CVLM + vehicle commNTS.

Figure 7: Cardiovascular effects of kynurenic acid into the commNTS combined with kynurenic acid into the CVLM.

(A) typical recording showing changes in pulsatile arterial pressure (PAP), mean arterial pressure (MAP), heart rate (HR), renal (RBF), mesenteric (MBF) and hindquarter (HBF) blood flows produced by injections of kynurenic acid (kyn, 50 mM) into the commNTS followed by bilateral injections of kyn (50 mM) into the CVLM. Arrows indicated the moment of the injections. (B) Mean arterial pressure (MAP), (C) heart rate (HR) and changes in (D) renal (RVR), (E) mesenteric (MVR) and (F) hindquarter (HVR) vascular resistances produced by injections of kyn (50 mM) or vehicle into the commNTS followed by bilateral injection of kyn (50 mM) or vehicle into the CVLM. The results are

represented as means ± SEM. ANOVA showed significant differences among the treatments for MAP [$F(3, 113) = 39.12$, $p<0.01$], HR [$F(3, 113) = 44.27$, $p<0.01$], renal [$F(3, 113) = 111.13$, $p<0.01$], mesenteric [$F(3, 113) = 149.32$, $p<0.01$] and hindquarter [$F(3, 113) = 99.92$, $p<0.01$] vascular resistances. N = 7/group. *Different from vehicle commNTS + vehicle CVLM.

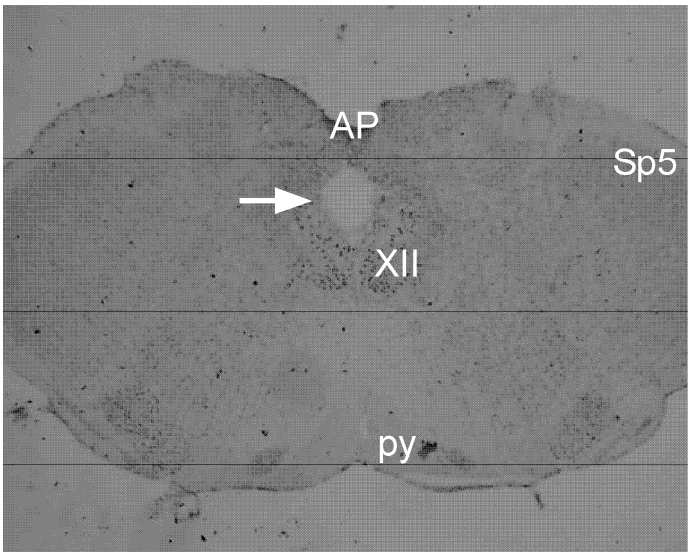


Figure 01

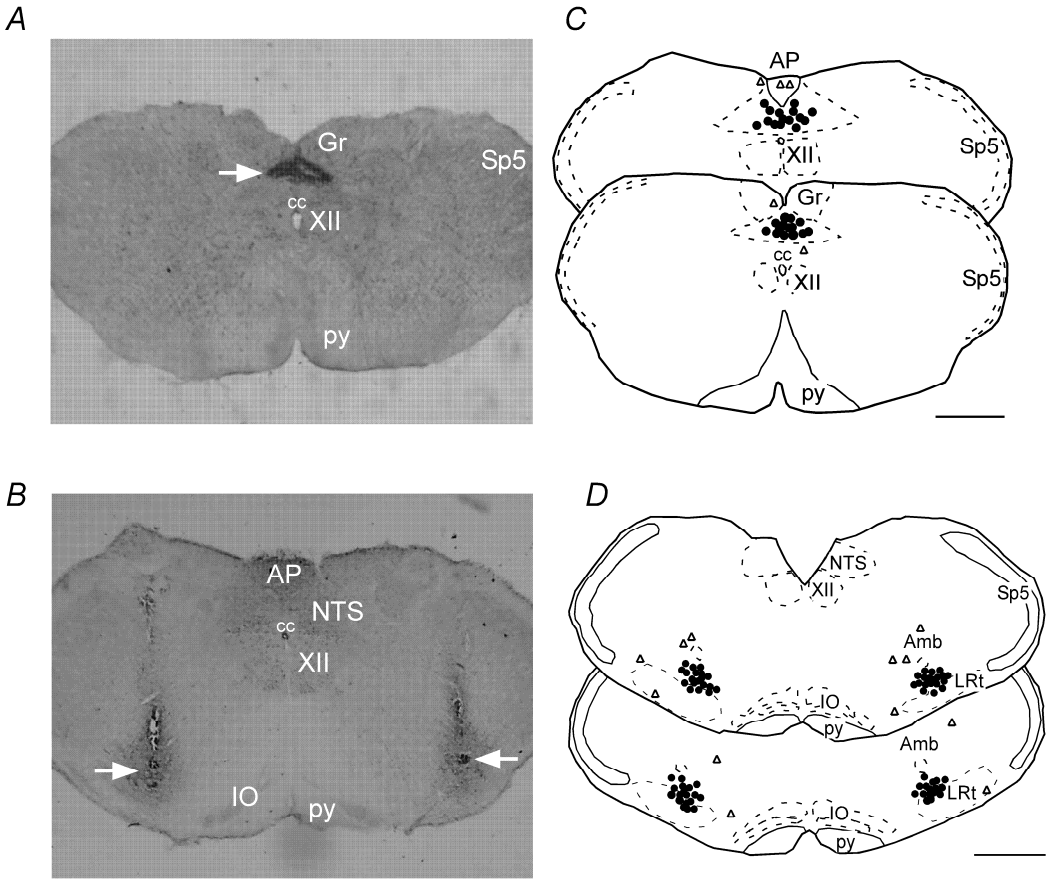

A
Gr
Sp5
cc
XII
py
C
AP
XII
Sp5
Gr
cc
XII
Sp5
py
B
AP
NTS
cc
XII
IO
py
D
NTS
XII
Sp5
Amb
LRt
IO
py
Amb
LRt
IO
py

Figure 02

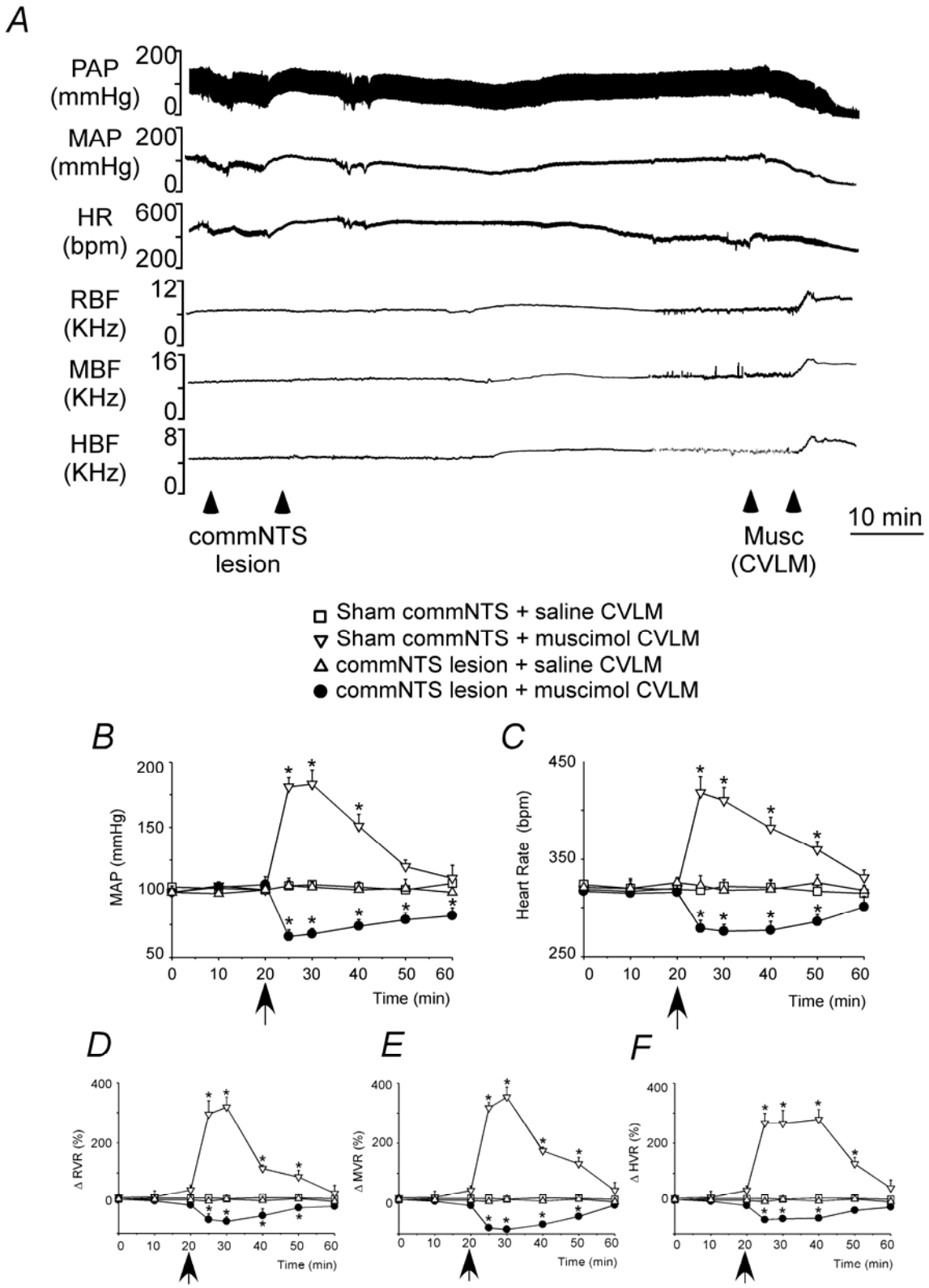

A
PAP (mmHg)
200
0
MAP (mmHg)
200
0
HR (bpm)
600
200
RBF (KHz)
12
0
MBF (KHz)
16
0
HBF (KHz)
8
0
commNTS lesion
Musc (CVLM)
10 min
Sham commNTS + saline CVLM
Sham commNTS + muscimol CVLM
commNTS lesion + saline CVLM
commNTS lesion + muscimol CVLM
B
MAP (mmHg)
200
150
100
50
0 10 20 30 40 50 60
Time (min)
C
Heart Rate (bpm)
450
350
250
0 10 20 30 40 50 60
Time (min)
D
Δ RVR (%)
400
200
0
0 10 20 30 40 50 60
Time (min)
E
Δ MVR (%)
400
200
0
0 10 20 30 40 50 60
Time (min)
F
Δ HVR (%)
400
200
0
0 10 20 30 40 50 60
Time (min)

Figure 03

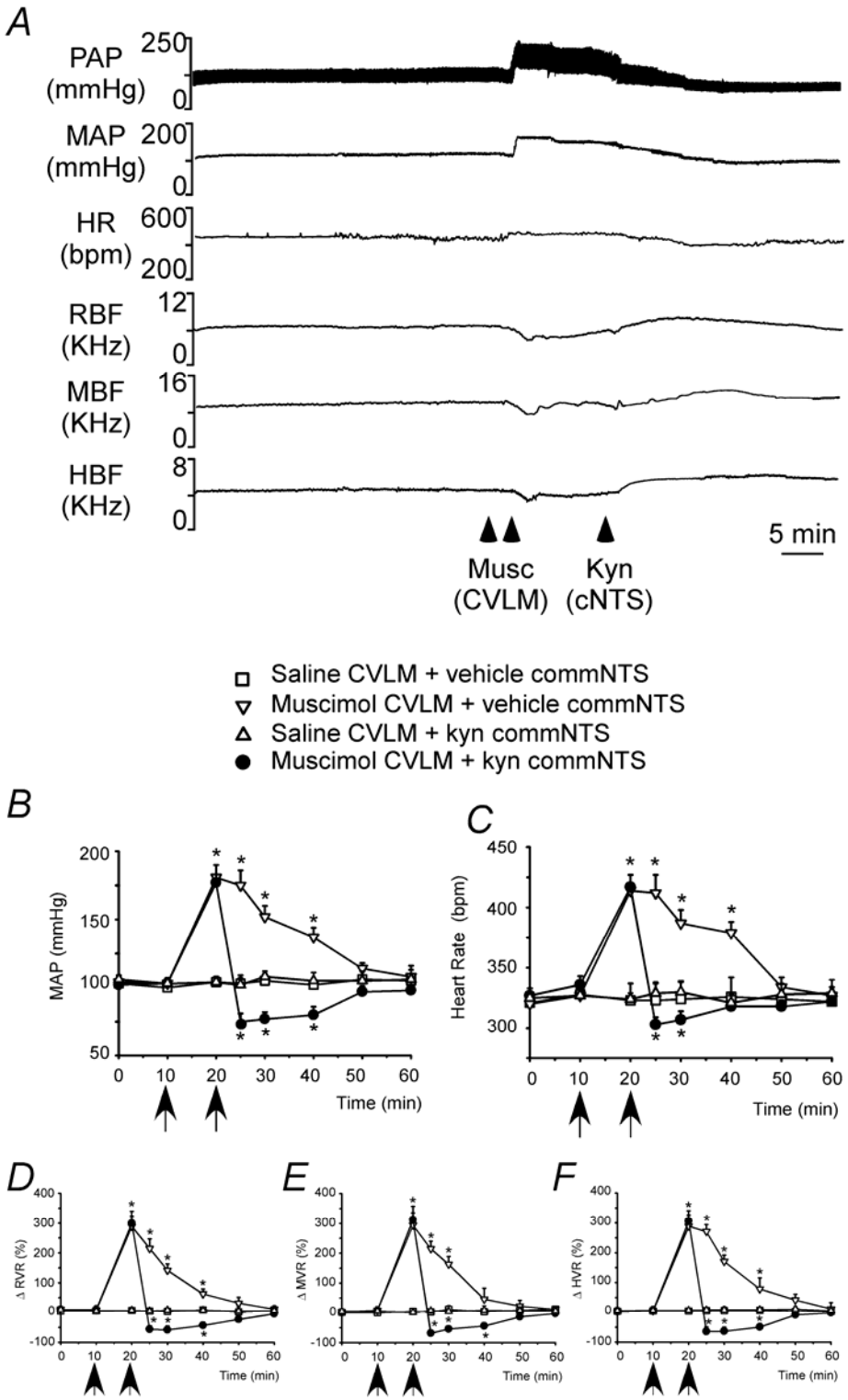

A
PAP (mmHg)
250
0
MAP (mmHg)
200
0
HR (bpm)
600
200
RBF (KHz)
12
0
MBF (KHz)
16
0
HBF (KHz)
8
0
5 min
Musc (CVLM)
Kyn (cNTS)
Saline CVLM + vehicle commNTS
Muscimol CVLM + vehicle commNTS
Saline CVLM + kyn commNTS
Muscimol CVLM + kyn commNTS
B
MAP (mmHg)
Time (min)
C
Heart Rate (bpm)
Time (min)
D
Δ RVR (%)
Time (min)
E
Δ MVR (%)
Time (min)
F
Δ HVR (%)
Time (min)

Figure 04

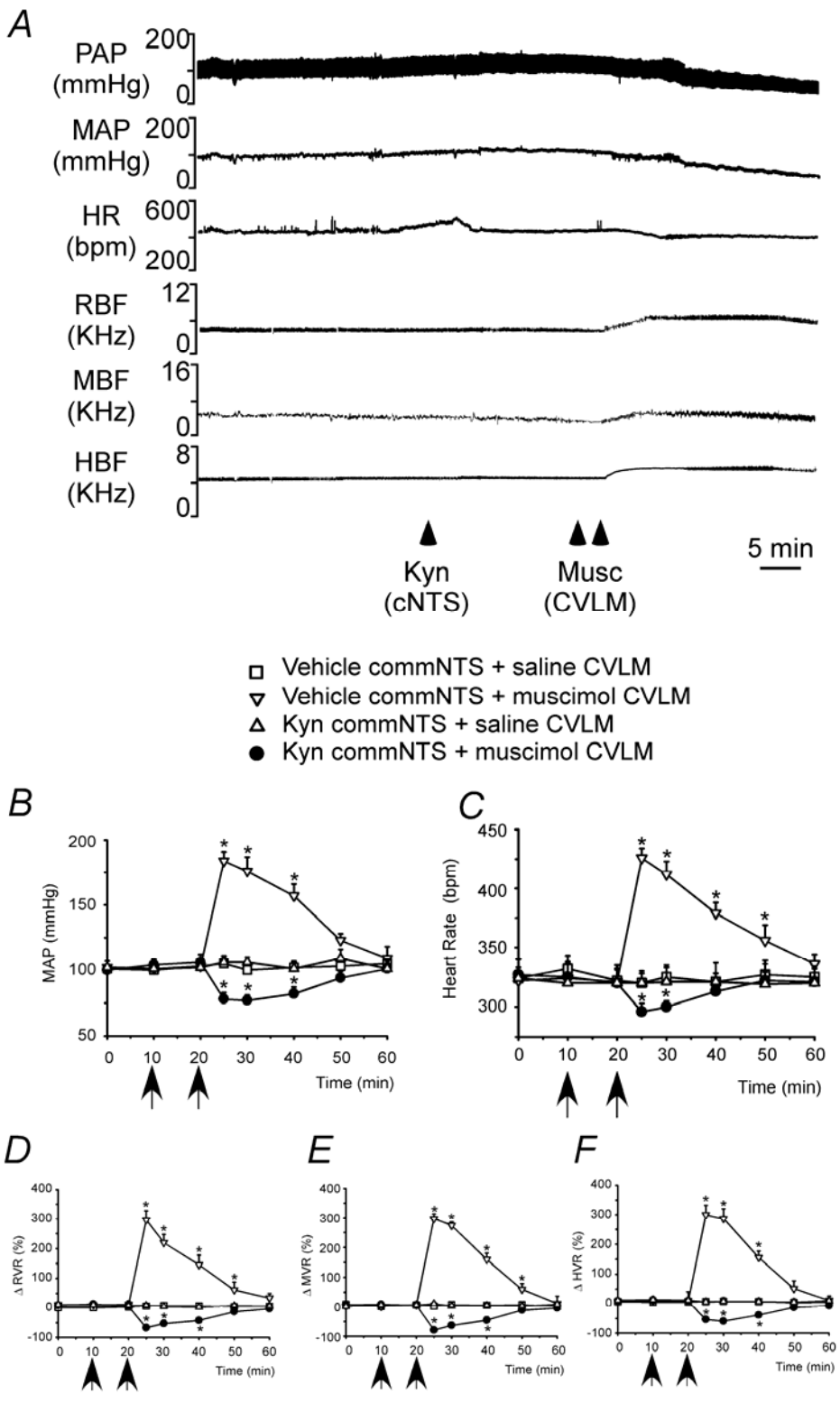

A
PAP (mmHg)
200
0
MAP (mmHg)
200
0
HR (bpm)
600
200
RBF (KHz)
12
0
MBF (KHz)
16
0
HBF (KHz)
8
0
Kyn (cNTS)
Musc (CVLM)
5 min
Vehicle commNTS + saline CVLM
Vehicle commNTS + muscimol CVLM
Kyn commNTS + saline CVLM
Kyn commNTS + muscimol CVLM
B
MAP (mmHg)
Time (min)
C
Heart Rate (bpm)
Time (min)
D
Δ RVR (%)
Time (min)
E
Δ MVR (%)
Time (min)
F
Δ HVR (%)
Time (min)

Figure 05

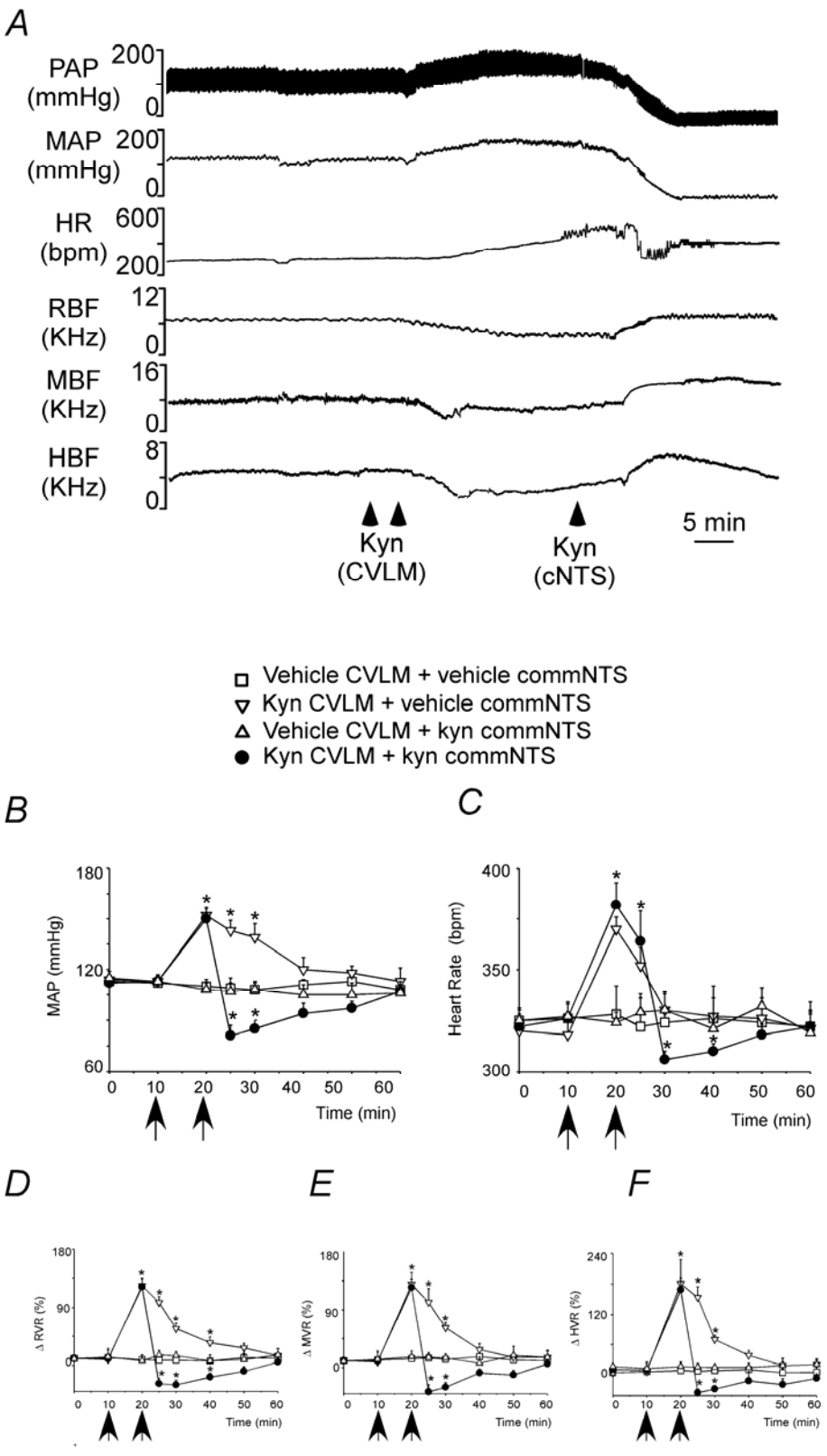

A
PAP (mmHg)
200
0
MAP (mmHg)
200
0
HR (bpm)
600
200
RBF (KHz)
12
0
MBF (KHz)
16
0
HBF (KHz)
8
0
Kyn (CVLM)
Kyn (cNTS)
5 min
Vehicle CVLM + vehicle commNTS
Kyn CVLM + vehicle commNTS
Vehicle CVLM + kyn commNTS
Kyn CVLM + kyn commNTS
B
MAP (mmHg)
180
120
60
0 10 20 30 40 50 60
Time (min)
C
Heart Rate (bpm)
400
350
300
0 10 20 30 40 50 60
Time (min)
D
Δ RVR (%)
180
90
0 10 20 30 40 50 60
Time (min)
E
Δ MVR (%)
180
90
0
0 10 20 30 40 50 60
Time (min)
F
Δ HVR (%)
240
120
0
0 10 20 30 40 50 60
Time (min)

Figure 06

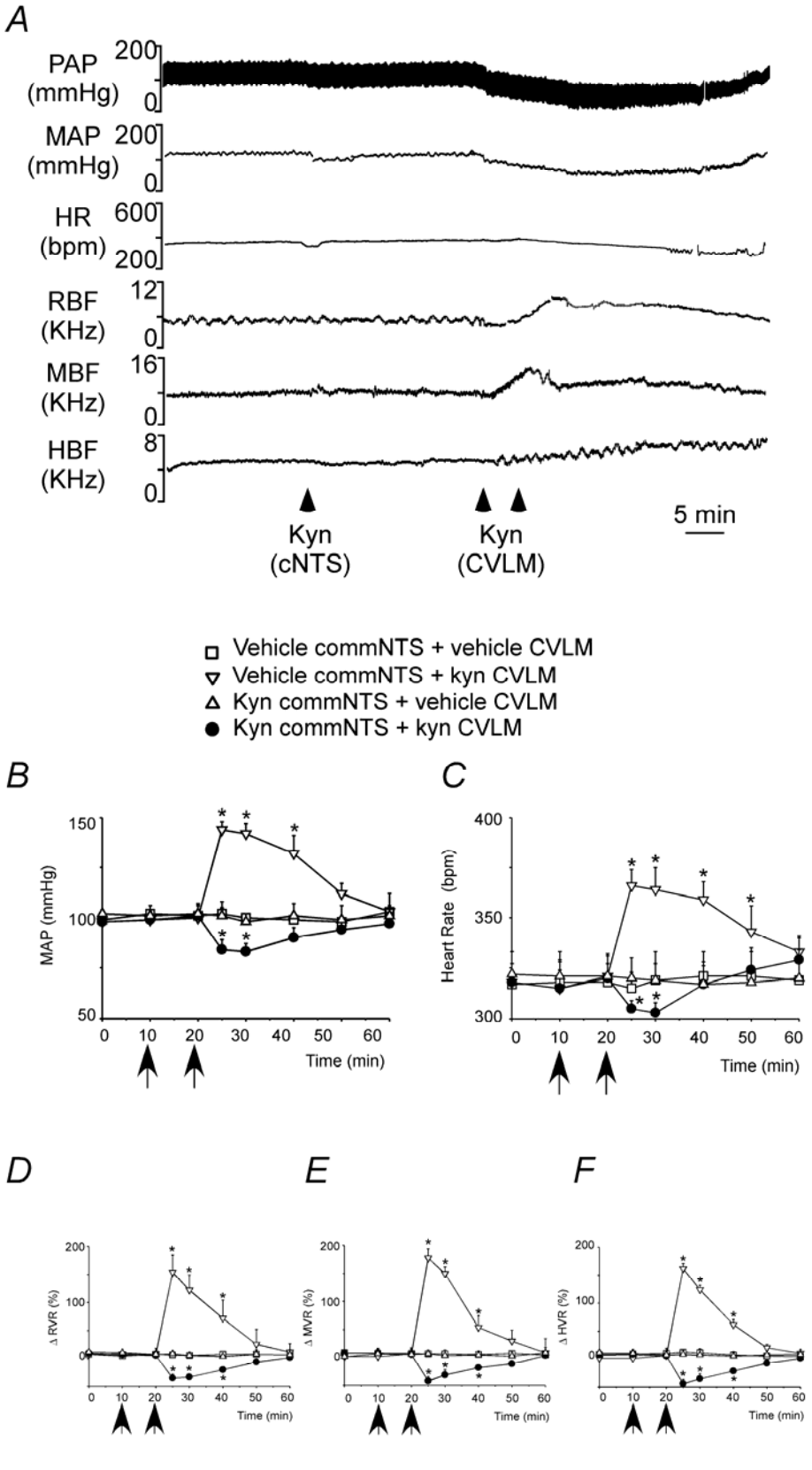

A
PAP (mmHg)
200
0
MAP (mmHg)
200
0
HR (bpm)
600
200
RBF (KHz)
12
0
MBF (KHz)
16
0
HBF (KHz)
8
0
Kyn (cNTS)
Kyn (CVLM)
5 min
Vehicle commNTS + vehicle CVLM
Vehicle commNTS + kyn CVLM
Kyn commNTS + vehicle CVLM
Kyn commNTS + kyn CVLM
B
MAP (mmHg)
150
100
50
0 10 20 30 40 50 60
Time (min)
C
Heart Rate (bpm)
400
350
300
0 10 20 30 40 50 60
Time (min)
D
Δ RVR (%)
200
100
0
0 10 20 30 40 50 60
Time (min)
E
Δ MVR (%)
200
100
0
0 10 20 30 40 50 60
Time (min)
F
Δ HVR (%)
200
100
0
0 10 20 30 40 50 60
Time (min)

Figure 07

Discussão e Conclusão

A principal novidade da primeira parte dessa tese mostra que o bloqueio dos receptores glutamatérgicos do NTS (região postremal ou comissural), juntamente com a inibição bilateral da região do CVL, reduz a pressão arterial e a resistência vascular abaixo dos valores controles. Do mesmo modo, a lesão eletrolítica da região comissural do NTS, juntamente com a inibição bilateral do CVL produz redução da pressão arterial e a resistência vascular abaixo dos valores controles. Vale ressaltar que, diferente da injeção bilateral de ácido quinurênico no NTS postremal, a lesão eletrolítica ou o bloqueio glutamatérgico da região comissural do NTS não produz alterações nos valores basais da pressão arterial e resistência vascular.

Portanto, os dois primeiros trabalhos sugerem que importantes mecanismos excitatórios existem no NTS (postremal e comissural) e também no CVL e estão envolvidos no controle das variáveis cardiovasculares. Vale ressaltar que esses mecanismos foram estudados numa determinada condição experimental, condição essa na qual realizamos inibição da região do CVL e bloqueios na região do NTS. Situações de inibição da região do CVL ou bloqueio da região do NTS são sinônimos de hipertensão. Quando realizamos a combinação de inibições (CVL e NTS), esperávamos que a hipertensão fosse ainda maior, o que relamente não aconteceu. Na sequência de inibições do NTS e do CVL, observamos uma redução e/ou manutenção dos níveis de pressão arterial próximo dos níveis basais. Portanto, nessa situação, quem estaria mantendo o tônus simpático? O NTS e CVL deixam de manter inibição sobre o RVL, e então, quais os mecanismos responsáveis para manter o controle excitação e inibição sobre o RVL? Certamente, nessa tese iremos tentar esclarecer alguns dos possíveis mecanismos responsáveis por esses efeitos, mas futuros experimentos serão necessários para completamente esclarecer esses fatos.

Importantes mecanismos cardiovasculares envolvendo o NTS, CVL e RVL têm sido exaustivamente propostos na literatura (Dampney, 1994; Colombari e cols., 2001; Guyenet, 2006).

Importantes mecanismos, envolvendo a região bulbar, estão relacionados a ativação de vários reflexos, como por exemplo o quimiorreflexo e o barorreflexo. Os quimiorreceptores periféricos promovem a ativação dos neurônios pré-motores simpáticos da região do RVL via, basicamente, 2 mecanismos excitatórios: um mecanismo excitatório direto entre o NTS e os neurônios do RVL (Urbanski e Sapru, 1988a; Colombari e cols., 1996; Koshiya e Guyenet, 1996) ou, indiretamente, via o grupamento noradrenérgico A5, localizado na ponte ventrolateral (Koshiya e Guyent, 1994; Guyenet, 2000). Por outro lado, os barorreceptores enviam aferências excitatórias para neurônios de segunda ordem, localizados na região postremal do NTS. Esses neurônios projetam-se para a região CVL, local da população de neurônios inibitórios (GABAérgicos), que por sua vez se projetam para a região do RVL (Dampney, 1994; Colombari e cols., 2001; Gordon e Sved, 2002; Guyente, 2006). O aminoácido excitatório L-glutamato parece ser o principal neurotransmissor liberado pelas aferências dos baro e quimiorreflexos (Talman e cols., 1980; Leone e Gordon, 1989; Ciriello e cols., 1994; Dampney, 1994; Gordon e Sved, 2002). O bloqueio do barorreflexo com a injeção bilateral do antagonista de receptores glutamatérgicos ionotrópicos, ácido quinurênico, no NTS promove uma desativação do mecanismo inibitório do CVL, resultando numa redução da inibição para os neurônios pré-motores simpáticos do RVL e, consequentemente, num aumento da atividade simpática e da pressão arterial. A injeção bilateral do agonista GABAérgico muscimol na região do CVL também promove uma redução da inibição para os neurônios do RVL, produzindo efeitos similares ao bloqueio glutamatérgicos na região do NTS (Willette e cols., 1987; Blessing, 1988; Cravo e cols., 1991; Moreira e cols., 2005; 2006; 2007). Embora os sinais do barorreflexo sejam importantes para inibir os neurônios pré-motores simpáticos do RVL, vale ressaltar que parte da inibição para os neurônios do RVL parece ser não dependente do barorreflexo (Cravo e cols., 1991). Isso pode explicar por que, em nossos experimentos, o aumento de pressão arterial e resistência vascular é muito maior com a injeção bilateral de muscimol no CVL em relação a injeção bilateral de ácido quinurênico no NTS. Certamente, nossos resultados em adição aos da literatura, nos permite sugerir

que os neurônios da região do CVL devem ser a fonte principal de inibição direta sobre o RVL, enquanto que a projeção do NTS para o RVL parece ser predominantemente excitatórias.

Independentemente, a injeção bilateral de muscimol no CVL ou a injeção bilateral de ácido quinurênico no NTS promove uma desinibição para a região do RVL e, consequentemente, um aumento da atividade simpática, resistência vascular e pressão arterial. Entretanto, quando esses tratamentos são feitos simultaneamente, isto é, injeção bilateral de muscimol no CVL seguido da injeção bilateral de ácido quinurênico no NTS postremal, a resistência vascular e pressão arterial reduzem para valores abaixo dos valores basais (controles). O mesmo efeito é também observado quando são feitas injeções simultâneas de muscimol no CVL, juntamente com a injeção de ácido quinurênico ou lesão eletrolítica na região caudal e medial (comissural) do NTS. A única diferença é que o bloqueio ou a lesão do NTScom sozinho não produz alterações na pressão arterial ou resistência vascular basais. Este dado por si é muito importante no cenário da literatura que invariavelmente cita lesões ou inibições do NTS como fonte de hipertensão. Diferentes trabalhos do nosso laboratório mostram que lesões ou inibições da porção comissural do NTS não promovem modificações na resistência periférica e consequentemente na pressão arterial basal. Ademais, em situações de hipertensão, tal lesão ou inibição do NTS comissural promove uma redução de atividade simpática associada com redução de níveis pressóricos (Sato e cols., 2001; 2002). Esses resultados também sugerem que o aumento de pressão arterial e resistência vascular produzido pela inibição apenas do CVL depende de mecanismos excitatórios localizados no NTS intermediário e/ou comissural. Os efeitos de aumento de pressão arterial e resistência vascular produzido apenas pelo bloqueio do NTS intermediário (postremal) dependem de mecanismos excitatórios localizados no CVL, associados evidentemente com a remoção de projeções do NTS para o CVL dependente de barorreceptores que são inibitórios sobre o RVL.

Esses resultados sugerem que o aumento de pressão arterial e resistência vascular produzido pela inibição apenas do CVL depende de mecanismos

excitatórios localizados no NTS. Os efeitos de aumento de pressão arterial e resistência vascular produzido apenas pelo bloqueio do NTS depende de mecanismos excitatórios localizados no CVL.

A existência de projeções excitatórias da região CVL para a região RVL do bulbo foi primeiramente proposta por Ito e Sved (Ito e Sved, 1997), como sendo parte de um modelo conceitual de interconexões entre os neurônios da região bulbar, os quais são responsáveis, em grande parte, pela manutenção das variáveis cardiovasculares. Esse modelo foi proposto considerando a observação que o nível de pressão arterial basal não foi alterado após o bloqueio dos receptores glutamatérgicos na região RVL do bulbo (Guyenet e cols., 1987; Kiely e cols., 1994; Sun, 1996; Ito e Sved , 1997). No mesmo trabalho, o bloqueio glutamatérgico da região RVL após a inibição da região CVL com muscimol reduziu a pressão arterial para níveis considerados espinais, sugerindo a eliminação do tônus simpático (Ito e Sved, 1997). Projeções excitatórias da região CVL para a região RVL do bulbo também foi proposto por Natarajan e Morisson (2000). Os autores sugerem que os neurônios localizados na área pressora caudal (APC) constituem uma das principais fontes de regulação da atividade dos neurônios excitatórios da região CVL do bulbo e que esses neurônios seria glutamatérgicos (Natarajan e Morisson, 2000).

Um importante ponto a ser lembrado nessa discussão diz respeito à especificidade do antagonista de receptores glutamatérgicos ionotrópicos ácido quinurênico. Devemos considerar que o ácido quinurênico antagoniza a maioria dos receptores glutamatérgicos ionotrópicos como o NMDA, kainato e AMPA (Collingridge e cols., 1989). A especificidade farmacológica da injeção de ácido quinurênico na dose utilizada nesse trabalho mostra os seguintes critérios de especificidade: 1) essa droga não produz efeito na transmissão GABAérgica e promove, principalmente o bloqueio dos receptores NMDA (Guyenet e cols., 1987; Glaum e cols., 1992; Koshiya e cols., 1993; Sun, 1996); 2) A atividade simpática não é afetada quando ocorre a injeção de ácido quinurênico na região RVL (Guyenet e cols., 1987; Koshiya e cols., 1993); 3) O análogo estrutural inativo

ácido xanturênico não produz nenhum efeito cardiovascular quando injetado na região CVL e RVL (Guyenet e cols., 1987; Koshiya e cols., 1993).

Sabe-se que mecanismos excitatórios partem de diferentes áreas encefálicas e são importantes para a manutenção da atividade basal dos neurônios do RVL (Ito e Sved, 1997; Sato e cols., 2003). Embora o bloqueio de receptores glutamatérgicos no RVL não produza efeitos significantes na pressão arterial e atividade simpática (Guyenet e cols., 1987; Ito e Sved, 1997; Horiuchi e cols., 2004), sabe-se que esse bloqueio reduz a pressão arterial após a inibição dos neurônios da região do CVL (Ito e Sved, 1997). Esses resultados sugerem que o aumento de pressão arterial produzido pela inibição do CVL é dependente da liberação de aminoácidos excitatórios (o L-glutamato seria o principal candidato) na região do RVL. De uma maneira similar, os presentes resultados sugerem que importantes projeções excitatórias (glutamatérgicas) para a região do RVL, para o controle de pressão arterial e resistência vascular, originam-se no NTS, confirmando os dados da literatura (Ito e Sved., 1997; Natarajan e Morrison, 1998; Heesch e cols., 2006).

A presença de mecanismos excitatórios no NTS foi sugerido em vários modelos experimentais. Esses mecanismos são sugeridos pela resposta pressora produzida pela injeção de L-glutamato no NTS de ratos acordados ou pelo efeito anti-hipertensivo da lesão eletrolítica no NTS (comissural) em ratos espontaneamente hipertensos (Machado e Bonagamba, 1992; Colombari e cols., 1994; Sato e cols., 1999; 2003). A injeção de L-glutamato no NTS, em ratos anestesiados, produz resposta depressora, mas a mesma injeção no NTS, após o bloqueio da região do CVL, produz resposta pressora, sugerindo que a injeção de L-glutamato no NTS pode ativar mecanismos excitatórios, mesmo em animais anestesiados (Urbanski e Sapru, 1988b).

Estudos anatômicos, imunohistoquímicos e eletrofisiológicos têm sugerido a existência de mecanismos excitatórios do NTS para o RVL e que estariam envolvidos na via do quimiorreflexo (Urbanski e Sapru, 1988a; 1988b; Koshiya e cols., 1993; Koshiya e Guyenet, 1996a; Moreira e cols., 2006).

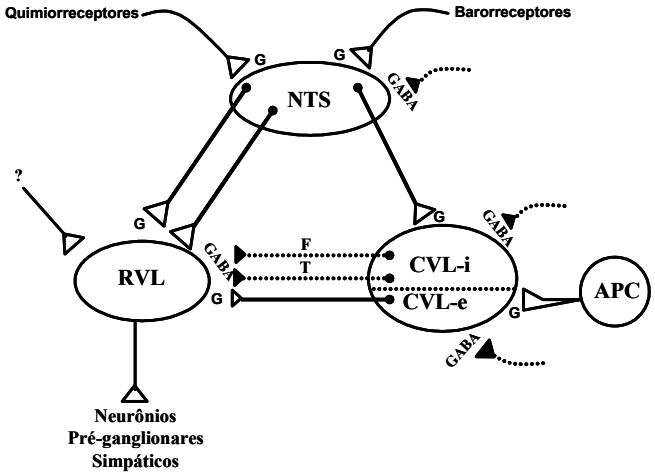
A injeção de ácido quinurênico no NTS bloqueia as respostas do quimiorreflexo em animais anestesiados (Zhang e Mifflin, 1993; Vardhan e cols., 1993; Chitravanshi e cols., 1994; Chitravanshi e Sapru, 1995), mas é necessário considerar que o quimiorreflexo necessita de um estímulo próprio para ser ativado. Nos nossos experimentos, durante o bloqueio da região do CVL com muscimol, um mecanismo não relacionado ao quimiorreflexo parece ativar os mecanismos excitatórios no NTS, causando aumento da resistência vascular e pressão arterial.

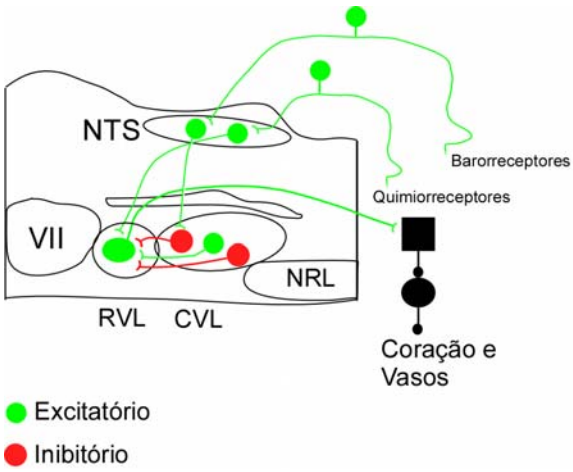
Dados da literatura mostram que a injeção de ácido quinurênico no RVL aboliu a resposta pressora produzida pela injeção de muscimol na região do CVL, indicando que projeções excitatórias para a região do RVL são originadas em outras regiões do SNC e não somente no NTS (Ito e Sved, 1997). Parece claro que, no nosso modelo experimental (inibição da região do CVL), a pressão arterial e a resistência vascular são dependentes de mecanismos glutamatérgicos no NTS. Por outro lado, a resposta pressora produzida pelo bloqueio glutamatérgico no NTS é abolido pela injeção de muscimol no CVL, sugerindo que mecanismos excitatórios existentes na região do CVL (bloqueados pelo muscimol) estão envolvidos na resposta pressora produzida pelo bloqueio glutamatérgico no NTS. Vale ressaltar nesses experimentos também que independente da sequência de bloqueios farmacológicos no NTS e CVL, nós observamos as mesmas respostas de queda de pressão e de resistência vascular para valores abaixo do basal.

Diante de tudo que foi discutido chegamos a conclusão de que um mecanismo ainda não muito bem elucidado é como os neurônios simpatoexcitatórios da região do RVL são ativados para promover a manutenção da pressão arterial e da atividade simpática. Parece existir um balanço entre vias inibitórias e vias excitatórias. Além dos mecanismos inibitórios para o controle dos neurônios do RVL, mais recentemente, tem-se discutido a presença e a importância dos mecanismos excitatórios no controle da atividade dos neurônios do RVL. Os resultados discutidos nessa primeira parte da tese mostram a possível existência de vias excitatórias da região do NTS (região postremal e comissural) bem como da região do CVL para o controle dos neurônios simpatoexcitatórios da região do RVL. Para melhor elucidar essas possibilidades, diferentes metodologias

de eletrofisiologia e técnicas anatômicas serão necessárias para comprovar a existência desses mecanismos. Uma outra questão em aberto é como essas vias excitatórias estariam funcionando numa situação fisiológica normal. Estariam também essas vias envolvidas nos reflexos barorreceptor e quimioreceptor? Por exemplo, qual seria a condição fisiológica em que estas vias glutamatergicas simpatoexcitatórias estariam funcionando? Uma situação importante seria como ocorre a ativação da atividade simpática durante ativação do quimiorreflexo periférico e/ou central? Existiria uma integração entre a ativação do quimiorreflexo central e periférico? Logo, uma série de perguntas surgiram durante a finalização dessa primeira parte. A partir disso, eu procurei responder parte delas durante a realização do meu estágio de doutorado sanduíche e que serão discutidos na segunda parte desta tese.

Portanto, com os nossos experimentos, nós podemos concluir e sugerir que a resposta pressora produzida pelo bloqueio do mecanismo inibitório para o RVL é dependente de projeções excitatórias provenientes da região do CVL e do NTS (região postremal e comissural). Esses mecanismos excitatórios são importantes para manutenção da pressão arterial e resistência vascular, pois o bloqueio simultâneo dos dois mecanismos resulta em queda da pressão arterial e resistência vascular abaixo dos valores basais. O esquema a seguir procura elucidar os possíveis mecanismos envolvidos no circuito bulbar responsáveis pelo controle das variáveis cardiovasculares.





Os esquemas acima procuram mostrar as possíveis interconexões entre NTS, região CVL e a região RVL. Neurônios do NTS enviam projeções excitatórias para a região CVL e RVL. Os neurônios da região CVL (CVL inibitório, vermelho) enviam projeções para a região RVL, promovendo a principal fonte de inibição dos neurônios dessa área. Por outro lado, a região CVL pode conter neurônios excitatórios (CVL exitatório, verde), os quais enviariam projeções para a região RVL e auxiliam na manutenção das variáveis cardiovasculares.

Introdução

O SNC possui mecanismos refinados de defesa contra elevados valores de dióxido de carbono (CO_2) e alteraçõs nos níveis de pH. Esses mecanismos são, basicamente, os mecanismos de controle da respiração, que promovem um ajuste no níveis de CO_2 para valores considerados fisiológicos (Feldman e cols., 2003). Há mais de 40 anos atrás, Mitchell e colaboradores propuseram uma hipótese de que os neurônios sensíveis a variações de CO_2 e pH estariam localizados na superfície ventral do bulbo (Mitchell e cols 1963a, 1963b). Essa hipótese rapidamente transformou-se em livro texto para as áreas de saúde. Embora um grande número de estudos foram realizados no campo da quimiossensibilidade, o verdadeiro papel dos quimiorreceptores centrais ainda é motivo de muitas controvérsias (Guyenet e col., 2005b; Richerson e cols., 2005). Atualmente, sabe-se que neurônios quimiossensíveis foram identificados em várias regiões do SNC, mas ainda a verdadeira contribuição dessas regiões no controle da respiração, em relação a variações nos valores de CO_2, ainda não foi completamente esclarecida. Recentemente, o campo da neurobiologia respiratória também tem entrado em diversas discussões a respeito do principal local no SNC responsável pelo controle da quimiorrecepção.

Quimiorreflexo central e periférico: possível interação bulbar das vias glutamatérgicas.

Situações de hipercapnia (aumento nas concentrações de CO_2) promovem um aumento na atividade simpática, mesmo em situações de remoção cirúrgica dos quimiorreceptores periféricos e dos barorreceptores (Millhorn, 1986; Moreira e cols., 2006). Isso sugere que os quimiorreceptores centrais seriam os responsáveis pelos efeitos excitatórios da hipercapnia (Millhorn, 1986; Oikawa et al., 2005). Em situações de anestesia, a ativação simpática promovida pelos quimiorreceptores centrais parece ser mediada em grande escala pelos neurônios bulboespinais do RVL, pois esses neurônios respondem a variações nas concentrações de CO_2 com um aumento na modulação respiratória sobre a

atividade simpática. Essas respostas são semelhantes às observadas nos nervos simpáticos lombar e esplâncnico (McAllen, 1987; Haselton and Guyenet, 1989, Moreira e cols., 2006) (Figura 1).

Como já citado anteriormente, a maioria dos quimiorreceptores centrais estão localizados na superfíce ventral do bulbo (Loeschcke, 1982). Portanto, os quimiorreceptores centrais estão, provavelmente, expostos a níveis de CO_2 semelhantes aos níveis de CO_2 arterial e venoso (Loeschcke, 1982; Kiley et al., 1985). Alguns dos quimiorreceptores centrais foram recentemente caracterizados no núcleo retrotrapezóide (NRT), uma estrutura localizada na porção rostral da coluna respiratória e embaixo da porção caudal do núcleo facial (Mulkey et al., 2004), (Figura 2).

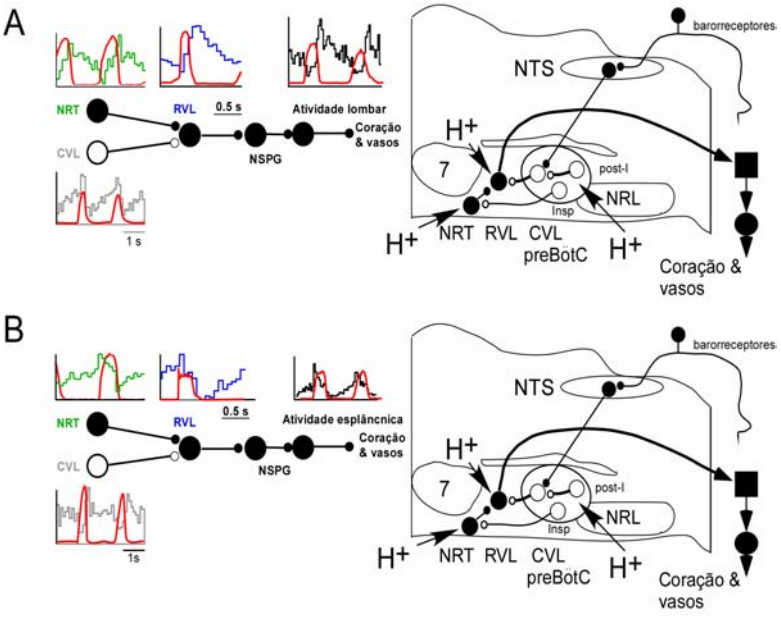


Figura 1: Relação entre a atividade simpática eferente e atividade respiratória.
A. Controle neural do coração e vasos sanguíneos. Os quatro histogramas relacionam a atividade neuronal com a atividade do nervo frênico (vermelho) em ratos vagotomizados e anestesiados com halotana. A modulação respiratória da atividade do nervo simpático lombar parece ser similar a modulação respiratória encontrada nos neurônios bulbo-espinais do RVL. Essa similaridade sugere que os centros respiratórios podem influenciar a atividade simpática eferente via região RVL. A modulação respiratória nos neurônios do RVL é, provavelmente, influenciada por interneurônios GABAérgicos na região do CVL. Pode-se notar que a modulação respiratória nos neurônios do CVL são, exatamente, o oposto da modulação encontrada nos neurônios do RVL. Neurônios glutamatérgicos localizados no NRT, podem contribuir para a modulação respiratória dos neurônios do RVL. O esquema acima representa uma possível representação da influência dos quimiorreceptores centrais na atividade simpática durante uma situação de hipercapnia.
B. Outro exemplo da modulação respiratória sobre a atividade simpática eferente. Nesse caso a modulação respiratória é sobre a atividade simpática do nervo esplâncnico.
Atividade dos neurônios do NRT (verde); atividade dos neurônios do RVL (azul); atividade simpática do nervo lombar ou esplâncnico (preto); atividade dos neurônios do CVL (cinza).

A quimiossensibilidade dessa região parece ser mediada pela expressão de canais de

potássio sensíveis a variações de pH, que se fecham durante a acidificação dessa região, promovendo excitação celular e disparo de potenciais de ação. Sabe-se também que os neurônios quimiossensíveis do NRT são glutamatérgicos, são modulados pela respiração em altas concentrações de CO_2 e projetam-se para a região ventrolateral do bulbo, incluindo a região do RVL (Mulkey et al., 2004).

O mecanismo neural que os quimiorreceptores centrais fazem parte são de extrema importância, pois eles são responsáveis pelo controle químico da respiração (Feldman e cols., 2003; Feldman e Del Negro, 2006). Esses receptores conseguem detectar alterações na pressão parcial de CO_2 do fluido extracelular, via mudanças no pH (Feldman e cols., 2003; Nattie, 2006). Sabe-se, também, que os quimiorreceptores periféricos, localizados no corpúsculo carotídeo, também auxiliam na manutenção dos níveis de CO_2 (Scheid e cols., 2001; Feldman e cols., 2003; Richerson, 2004; Putnam e cols., 2004; Prabhakar e Peng, 2004; Bin-Jaliah e cols., 2004). A integração entre os quimiorreceptores centrais e periféricos ainda não está muito bem esclarecida, exceto por se saber que essa integração ocorre, praticamente, na região pontina e bulbar (Feldman e cols., 2003).

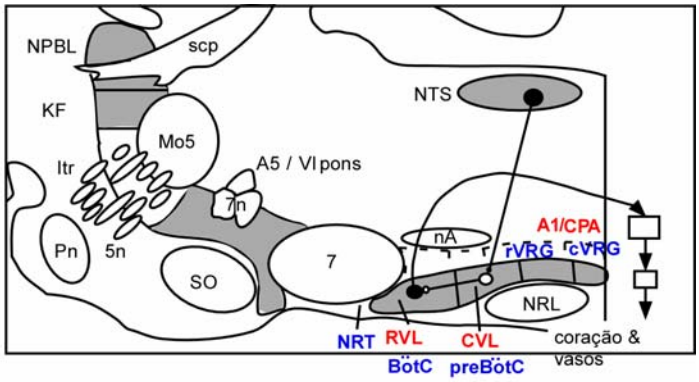


Figura 2: Principais regiões cardio-respiratórias do bulbo e da ponte.
O painel representado consiste um corte sagital (aproximadamente 1,9 mm lateral à linha média) de um rato. A via do barorreflexo foi desenhada apenas para orientação. Círculo preto: transmissão glutamatérgica; círculo aberto: transmissão GABAérgica.
A mesma região no bulbo ventrolateral pode ser dividido em três partes rostrocaudais referentes ao controle cardiovascular (vermelho) e cinco partes rostrocaudais referentes ao controle respiratório (azul).

Como já descrito anteriormente, a teoria mais aceita é que os quimiorreceptores centrais são sensíveis a alterações de pH (Loeschcke, 1982; Putnam e cols., 2004; Richerson e cols., 2005; Guyenet e cols., 2005b). A proporção de neurônios bulbares, *in vitro,* sensíveis a alterações de pH varia de 15% até mais de 90% dependendo da

região estudada, da preparação experimental e dos critérios utilizados para se definir quimiossensibilidade (Dean e cols., 1989; Kawai e cols., 1996; Richerson, 2004; Mulkey e cols., 2004; Ritucci e cols., 2005). Logo, pode-se concluir que os quimiorreceptores centrais são essenciais para o sistema respiratório (Nattie, 2001; Feldman e cols., 2003; Nattie, 2006).

O NRT contém características que definem essa região como sendo uma região, classicamente, quimiossensível (Smith e cols., 1989; Ellenberger e Feldman, 1990; Nattie e cols., 2001; Cream e cols., 2002; Mulkey e cols., 2004; Ritucci e cols., 2005; Guyenet e cols., 2005a; Takakura e cols., 2006). Os neurônios do NRT respondem a variações de CO_2 mesmo na ausência dos quimiorreceptores periféricos, são classificados como células excitatórias (glutamatérgicas) e projetam-se para centros respiratórios (Mulkey e cols., 2004; Ritucci e cols., 2005; Guyenet e cols., 2005a). As respostas dos neurônios do NRT para as variações de CO_2 são muito maiores do que as células vizinhas a essa região, como por exemplo os núcleos da rafe (Mulkey e cols., 2004; Ritucci e cols., 2005; Guyenet e cols., 2005a).

Os quimiorreceptores periféricos detectam principalmente situações de redução da pressão parcial de O_2 (hipóxia). Eles também detectam alterações na pressão parcial de CO_2 ainda mais rápido do que os quimiorreceptores centrais (Nattie, 2006; Smith e cols., 2006). A estimulação dos quimiorreceptores periféricos produz bradicardia e ativação do sistema nervoso simpático (Paton e cols, 2001; Braga e cols., 2006; Moreira e cols., 2006).

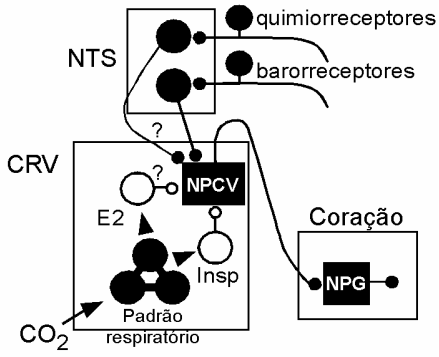


Figura 3: Controle respiratório dos neurônios cardio-vagais. Neurônios pré-ganglionares cardiovagais (NPCV) estão, continuamente, sendo ativados pelo baroreceptores arteriais. A modulação respiratória é mediada em grande escala pela inibição periódica dos neurônios (inspiratórios (insp) e expiratórios (E2) da coluna respiratória ventral (CRV). Esses neurônios contribuem na arritimia e causam uma aceleração cardíaca durante a inspiração. A bradicardia associada ao quimiorreflexo pode ser devido a um efeito excitatório direto do NTS sobre os neurônios cardio-vagais.

A presença da resposta vagal (bradicardia) indica que os neurônios cardiovagais são ativados pelo quimiorreceptores periféricos (Figura 3). Durante a

ativação desse mecanismo, assume-se que tenha uma arritmia respiratória (aumento da inibição dos neurônios pré-ganglionares durante inspiração). Esse mecanismo pode não ser responsável pela hipóxia induzindo bradicardia, pois a estimulação dos quimiorreceptores periféricos aumentam a atividade inspiratória, que por sua vez inibiria os neurônios cardio-vagais, causando uma taquicardia, mas não uma bradicardia. Portanto, o mecanismo que os quimiorreceptores periféricos produzem bradicardia permanece ainda desconhecido.

A ativação simpática produzida pela estimulação dos quimiorreceptores periféricos ocorre em sincronia com a atividade do nervo frênico (Figura 4). Como e por que os quimiorreceptores periféricos produzem um aumento da atividade simpática? Seria a explicação clássica (via neural dos quimiorreceptores periféricos: NTS – RVL) ou então seria por causa da sua modulação respiratória. Em várias espécies, o padrão respiratório da atividade simpática, produzido pela ativação dos quimiorreceptores periféricos, parece ser praticamente o mesmo padrão observado quando se tem a ativação dos quimiorreceptores centrais e, possivelmente, envolveria os mesmos mecanismos neurais já conhecidos (Figura 4) (Sun, 1995).

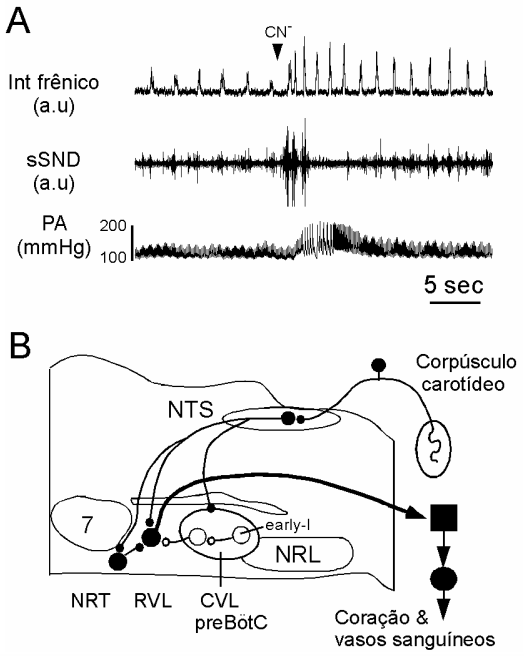


Figure 4: Quimiorreflexo periférico.
A. Estimulação dos quimiorreceptores periféricos com cianeto de sódio produz aumento da atividade do nervo frênico, da atividade simpática do nervo esplâncnico e da pressão arterial. A ativação do nervo esplâncnico ocorre em sincrônia com o nervo frênico (modulação respiratória). B. Possível circuito representativo da situação descrita em A. O núcleo do trato solitário (NTS) contém neurônios glutamatérgicos que projetam-se diretamente para o RVL ou indiretamente via a região do CVL.

Sabe-se que os neurônios bulbo-espinais do RVL não são ativados pelos quimiorreceptores periféricos após o bloqueio dos receptores glutamatérgicos da região do RVL (Sun e Reis, 1995), mas uma grande ativação persiste após a inibição da região do CVL, embora a ativação não ocorra mais em sincronia com a respiração (Koshiya e Guyente, 1996b; Guyenet, 2000). Logo, essa evidência sugere que durante a ativação dos

quimiorreceptores periféricos, a modulação respiratória dos neurônios do RVL seja mediada via região do CVL (Figura 4). Esse processo não é responsável pelo aumento total da atividade simpática que, provavelmente, é devido a uma via excitatória do NTS para a região do RVL (Figura 4). A via entre os quimiorreceptores periféricos e RVL envolve uma sinapse glutamatérgica localizada no NTScom (Koshiya e Guyenet, 1996a; Sun e Reis, 1995; Takakura e cols., 2006). Os neurônios do NTScom projetam-se diretamente para a região do RVL via uma projeção excitatória (Colombari e cols., 1996; Koshiya e Guyenet, 1996a; Blessing, 1997; Paton, 1999). Possivelmente, interneurônios, localizados na região do NRT, podem fazer parte desse circuito (Takakura e cols., 2006; Rosin e cols., 2006) (Figura 4).

A atividade simpática é ativada pela estimulação tanto dos quimiorreceptores centrais como periféricos (Koshiya e cols., 1993; Koshiya e Guyente, 1996a; 1996b; Guyenet, 2006; Moreira e cols., 2006). O modo pelo qual os quimiorreceptores centrais ativam a respiração e a atividade simpática ainda não está muito bem esclarecido, principalmente em termos celulares e moleculares. Entretanto, o que se sabe é que esse mecanismo envolve a ativação de uma rede de neurônios na região bulbar (Guyenet, 2000). Há uma teoria que defende a idéia de que a quimiorrecepção central parece estar amplamente distribuída no SNC, em especial no bulbo e ponte, resultando num efeito cumulativo do pH em vários neurônios (Feldman e cols., 2003; Richerson, 2004; Nattie, 2001; 2006; Nattie e Li, 2006). A existência de uma rede neuronal distribuída no SNC é sugerida por evidências *in vivo*, mostrando que a acidificação de várias partes do bulbo, incluindo o NTS, a coluna respiratória ventral, o NRT e os núcleos da rafe aumentam os padrões de atividade respiratória (Nattie, 2001; Richerson, 2004; Mulkey e cols., 2004; Mandel e Schreihofer, 2006). Em contrapartida, existe uma outra visão sobre os quimiorreceptores centrais, mostrando que, ao contrário da ampla distribuição dessas células no bulbo, um conjunto de neurônios localizados no NRT, ou NTS ou na rafe parece mediar os efeitos do pH no controle cardio-respiratório (Guyenet e cols., 2005a; 2005b; Li e Nattie, 2002; Richerson e cols., 2005; Takakura e cols., 2006).

O fato de os quimiorreceptores centrais estarem localizados em várias partes do bulbo, com diferentes graus de sensibilidade, aumentam as possibilidades de que vários componentes do sistema nervoso simpático possam estar envolvidos na quimiorrecepção. A idéia clássica de que uma situação de hipercapnia promove um aumento da atividade simpática via quimiorreceptores localizados exclusivamente nos neurônios respiratórios não foi ainda demonstrada experimentalmente. A região do locus coeruleus é, visivelmente, sensível ao pH *in vitro* e *in vivo* (Pineda e Aghajanian, 1997). A literatura também mostra que os neurônios do RVL podem ser ativados por acidificação ou, ainda, que o CO_2 ativaria os neurônios do RVL via neurônios sensíveis ao pH, mas que tecnicamente não fazem parte da rede respiratória (Haselton e Guyenet, 1989; Moreira e cols., 2006).

Como já citado anteriormente, na ausência das aferências dos quimiorreceptores periféricos, uma situação de hipercapnia promove o aumento da atividade simpática para coração e vasos sanguíneos (Hanna e cols., 1988). O efeito do CO_2 é normalmente atribuído a uma sequência de eventos. A acidificação do fluido extracelular promove a estimulação dos quimiorreceptores centrais; esses receptores ativam os neurônios que controlam o padrão respiratório que, por sua vez, enviam informações para os neurônios simpatoexcitatórios do RVL (Millhorn e Eldrige, 1986; Richter e Spyer, 1990; Moreira e cols., 2006). Essa teoria está fundamentada na evidência de que a ativação simpática promovida pelos quimiorreceptores centrais ocorre em sincronia com o ciclo respiratório (Millhorn, 1986; Millhorn e Eldrige, 1986; Guyenet e cols., 1990; Moreira e cols., 2006). Lesões químicas ou eletrolíticas na superfíce ventral do bulbo promove uma atenuação da atividade do nervo frênico e também na modulação respiratória na atividade simpática (Hanna e cols., 1979; Millhorn, 1986; Millhorn e Eldrige, 1986). Durante a estimulação dos quimiorreceptores periféricos, os neurônios simpatoexcitatórios do RVL exibem um padrão de atividade respiratória central. Essa atividade é similar aos neurônios pré-ganglionares simpáticos (McAllen, 1987; Haselton e Guyenet, 1989; Darnall e Guyenet, 1990; Guyenet e cols., 1990; Miyawaki e cols., 1995) (Figura 1).

Sabe-se que a região pontina e bulbar contêm múltiplas regiões quimiossensíveis, como já descrito anteriormente (Nattie e Li, 1996; Feldman e cols., 2003; Hodges e cols., 2004; Mulkey e cosl., 2004; Putnam e cols., 2004; Richerson e cols., 2005; Pineda e Aghajanian, 1997). A observação de que os neurônios simpatoexcitatórios do RVL são ritmicamente modulados pela respiração é insuficiente para concluir que esses neurônios promovem um aumento na atividade simpática durante a estimulação dos quimiorreceptores centrais. O fato de a atividade simpática e os neurônios simpatoexcitatórios do RVL serem ativados em sincronia com a respiração, durante a estimulação dos quimiorreceptores centrais, não se justifica que essa sincronia seja resultante de uma via excitatória para os neurônios do RVL.

Portanto, um dos objetivos da segunda parte dessa tese é tentar provar uma série de dúvidas existentes sobre o principal mecanismo pelo qual os quimiorreceptores centrais promovem um aumento na atividade simpática. Como se comportariam os neurônios simpatoexcitatórios do RVL durante a ativação dos quimiorreceptores centrais (aumento das concentrações de CO2: 5% até 10%) e se essa ativação envolve a participação de sinapses e vias glutamatérgicas. Um outro objetivo dessa tese será tentar esclarecer uma possível integração entre os quimiorreceptores periféricos e centrais. Nossa hipótese é que essa integração ocorra na região ventrolateral do bulbo, em especial no NRT e envolva a participação de vias excitatórias glutamatérgicas, uma vez que o NRT é um importante centro cardio-respiratório. Para a realização desses protocolos, procuramos abordar técnicas de eletrofisiologia e marcações neuronais.

Peripheral chemoreceptor inputs to retrotrapezoid nucleus (RTN) CO2-sensitive neurons in rats

Ana Carolina Thomaz Takakura, Thiago Santos Moreira, Eduardo Colombari, Gavin H. West, Ruth L. Stornetta and Patrice G. Guyenet

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Peripheral chemoreceptor inputs to retrotrapezoid nucleus (RTN) CO_2-sensitive neurons in rats

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The rat retrotrapezoid nucleus (RTN) contains pH-sensitive neurons that are putative central chemoreceptors. Here, we examined whether these neurons respond to peripheral chemoreceptor stimulation and whether the input is direct from the solitary tract nucleus (NTS) or indirect via the respiratory network. A dense neuronal projection from commissural NTS (commNTS) to RTN was revealed using the anterograde tracer biotinylated dextran amine (BDA). Within RTN, 51% of BDA-labelled axonal varicosities contained detectable levels of vesicular glutamate transporter-2 (VGLUT2) but only 5% contained glutamic acid decarboxylase-67 (GAD67). Awake rats were exposed to hypoxia ($n = 6$) or normoxia ($n = 5$) 1 week after injection of the retrograde tracer cholera toxin B (CTB) into RTN. Hypoxia-activated neurons were identified by the presence of Fos-immunoreactive nuclei. CommNTS neurons immunoreactive for both Fos and CTB were found only in hypoxia-treated rats. VGLUT2 mRNA was detected in 92 ± 13% of these neurons whereas only 12 ± 9% contained GAD67 mRNA. In urethane–chloralose-anaesthetized rats, bilateral inhibition of the RTN with muscimol eliminated the phrenic nerve discharge (PND) at rest, during hyperoxic hypercapnia (10% CO_2), and during peripheral chemoreceptor stimulation (hypoxia and/or I.V. sodium cyanide, NaCN). RTN CO_2-activated neurons were recorded extracellularly in anaesthetized intact or vagotomized rats. These neurons were strongly activated by hypoxia (10–15% O_2; 30 s) or by NaCN. Hypoxia and NaCN were ineffective in rats with carotid chemoreceptor denervation. Bilateral injection of muscimol into the ventral respiratory column 1.5 mm caudal to RTN eliminated PND and the respiratory modulation of RTN neurons. Muscimol did not change the threshold and sensitivity of RTN neurons to hyperoxic hypercapnia nor their activation by peripheral chemoreceptor stimulation. In conclusion, RTN neurons respond to brain P_{CO_2} presumably via their intrinsic chemosensitivity and to carotid chemoreceptor activation via a direct glutamatergic pathway from commNTS that bypasses the respiratory network. RTN neurons probably contribute a portion of the chemical drive to breathe.

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The chemical drive to breathe relies on central chemoreceptors that detect brain extracellular fluid P_{CO_2} via pH, and on carotid body chemoreceptors that respond to arterial P_{CO_2} in a P_{O_2}- and glucose-dependent manner (Scheid *et al.* 2001; Feldman *et al.* 2003; Richerson, 2004; Putnam *et al.* 2004; Prabhakar & Peng, 2004; Bin-Jaliah *et al.* 2004). The way in which central and peripheral chemoreceptor information is integrated at the cellular and network levels is unclear except for the fact that the process occurs within the pontomedullary region (Feldman *et al.* 2003). The question is made even more complex by current uncertainties regarding the very nature of central chemoreceptors.

Although substances released from glia or non-neuronal cells have repeatedly been invoked to account for the sensitivity of brainstem neurons to extracellular fluid P_{CO_2} (Erlichman *et al.* 1998; Gourine *et al.* 2005; Guyenet *et al.* 2005*b*), the dominant theory is that neuronal pH-sensitive conductances underlie central chemoreception

A. C. T. Takakura and T. S. Moreira contributed equally to this study.

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(Loeschcke, 1982; Putnam *et al.* 2004; Richerson *et al.* 2005; Guyenet *et al.* 2005*b*). The proportion of brainstem neurons that respond to pH or P_{CO_2} *in vitro* varies from 15% to more than 90% depending on the brain region, the preparation and the criteria that is applied to define the cells as chemosensitive (Dean *et al.* 1989; Kawai *et al.* 1996; Richerson *et al.* 2001; Mulkey *et al.* 2004; Ritucci *et al.* 2005). In light of this evidence, central chemosensitivity could be viewed as an emergent property of the respiratory network that cannot be assigned to any particular component of the system. Yet, other evidence suggests that central chemoreception does rely on specialized neurons that drive a respiratory motor pattern generator that has no or inadequate sensitivity to pH on its own (Nattie, 2001; Feldman *et al.* 2003). Congenital central hypoventilation syndrome, a disease in which patients have absent or greatly diminished central hypercapnic ventilatory chemosensitivity, argues in favour of the existence of central neurons specialized for chemoreception (Spengler *et al.* 2001; Gaultier & Gallego, 2005). The highly differentiated responsiveness of brainstem neurons to hypercapnia *in vivo* provides further evidence (Guyenet *et al.* 2005*b*).

The retrotrapezoid nucleus (RTN) contains very superficial propriobulbar neurons that have properties consistent with such specialized chemoreceptors (Smith *et al.* 1989; Ellenberger & Feldman, 1990; Nattie *et al.* 1991; Cream *et al.* 2002; Mulkey *et al.* 2004; Ritucci *et al.* 2005; Guyenet *et al.* 2005*a*). Their response to P_{CO_2} is far greater than that of surrounding cells, *in vivo* and in slices (Mulkey *et al.* 2004; Ritucci *et al.* 2005; Guyenet *et al.* 2005*a*). These CO_2-responsive cells are glutamatergic and have axonal projections that are anatomically appropriate to be driving the respiratory network (Mulkey *et al.* 2004).

The present study is designed to test whether RTN is an important site of integration between central and peripheral chemoreception. Our working hypothesis is that the intrinsic response of RTN neurons to pH is enhanced by excitatory inputs from peripheral chemoreceptors and that the resulting activity of these neurons could be encoding some of the chemical drive to breathe.

The results demonstrate that RTN neurons receive a strong excitatory input from carotid chemoreceptor afferents that bypasses the respiratory network and probably involves a single intervening glutamatergic neuron located in the nucleus of the solitary tract (NTS). Based on this and prior evidence, we conclude that RTN neurons have the capability of detecting brain P_{CO_2} directly and that they also respond to arterial blood gas composition via very direct neural inputs from peripheral chemoreceptors. RTN neurons could therefore be a source of integrated chemical drive to some aspect of the respiratory circuitry.

Methods

Animals

The experiments were performed on a total of 64 male Sprague-Dawley rats (Taconic; Germantown, NY, USA) weighing 250–350 g. Fifty rats were used in electrophysiological experiments, the rest for anatomy. Procedures were in accordance with NIH Animal Care and Use Guidelines and were approved by the University of Virginia's Animal Care and Use Committee.

Surgery and anaesthesia

General anaesthesia was induced with 5% halothane in 100% oxygen. The rats received a tracheostomy and artificial ventilation with 1.4–1.5% halothane in 100% oxygen was maintained throughout surgery. All rats were subjected to the following previously described surgical procedures: femoral artery cannulation for arterial pressure (AP) measurement, bladder cannulation to ease urination, femoral vein cannulation for administration of fluids and drugs, removal of the occipital plate to insert a recording electrode into the medulla oblongata via a dorsal transcerebellar approach, and skin incision over the lower jaw for placement of a bipolar stimulating electrode next to the mandibular branch of the facial nerve (Guyenet *et al.* 2005*a*). The phrenic nerve was accessed by a dorsolateral approach after retraction of the right shoulder blade. A bilateral vagotomy in the neck was performed in 11 rats. Six intact rats were additionally subjected to bilateral carotid body denervation.

Upon completion of surgical procedures, halothane was replaced by a mixture of urethane ($0.5\,g\,kg^{-1}$) and α-chloralose ($60\,mg\,kg^{-1}$) slowly administered I.V. All rats were ventilated with 100% oxygen throughout the experiment except during the hypoxia protocols. The O_2 concentration of the breathing mixture was monitored with a P_{O_2}-sensitive electrode located at the intake of the ventilator. Rectal temperature (maintained at 37°C) and end-tidal CO_2 were monitored throughout the experiment with a capnometer (Columbus Instruments, Ohio, USA) that was calibrated twice per experiment against a calibrated CO_2–N_2 mix. This instrument provided a reading of $< 0.1\%$ CO_2 during inspiration in animals breathing 100% oxygen and an asymptotic, nearly horizontal reading during expiration. We previously showed that the capnometer readings closely approximate arterial P_{CO_2} (Guyenet *et al.* 2005*a*). After injection of the intravenous anaesthetic mixture, the adequacy of anaesthesia was monitored during a 20 min stabilization period by testing for absence of withdrawal response, lack of BP change and lack of change in PND rate or amplitude to firm toe pinch. After these criteria were satisfied, the muscle relaxant pancuronium was administered at

the initial dose of 1 mg kg^{-1} I.V. and the adequacy of anaesthesia was thereafter gauged solely by the lack of increase in BP and PND rate or amplitude to firm toe pinch. Approximately hourly supplements of one-third of the initial dose of chloralose–urethane were needed to satisfy these criteria during the course of the recording period (3–4 h).

In vivo recordings of physiological variables and neuronal activity

Arterial pressure (AP), the mass discharge of the phrenic nerve (PND), tracheal CO_2 and single units were recorded as previously described (Guyenet *et al.* 2005*a*). Before searching for RTN neurons, ventilation was adjusted to lower end-expiratory CO_2 to 4% at steady-state (60–80 cycles s^{-1}; tidal volume 1.2–1.4 ml (100 g)$^{-1}$). These conditions were selected because 4% end-expiratory CO_2 was below the firing threshold of both RTN units and the PND. Variable amounts of pure CO_2 were then added to the breathing mixture to adjust end-expiratory CO_2 to the desired level. When searching for RTN units, end-expiratory CO_2 was set at 6.5–7% in order to insure that both PND and the CO_2-sensitive neurons of RTN were active. RTN neurons were recorded exclusively under the caudal end of the facial motor nucleus in a region that matches the prior definition of RTN in rats (Cream *et al.* 2002; Weston *et al.* 2004; Guyenet *et al.* 2005*a*). As in prior work, the caudal and ventral boundaries of the facial motor nucleus were identified in each rat by the large (up to 5 mV) negative antidromic field potential generated in the facial motor nucleus by stimulating the mandibular branch of the facial nerve (for details see Brown & Guyenet, 1985). RTN CO_2-activated neurons were encountered between 200 and 350 μm below the lower edge of the facial motor nucleus, 1.6–1.9 mm lateral to the midline and from 100 μm caudal to 400 μm rostral to the caudal end of the facial field potential (Mulkey *et al.* 2004; Guyenet *et al.* 2005*a*). Prior single neuron labelling experiments have indicated that this region lies between coronal planes Bregma −11.7 and Bregma −11.2 mm of the Paxinos and Watson atlas (Paxinos & Watson, 1998; Mulkey *et al.* 2004; Guyenet *et al.* 2005*a*). Most recordings were made on the left side of the brain. The RTN also contains presympathetic barosensitive neurons located on average dorso-medial to the CO_2-sensitive neurons (Mulkey *et al.* 2004). These neurons cannot be silenced by hypocapnia and their discharge rate increases by at most 80% between 4 and 10% end-expiratory CO_2. These cells were ignored in the present study.

All analog data (end-expiratory CO_2, PND, unit activity, AP) were stored on a microcomputer via a micro1401 digitizer from Cambridge Electronics Design (CED, Cambridge, UK) and were processed off-line using version 5 of the Spike 2 software (CED). Processing included action potential discrimination and binning, neuronal discharge rate measurement, and PND 'integration' (iPND) consisting of rectification and smoothing (τ, 0.015 s). Neural minute × volume (mvPND, a measure of the total phrenic nerve discharge per unit of time) was determined by averaging iPND over 50 s (vagotomized rats) or during 20 respiratory cycles (rats with intact vagus nerves) and normalizing the result by assigning a value of 0 to the dependent variable recorded at low levels of end-expiratory CO_2 (below threshold) and a value of 1 at the highest level of P_{CO_2} investigated (between 9.5 and 10%). The CED software was also used for acquisition of peri-event histograms of neuronal activity and peri-event averages of iPND or tracheal CO_2. The peri-event histograms of neuronal single-unit activity were triggered either on iPND or on the tracheal CO_2 trace and represented the summation of at least 100 respiratory cycles (350–800 action potentials per histogram).

The steady-state relationship between RTN neuronal activity and end-expiratory CO_2 was obtained by stepping the inspired CO_2 level to various values for a minimum of 3 min and up to 5 min. The mean discharge rate of the neuron was measured during the last 30 s of each step at which time end-expiratory CO_2 and the discharge of the neuron appeared to have reached equilibrium. End-expiratory CO_2 was measured by averaging the maximum values recorded from 10 consecutive breaths at the midpoint of the time interval sampled.

Stimulation of carotid chemoreceptor was done with bolus injections of NaCN (50 μg kg^{-1}, I.V.) or by switching the breathing mixture from 100% O_2 to 10–15% O_2 balanced with N_2 for 30 s using an electronic valve. Evidence that the hypoxic stimulus activated neurons via stimulation of carotid chemoreceptors was obtained by demonstrating that denervation of these receptors eliminated the excitatory effect of the hypoxic stimulus on PND and the activity of hypoxia-responsive RTN neurons.

Intraparenchymal injections

Muscimol (Sigma Chemicals Co.; 1.75 mM in sterile saline pH 7.4) was pressure injected (30 nl in 5 s) bilaterally through single-barrelled glass pipettes (20 μm tip diameter). The muscimol solution contained a 5% dilution of fluorescent latex microbeads (Lumafluor, New City, NY, USA) for later histological identification of the injection sites (Guyenet *et al.* 1990). These glass pipettes also allowed recordings of field potentials and multiunit brain activity, properties that were used to direct the electrode tip to the desired sites. Injections into RTN were thus guided by recording the facial field potential and were placed 200 μm below the lower edge of the field, 1.6–1.9 mm lateral to the midline and 200–300 μm

rostral to the caudal end of the field. Injections into the midline raphe were done bilaterally 300 μm lateral to the midline at the same coronal level and depth as RTN. Injections into the rostral ventral respiratory group (rVRG) were guided by locating inspiratory-related multiunit activity. The target region was found 400 μm rostral to the calamus scriptorius, 1.8 mm lateral to midline and 1.9–2.2 mm below the dorsal surface of the brainstem using electrodes angled 20 deg forward. The electrophysiological recordings were made on one side only and the second injection was placed 1–2 min later in the symmetric brain location based on the stereotaxic coordinates of the first one. The classification of respiratory neurons was based on the timing of their discharge in relation to that of the phrenic nerve using accepted nomenclature (Feldman & McCrimmon, 1999).

Tracer injections

Tracer injections were made while the rats were anaesthetized with a mixture of ketamine (75 mg kg^{-1}), xylazine (5 mg kg^{-1}), and acepromazine (1 mg kg^{-1}) administered I.M. Surgery used standard aseptic methods, and after surgery, the rats were treated with the antibiotic ampicillin (100 mg kg^{-1}) and the analgesic ketorolac (0.6 mg kg^{-1}, S.C.).

A group of seven rats received iontophoretic injections of the anterograde tracer biotinylated dextran amine (BDA-lysine fixable, MW 10000; 10% w/v in 10 mM phosphate buffer, pH 7.4; Molecular Probes) into the commissural part of the nucleus of the solitary tract (commNTS) (20 μm tip diameter glass pipettes; 2 μA positive current pulses, 5 s duration every 10 s for 10 min). These injections were made 0.4 mm caudal to the calamus scriptorius, in the midline and 0.3–0.5 mm below the dorsal surface of the brainstem. These rats were allowed to survive 7–10 days following which they were anaesthetized with pentobarbital (60 mg kg^{-1}, I.P.) and perfused transcardially with fixative as described below.

Another group of 11 rats received an iontophoretic injection of cholera toxin B (1% CTB in 0.2 M phosphate buffer, pH 7.35; List Biological Laboratories, Campbell, CA, USA; 20 μm tip diameter glass pipettes; 2 μA positive current pulses, 5 s duration every 10 s for 10 min) into the left RTN in order to retrogradely label commNTS neurons that innervate RTN. These injections were made below the facial motor nucleus under electrophysiological guidance as described above (200 μm below the ventral boundary of the facial nucleus, 1.6–2.0 mm lateral to the midline, between Bregma −11.6 and −11.2 mm). Seven to ten days following the CTB deposits, six of the rats were exposed for 3 h to a hypoxic breathing mixture (8% O_2, balanced with N_2) in a small flow-through environmental chamber. The rest of the rats were exposed to room air under the same conditions. Except in one case, the experiments were run in pairs consisting of one rat exposed to hypoxia and the other to normoxia. The pair of animals were anaesthetized with pentobarbital and perfusion fixed immediately after the 3 h of exposure to hypoxia or normoxia.

Histology

The rats were deeply anaesthetized with 60 mg kg^{-1} pentobarbital I.P. then injected with heparin (500 units, intracardially) and finally perfused through the ascending aorta with 150 ml of phosphate-buffered saline (pH 7.4) followed by 4% phosphate-buffered (0.1 M; pH 7.4) paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA). The brain was removed and stored in the perfusion fixative for 24–48 h at 4°C. Series of coronal sections (30 μm) from the brain were cut using a vibrating microtome and stored in cryoprotectant solution (20% glycerol plus 30% ethylene glycol in 50 mM phosphate buffer, pH 7.4) at −20°C for up to 2 weeks awaiting histological processing.

All histochemical procedures were done using free-floating sections according to previously described protocols. BDA was detected using either streptavidin-Cy3 (1 : 200; 60 min; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or avidin–Alexa 488 (1 : 200; 60 min; Molecular Probes) (Stocker *et al.* 2006). Vesicular glutamate transporter 2 (VGLUT2) and glutamic acid decarboxylase 67 (GAD67) were detected by immunofluorescence using a guinea-pig anti-VGLUT2 antibody (AB 5907; Chemicon International, Temecula, CA, USA; dilution 1 : 2500 dilution) or a rabbit anti-GAD67 antibody (AB5992; Chemicon International; 1 : 2500 dilution; 24–48 h incubation in Tris-buffered saline with 10% horse serum and 0.1% Triton X-100) (Stocker *et al.* 2006). The VGLUT2 antibody was revealed with Alexa-488-tagged goat anti-guinea-pig IgG (1 : 200, Molecular Probes; 60 min). The GAD67 antibody was revealed with Alexa 488-tagged goat anti-rabbit IgG (1 : 200, Molecular Probes; 60 min).

Fos immunoreactivity was detected using a rabbit anti-c-Fos antibody (sc-52, Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1 : 1500) followed by a Cy-3-conjugated goat anti-rabbit IgG (Jackson; 1 : 200). CTB was detected with a goat anti-CTB antibody (List Biological Laboratories, Campbell, CA, USA; 1 : 2000) followed by Alexa 488-conjugated donkey anti-goat IgG (Molecular Probes; 1 : 250).

GAD67 mRNA was detected using a 3.2 kb digoxigenin-labelled cRNA probe exactly as previously described (Stornetta & Guyenet, 1999). VGLUT2 mRNA was detected using a 3.4 kb probe, also according to previously described methods (Stornetta *et al.* 2003*a*). Digoxigenin was revealed with a sheep

polyclonal anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche Molecular Biochemicals, Indianapolis, IN, USA) and alkaline phosphatase was reacted with nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (BCIP). Previous testing has established the specificity of our probes (Stornetta *et al.* 2002). Absence of labelling in facial, hypoglossal and nucleus ambiguus motor neurons was taken as the quality standard since these cells are the most prone to exhibit non-specific NBT/BCIP reaction product under suboptimal conditions. Probe hybridization was always carried out before any immunohistochemistry, i.e. before detection of Fos immunoreactivity and or CTB. Following hybridization, all primary antibodies were applied, then the alkaline phosphatase colourimetric reaction was performed and, finally, fluorescent secondary antibodies were applied. Finally, the sections were mounted in sequential rostrocaudal order onto slides, dried and covered with Vectashield (Vector Laboratories, Burlingame, CA, USA). Coverslips were affixed with nail polish.

Antibody specificity

No labelling was observed when the primary antibodies were omitted. The VGLUT2 antibody was raised against a peptide which, when preincubated with the primary antibody, eliminated immunoreactivity (Stocker *et al.* 2006). This antibody labelled terminals only. The GAD67 antibody also labelled nerve terminals exclusively. The antigen used to raise this antibody was derived from a cloned feline DNA expressed in *E. coli* and has been characterized previously (Kaufman *et al.* 1991). The anti-digoxigenin antibody produced no reaction product in the absence of digoxigenin-labelled RNA probes. Riboprobe immunolabelling was confined to neuronal cell bodies exclusive of nuclei.

Cell mapping, cell counting and imaging

A conventional multifunction microscope (brightfield, darkfield and epifluorescence) was used for all observations except when indicated. The computer-assisted mapping technique made use of a motor-driven microscope stage controlled by the Neurolucida software and has been described in detail previously (Stornetta & Guyenet, 1999). The Neurolucida files were exported to NeuroExplorer software (MicroBrightfield, Colchester, VT, USA) to count the various types of neuronal profiles within a defined area of the reticular formation.

Section alignment between brains was done relative to a reference section. To align sections around RTN level, the most caudal section containing an identifiable cluster of facial motor neurons was identified in each brain and assigned the level 11.6 mm caudal to Bregma (Bregma −11.6 mm) according to the atlas of Paxinos & Watson (1998). Levels rostral or caudal to this reference section were determined by adding a distance corresponding to the interval between sections multiplied by the number of intervening sections. The same method was also used to identify the Bregma level of the muscimol injections targeted to the rVRG. When the object of the experiment was to locate neurons or injection sites at commissural NTS level, the reference section used to align all others was the one closest to mid-area postrema level (Bregma −13.8 mm).

To count the number of BDA-labelled varicosities located in RTN following tracer injection into commNTS, a 200 μm-wide rectangle extending from the medial edge of the trigeminal tract to the lateral edge of the pyramidal tract was placed tangentially to the ventral medullary surface. BDA-labelled axonal varicosities were counted within this rectangle at four levels of the RTN.

The Neurolucida files were exported to the Canvas 9 software drawing program for final modifications. Photographs were taken with a 12-bit colour CCD camera (CoolSnap, Roper Scientific, Tuscon, AZ, USA; resolution 1392 pixels × 1042 pixels).

An Olympus IX81 DSU spinning disk confocal microscope (Olympus America, Inc., Melville, NY, USA) was used to test for colocalization of BDA and either VGLUT2 or GAD67 immunoreactivity in axonal varicosities located in the RTN region. Images were captured with a SensiCam QE 12-bit CCD camera (resolution 1376 pixels × 1040 pixels, Cooke Corp., Auburn Hills, MI, USA). IPLab software (Scanalytics, Rockville, MD, USA) was used for merging of colour channels in photographs of dual labelling experiments.

The neuroanatomical nomenclature is after Paxinos & Watson (1998).

Statistics

Statistical analysis was done with Sigma Stat version 3.0 (Jandel Corporation, Point Richmond, CA, USA). Data are reported as means ± standard error of the mean. A *t* test, (paired or unpaired) and one- or two-way parametric ANOVA followed by the Newman-Keul multiple comparisons test were used as appropriate. Significance was set at $P < 0.05$.

Results

Hypoxia activates commNTS glutamatergic neurons that innervate RTN

Carotid chemoreceptor afferents terminate predominantly in commNTS. To test whether commNTS innervates RTN,

the anterograde tracer BDA was injected by iontophoresis into commNTS in seven rats. Five BDA injections out of seven were correctly placed (Fig. 1*A*). In each of these five cases, the BDA injection was centred at the level of the calamus scriptorius and labelled neurons were confined to the commNTS. One week after injection, numerous BDA-labelled axonal varicosities or terminals (putative synapses) were present in the RTN, defined here as the region where the cell bodies and dendrites of previously identified CO_2-activated neurons are located. Figure 1*B* is a computer-assisted plot of the BDA-labelled varicosities that were detected in a representative coronal section located close to Bregma −11.4 mm (200 μm rostral to the caudal end of the facial motor nucleus). The plotting was limited to the ventral third of the brain. BDA-labelled varicosities were found throughout the ventral medulla albeit at variable densities. As seen in the inset, these putative synapses were especially numerous under the medial half of the facial motor nucleus in very close proximity to the ventral medullary surface. The number of BDA-labelled axonal varicosities present within RTN (defined by the rectangle shown in Fig. 1*B*; see Methods for details) were counted in four equidistant sections per rat and the resulting distribution histogram is shown in Fig. 1*C*. The number of BDA-labelled varicosities within the defined area was greatest ($P < 0.05$ by repeated measure ANOVA) close to the caudal end of the facial motor nucleus and dropped considerably caudal to that level (Fig. 1*C*). The area with the maximum density of varicosities approximates the region where we find the greatest concentration of CO_2-responsive neurons.

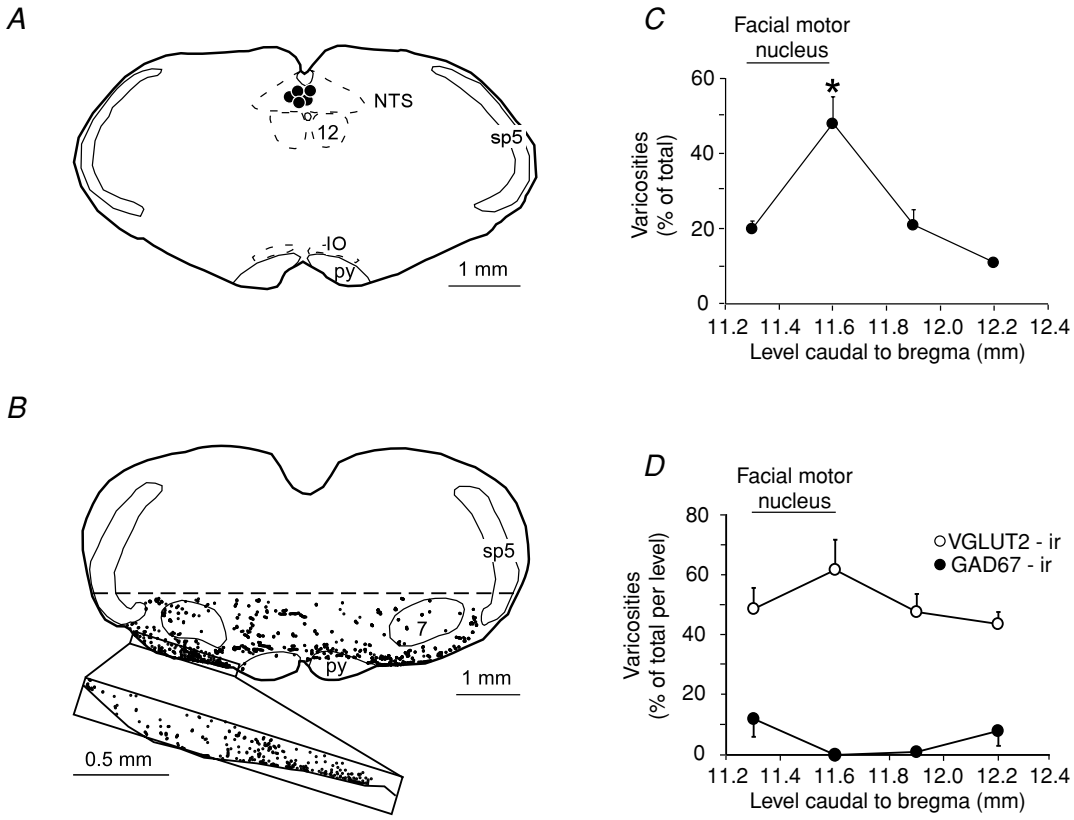

Figure 1. Projections from commissural NTS to RTN
A, computer-assisted plot of 5 iontophoretic injections of biotinylated dextran amine (BDA) that were confined to commNTS. All injection sites were within 200 μm of the level represented (Bregma −13.8 mm according to the atlas of Paxinos and Watson). This plane corresponds to the caudal edge of the area postrema. *B*, representative coronal section at Bregma −11.4 mm (200 μm rostral to the posterior edge of the facial motor nucleus (7). Each dot represents an axonal varicosity assumed to be a synapse or a release site. Plotting was confined to the lower third of the section below the dotted line. The enlarged inset is the area that was selected for counting the number of varicosities. *C*, mean number of varicosities present in the box defined in *B* at four levels of the RTN region (*$P < 0.05$ from other levels represented; ANOVA for repeated measures; 5 rats). *D*, proportion of BDA-immunoreactive varicosities that contained VGLUT2 or GAD67 immunoreactivity. The terminals were examined by confocal microscopy selectively within the marginal layer of RTN. IO, inferior olive; NTS, nucleus of the solitary tract; py, pyramidal tract; sp5, spinal tract of trigeminal nerve; 12, hypoglossal nucleus.

The next experiments were designed to test whether the projection from commNTS to RTN neurons is predominantly glutamatergic or GABAergic. Sections from the same five brains were reacted for simultaneous detection of BDA and either VGLUT2 or GAD67 immunoreactivity and the material was examined by confocal microscopy. We focused our observations on the marginal layer of RTN, a readily identified 50 μm-thick region of the ventral medulla that lies closest to the ventral medullary surface under the spinocerebellar tract. This choice was made because the marginal layer contains an especially dense input from commNTS (Fig. 1*B*) and because the CO_2-activated neurons of RTN have profuse dendrites within the marginal layer regardless of whether their cell bodies reside within or dorsal to this region (Mulkey *et al.* 2004; Weston *et al.* 2004). The BDA-labelled varicosities present within this region of RTN were rarely immunoreactive for GAD67 but they were commonly VGLUT2-immunoreactive (Fig. 2). An estimate of the percentage of excitatory (VGLUT2-ir) *versus* inhibitory (GAD67-ir) BDA-labelled varicosities was obtained by sampling the marginal layer with a confocal microscope in each of the five rats. Four equidistant sections between Bregma −11.3 and Bregma −12.2 mm were selected. Within this region an average of 51% of the total number of BDA-labelled varicosities sampled were immunoreactive for VGLUT2 (243 varicosities) whereas only 5% of 212 sampled varicosities were GAD67-ir. The proportion of BDA-positive varicosities containing VGLUT2- *versus* GAD67-immunoreactivity was approximately the same at the four levels investigated (Fig. 1*D*). Each level also contained numerous BDA-labelled axonal varicosities in which no other immunoreactivity could be detected.

The next series of experiments was designed to test whether the glutamatergic projection from commNTS to RTN includes neurons that are activated by systemic hypoxia. CommNTS with projections to RTN were prelabelled with CTB 1 week before the rats were exposed to hypoxia (8% O_2, 6 rats) or to normal air (5 rats). Fos immunoreactivity was used to identify commNTS neurons that were activated by hypoxia. CommNTS neurons were classified as glutamatergic or GABAergic based on whether they contained VGLUT2 mRNA or GAD67 mRNA.

The CTB injections, placed with electrophysiological guidance under the caudal end of the facial motor nucleus, were centred 100–300 μm dorsal to the ventral medullary surface 2–400 μm rostral to the caudal end of the facial motor nucleus. Figure 3*A* depicts the location of the CTB injections in the six rats that were exposed to hypoxia. Fos immunoreactivity was absent in the NTS of the five control rats exposed to room air and these rats were not examined further. One series of 30 μm-thick coronal sections (180 μm apart) was selected from the brain of each hypoxia-treated rat and reacted for simultaneous detection of Fos immunoreactivity, VGLUT2 mRNA and CTB immunoreactivity. An adjacent series of sections from the same six brains was reacted for detection of Fos immunoreactivity, GAD67 mRNA and CTB immunoreactivity. As illustrated in Fig. 4, neurons immunoreactive for both Fos and CTB commonly contained VGLUT2 mRNA (Fig. 4*A*–*C*) whereas they typically did not contain GAD67 mRNA (Fig. 4*D*–*F*). For quantification purposes, the coronal sections located at mid-area postrema level and 180, 360, 540 and 720 μm caudal to that plane were selected and commNTS neurons containing pertinent combinations of Fos immunoreactivity, CTB

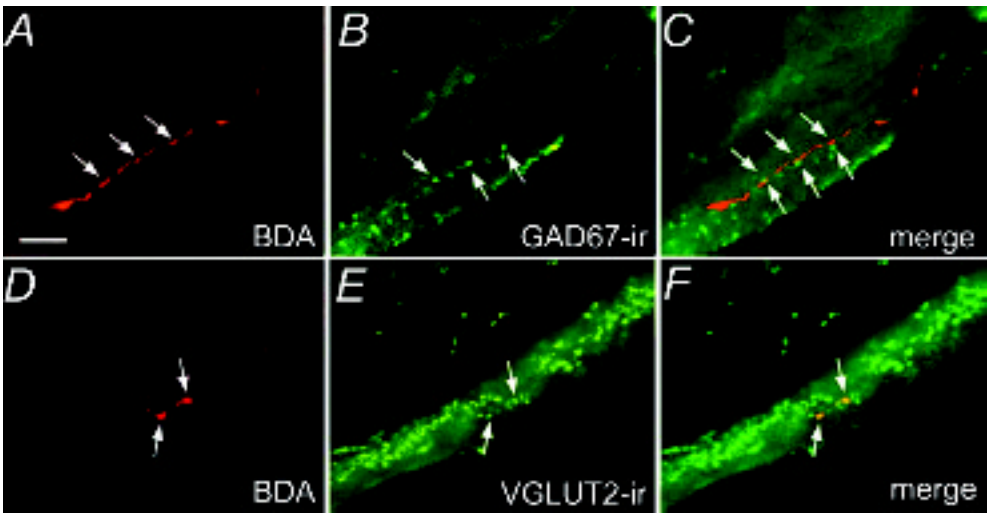

Figure 2. BDA-labelled varicosities within the marginal layer of RTN are predominantly glutamatergic
A–*C*, example of BDA-labelled axonal varicosities (red) that were not immunoreactive for GAD67 (still red, i.e. not double-labelled in the merged panel). *D*–*F*, example of BDA-labelled axonal varicosities that were immunoreactive for VGLUT2 (green in *E*, orange in the overlay). Scale bar, 50 μm, in *A*, applies to all panels.

immunoreactivity and VGLUT2 mRNA were mapped and counted. CommNTS neurons with the same marker combination were summed across the five coronal sections in each rat and the single resulting number was averaged across the six rats. As shown in Fig. 3*B* and *C*, the vast majority of the commNTS neurons that were immunoreactive for both Fos and CTB contained VGLUT2 mRNA ($92 \pm 13\%$) whereas only a small proportion of the same class of neurons (Fos- and CTB-positive) contained GAD67 mRNA ($12 \pm 9\%$; Fig. 3*D*).

Carotid chemoreceptor stimulation activates RTN neurons

As in prior work, RTN neurons were defined by their location (2–350 μm below the inferior margin of the antidromic facial field potential) and by the fact that they were silent below a threshold level of CO_2 and increasingly active at higher levels of CO_2. An example of one cell recorded in a rat with intact vagus nerves is shown in Fig. 5*A*. As under halothane anaesthesia, RTN cells became respiratory modulated only at higher levels

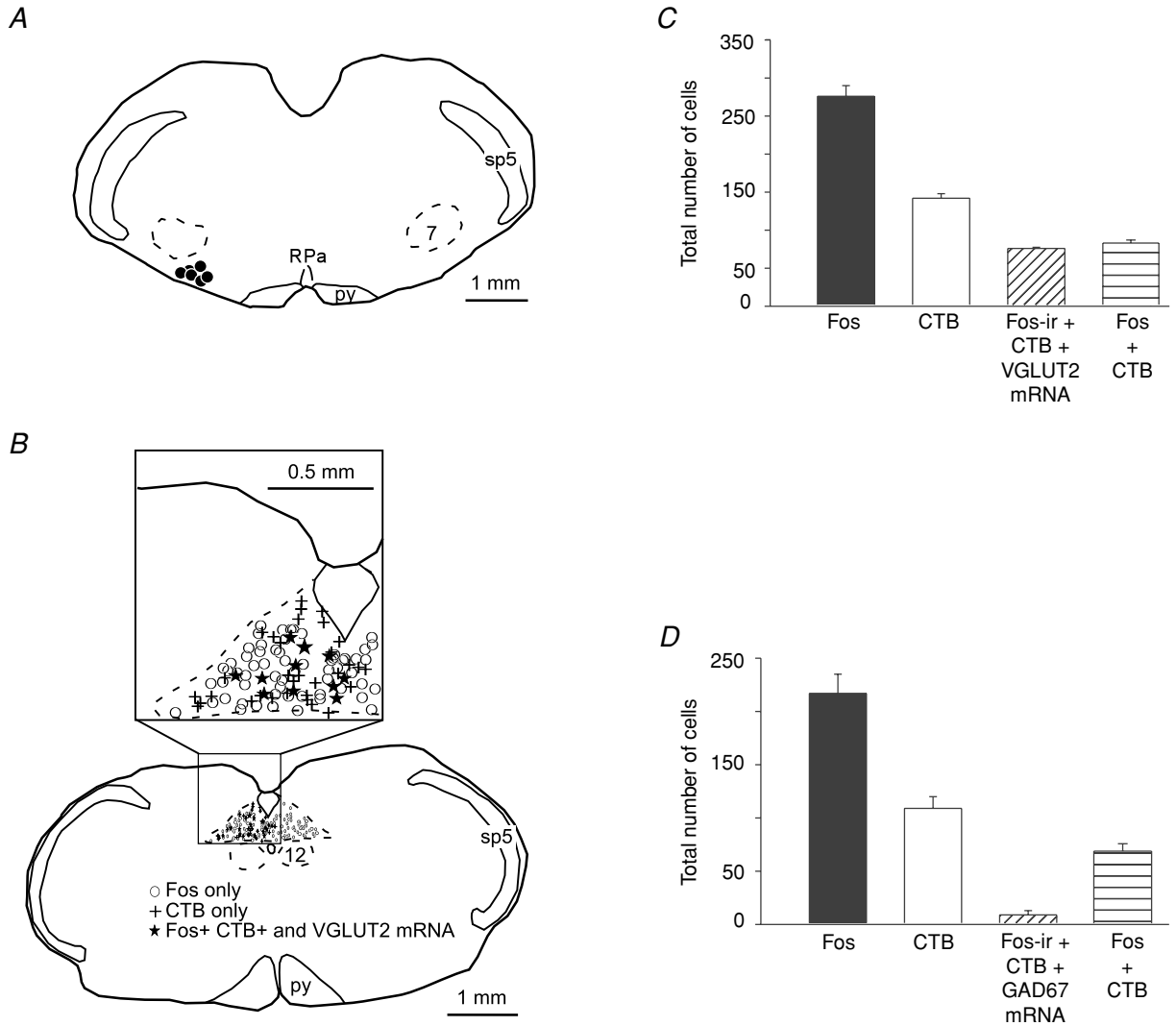

Figure 3. Hypoxia-activated commNTS neurons with RTN projections are predominantly glutamatergic
A, computer-assisted plots of cholera toxin B injection sites in six rats. The deposits were placed below the caudal end of the facial motor nucleus (7) under electrophysiological guidance. Each deposit was found within 200 μm of the coronal plane represented (Bregma −11.4 mm). *B*, computer-assisted plot of three pertinent marker combinations detected in commNTS neurons (Bregma level −14.08 mm). *C*, total number of commNTS neurons (mean ± S.E.M. of 6 rats) detected in four sections per brain (Fos, all Fos-ir neurons irrespective of other markers; CTB, all CTB-ir neurons irrespective of other markers). Most hypoxia-sensitive neurons with RTN projections contained VGLUT2 mRNA. *D*, total number of commNTS neurons (mean ± S.E.M. of 6 rats) detected in four sections per brain (Fos-ir, all Fos-ir neurons irrespective of other markers; CTB, all CTB-ir neurons irrespective of other markers). Few hypoxia-sensitive neurons with RTN projections contained GAD67 mRNA. RPa, raphe pallidus; for other abbreviations see Fig. 1.

of central respiratory drive (Fig. 5*A*, insets) but their firing probability did not reach zero at any period of the cycle even at 10% end-expiratory CO_2, the highest level examined (Fig. 5*B*). The particular cell shown in Fig. 5*B* displayed one of several respiratory patterns that we previously observed in halothane-anaesthetized rats (Guyenet *et al.* 2005*a*). This particular pattern was interpreted previously as the result of inhibitory volleys during post-inspiration and late expiration (Guyenet *et al.* 2005*a*). The full range of respiratory patterns previously identified under halothane was present under chloralose–urethane anaesthesia including the common pattern consisting of early inspiratory and post-inspiratory inhibition, an example of which is shown in Fig. 9*B*1. However, the respiratory modulation of RTN units was generally less pronounced under chloralose–urethane anaesthesia than under halothane judging by the fact that in a high proportion of the neurons (46%; 13 of 28 sampled neurons) less than 15% difference was observed between the presumed nadir and apex of the PND-triggered histograms even when the CO_2 level was above 9%.

At steady state, the discharge rate of RTN neurons was a linear function of end-expiratory CO_2 at first and then exhibited incomplete saturation at higher levels of CO_2 (Figs 5C and 6). PND also exhibited a somewhat curvilinear relationship to CO_2 (Figs 5*C* and 6) although the saturation was less marked than under halothane. On average, the discharge rate of RTN units increased by 2.2 ± 0.4 Hz for each 1% increase in end-expiratory CO_2 during the linear portion of the relationship and reached a mean level close to 10 Hz at 10% end-expiratory CO_2 (Fig. 6*B*). The CO_2 threshold of RTN neurons was not statistically different in vagotomized *versus* intact rats (Fig. 6*B*) unlike that of the PND, which was significantly lower in vagotomized rats (Fig. 6). Consequently, the CO_2 threshold of RTN neurons was lower than that of PND in intact rats (mean threshold of 41 RTN neurons in 23 rats: $5.1 \pm 0.2\%$; mean PND threshold: $6.4 \pm 0.3\%$; $P < 0.05$) whereas these threshold values were closer in vagotomized rats (mean CO_2 threshold of 20 RTN neurons in 11 rats: $5.0 \pm 0.3\%$; mean PND threshold: $5.3 \pm 0.5\%$; $P > 0.05$) (Fig. 6).

Stimulation of carotid chemoreceptors with brief periods of hypoxia (30 s of 10–13% O_2) or intravenous injection of NaCN (50 μg kg^{-1}) was performed in 16 rats with intact vagus nerves, 10 of which had intact carotid nerves and the rest were denervated bilaterally. In rats with intact peripheral chemoreceptors, hypoxia or cyanide increased PND activity and activated every RTN neuron sampled (hypoxia and NaCN: 41 neurons; Fig. 7*A* and *C*). During these tests, the CO_2 level was set slightly above the PND threshold, i.e. close to 6.5% CO_2. The stimulatory effects of hypoxia and cyanide on both PND and RTN neurons was absent in rats with carotid body denervation (Fig. 7*B* and *D*). The sensitivity of RTN neurons to CO_2 was unaffected by carotid body denervation (control rats: 2.3 ± 0.3 Hz per 1% rise in end-expiratory CO_2; chemodenervated rats: 2.2 ± 0.4 Hz; n.s.). On average, carotid body denervation had no effect on the CO_2 threshold measured under steady-state conditions (control rats: $4.7 \pm 0.3\%$; chemodenervated rats: $5.2 \pm 0.2\%$; n.s.).

To determine more precisely how peripheral chemoreceptor inputs and central chemosensitivity are integrated

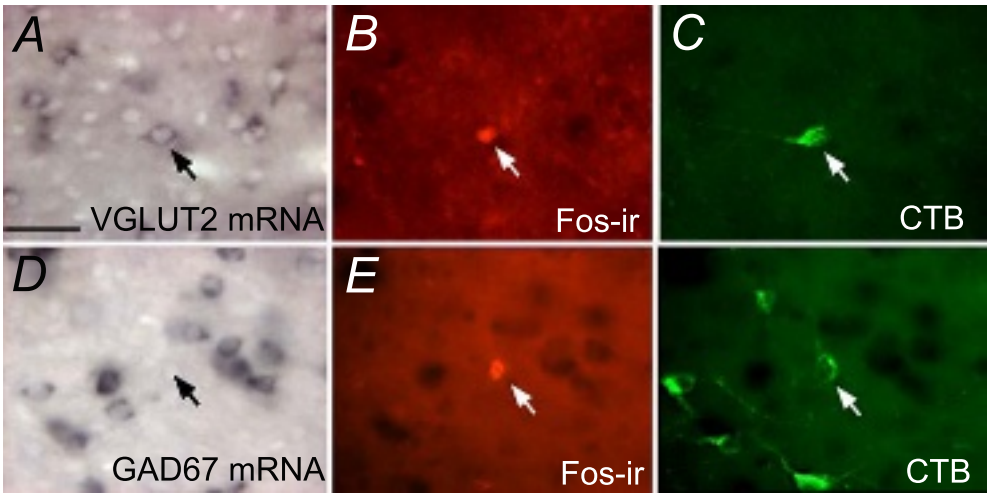

Figure 4. Hypoxia-activated commNTS neurons with RTN projections contain VGLUT mRNA
A–C, example of a neuron retrogradely labelled from RTN (CTB-immunoreactive) and located in commNTS that was activated by hypoxia (Fos-ir) and contained VGLUT2 mRNA. *D–F*, example of a CTB-labelled neuron located in commNTS that was activated by hypoxia (Fos-ir) but lacked GAD67 mRNA, unlike many of the surrounding cells. Scale bar, 20 μm in *A*, applies to all panels.

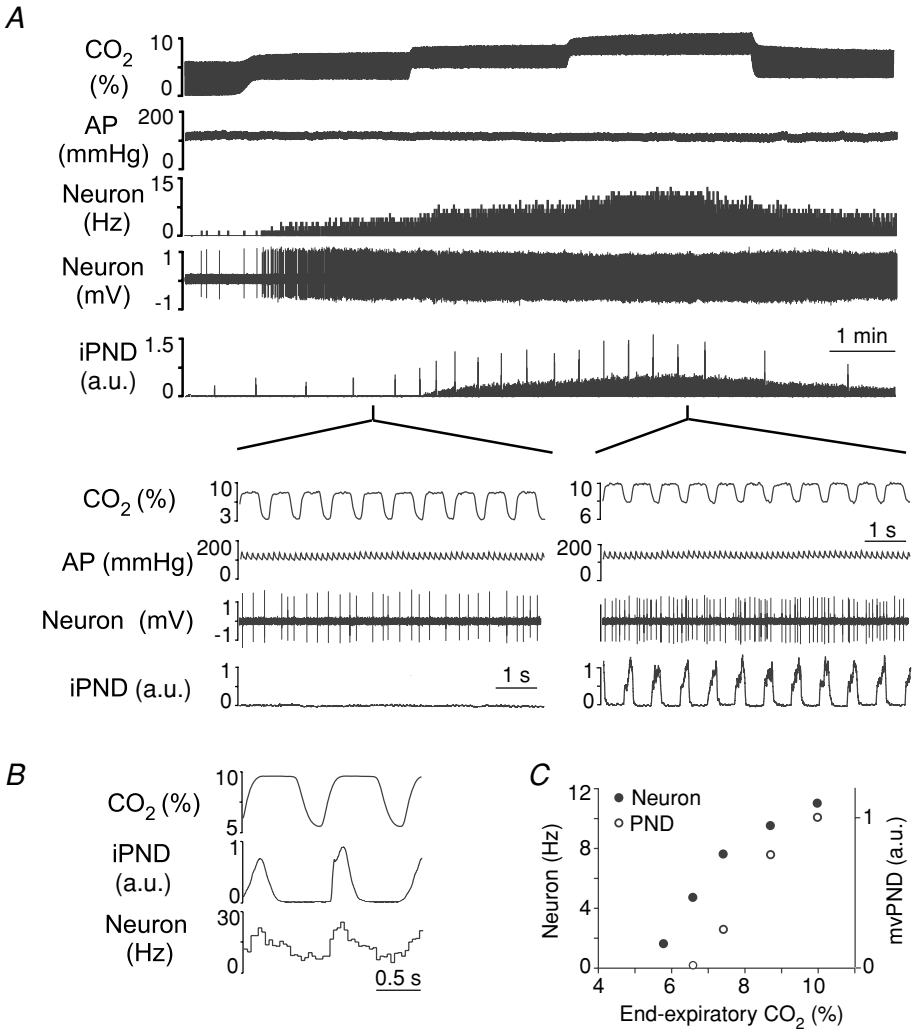

Figure 5. General electrophysiological characteristics of RTN neurons
A, example of one RTN neuron exposed to various levels of end-expiratory CO_2 in a rat with intact vagus nerves. The large upward deflections in the iPND trace represent augmented breaths commonly observed under chloralose–urethane anaesthesia. The insets illustrate the regularity of the cell discharge at low CO_2 level (left inset) and its respiratory entrainment at higher levels of CO_2 when the phrenic nerve was active (right inset). *B*, PND-triggered activity histogram showing that the RTN neuron is respiratory modulated but not respiratory phasic at high levels of CO_2. *C*, relationship between neuronal discharge rate and end-expiratory CO_2 at steady state for the neuron represented in *A*. The same graph also shows the relationship between mvPND (neural minute × volume) and end-expiratory CO_2. The high PND CO_2 threshold was characteristic or rats with intact vagus nerves.

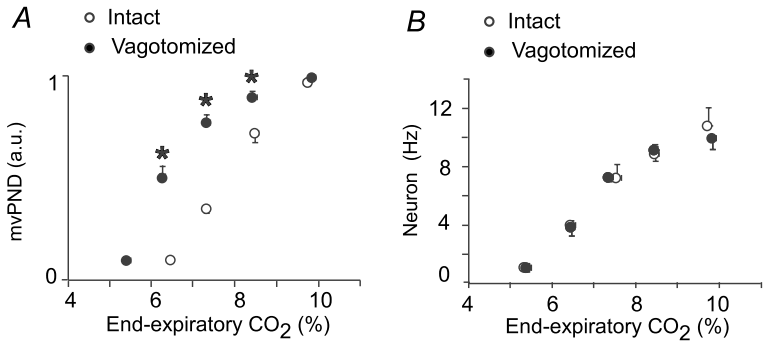

Figure 6. General electrophysiological characteristics of RTN neurons: group data
A, relationship between mvPND (neural minute × volume) and end-expiratory CO_2 at steady state in intact (8 rats) and in vagotomized rats (6 rats). *Significantly different from intact rats ($P < 0.05$; two-way ANOVA). *B*, relationship between neuronal discharge rate and end-expiratory CO_2 at steady state in 8 rats with intact vagus nerves (21 neurons) and in 6 vagotomized rats (11 neurons). There was no difference.

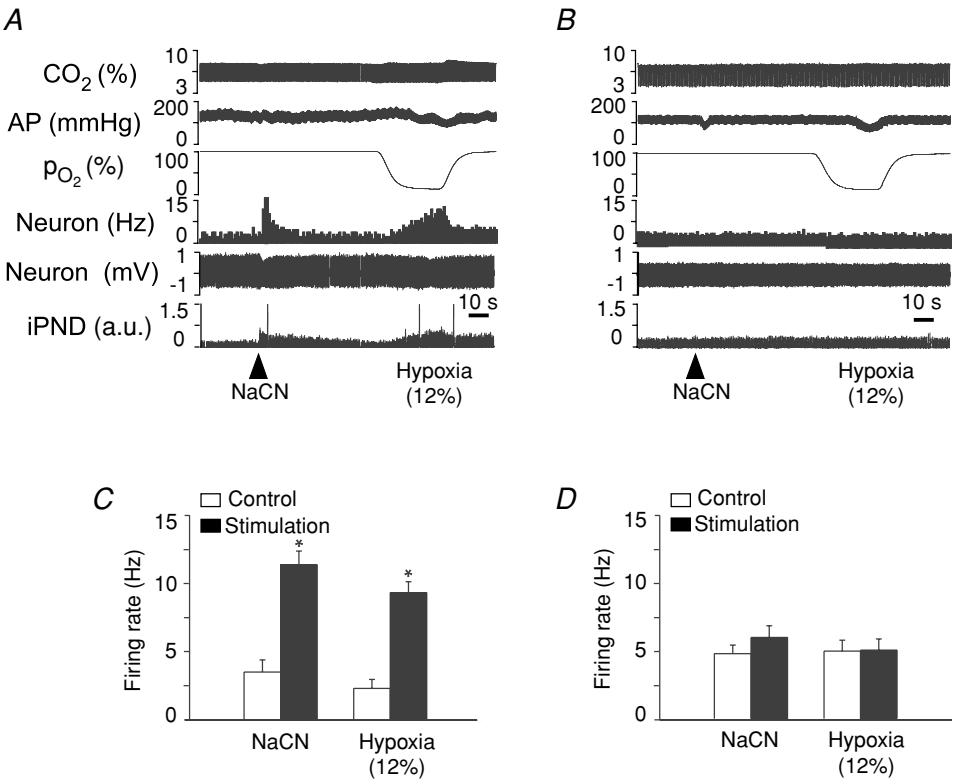

Figure 7. RTN neurons are activated by hypoxia and cyanide
A, response of one RTN neuron to I.V. injection of 50 μg kg^{-1} of cyanide and to hypoxia (12% O_2) in a rat with intact carotid chemoreceptors. *B*, lack of effect of the same stimuli on PND and an RTN neuron in a rat subjected to carotid chemoreceptor denervation. *C*, group data for rats with intact carotid chemoreceptors (15 neurons from 10 rats). In these tests, the resting discharge rate of RTN neurons was kept at 2–5 Hz by adjusting end-expiratory CO_2. *Significant difference from discharge at rest ($P < 0.05$; paired *t* test). *D*, group data for rats subjected to carotid chemoreceptor denervation (13 neurons from 6 rats). Carotid body stimulation produced no effect.

at RTN level, we measured the steady-state relationship between RTN neuron discharge rate and end-expiratory CO_2 during long exposures to three levels of oxygen (100%, 15% and 21% O_2 balanced with N_2; 20 min per O_2 level). These measurements were made sequentially in the order indicated in six cells from five rats with intact vagus nerves (Fig. 8). The effects of more severe hypoxia could not be tested because excessive hypotension occurred with

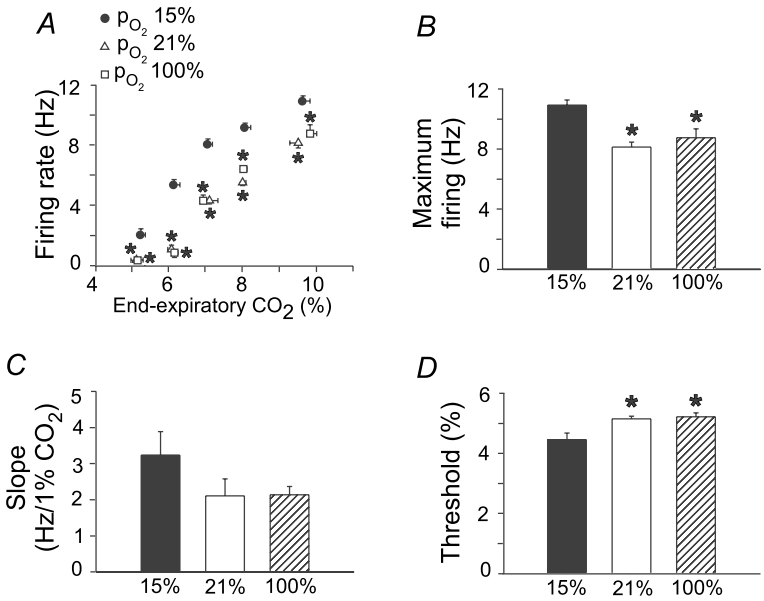

Figure 8. Effect of steady-state hypoxia on the CO_2-responsiveness of RTN neurons
Single RTN neurons were exposed to various steady-state levels of CO_2 under hyperoxia according to the protocol shown in Fig. 5*A*. The procedure was then repeated while oxygen in the breathing mixture was reduced to 15%. The test was repeated a second and final time while oxygen in the breathing mixture was set at 21%. *A*, relationship between firing rate at steady-state and end-expiratory CO_2 for 5 RTN neurons subjected to this protocol. *$P < 0.05$ from the corresponding 15% O_2 data points by two-way ANOVA for repeated measures. *B*, maximum firing rate of the cells *$P < 0.05$ from the 15% group by one-way repeated measure ANOVA. *C*, slope of the linear part of the relationship between firing rate and end-expiratory CO_2 (n.s.). *D*, CO_2 threshold of RTN neurons. *$P < 0.05$ from the 15% group by one-way repeated measure ANOVA.

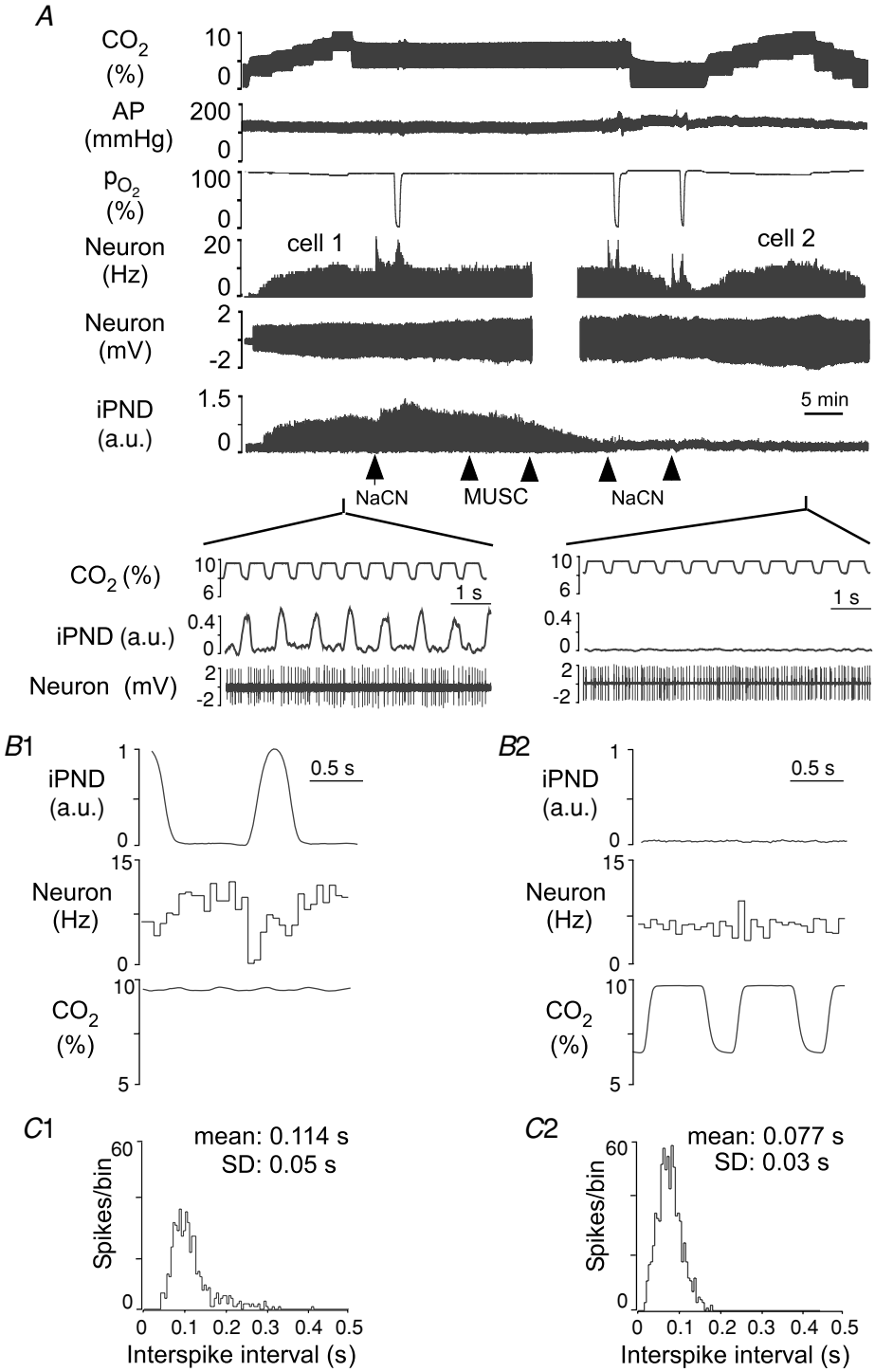

Figure 9. Bilateral injections of muscimol into the ventral respiratory column do not change the response of RTN neurons to central and peripheral chemoreceptor stimulation

A, a single RTN neuron (cell 1) was exposed to various concentrations of CO_2 under hyperoxia, then tested for its response to 12% hypoxia and cyanide (vagotomized rat). Muscimol was then injected into the ventrolateral medulla on both sides causing a gradual disappearance of PND. The mechanical disturbance caused by the injections typically caused the loss of the first RTN neuron and the search was resumed for another active neuron in the immediate vicinity of the first (cell 2). Muscimol eliminated PND at rest and during stimulation of peripheral or central chemoreceptors but cell 2 responded normally to these stimuli. The insets show that cell 2, recorded after muscimol, had a very regular discharge rate even at high levels of CO_2 whereas cell 1 was respiratory modulated. *B*1, peri-event histogram of the discharge of cell 1 at high CO_2. PND served as trigger. This neuron was inhibited

long duration exposure to hypoxia below 15% O_2. In the presence of 15% O_2, the CO_2 threshold of RTN neurons was significantly reduced (hypoxia: $4.5 \pm 0.6\%$ CO_2; hyperoxia: $5.3 \pm 0.3\%$ CO_2; $P < 0.05$) but the sensitivity of the cells to CO_2 was not changed (hypoxia: 3.3 ± 1.1 Hz per 1% rise in end-expiratory CO_2; hyperoxia: 2.3 ± 0.5; n.s.; Fig. 8). 'Normoxia' (21% O_2) had no effect on the CO_2 threshold of RTN neurons (normoxia: $5.2 \pm 0.4\%$ CO_2; hyperoxia: $5.3 \pm 0.3\%$ CO_2; n.s.) nor on their CO_2 sensitivity (normoxia: 2.1 ± 0.2 Hz per 1% rise in end-expiratory CO_2; hyperoxia: 2.3 ± 0.5 Hz; NS; Fig. 8).

Injection of muscimol in the rostral ventral respiratory group does not change the sensitivity of RTN neurons to central or peripheral chemoreceptor stimulation

The anatomical experiments described in the first paragraph of the Results section suggested that the activation of RTN neurons by peripheral chemoreceptor stimulation could be due to a direct excitatory input from commNTS. Our prior work on RTN neurons is consistent with the notion that their response to CO_2 under hyperoxia is due to their intrinsic chemosensitivity (Mulkey *et al.* 2004; Guyenet *et al.* 2005*a*). If both hypotheses are correct, the response of RTN neurons to hyperoxic hypercapnia and to peripheral chemoreceptor stimulation should persist after inactivation of the central respiratory pattern generator. The $GABA_A$ receptor agonist muscimol was selected for this purpose. In six vagotomized rats, bilateral injections of muscimol (1.75 mM, 30 nl per side) were placed under electrophysiological guidance into a region of the VRG from where strong inspiratory-related multiunit activity could be recorded. Muscimol injection into this region eliminated the PND for up to 2 h (Fig. 9*A* and *B*). Upon histological examination, the centres of the injection sites were found close to 1.5 mm posterior to the caudal end of the facial motor nucleus (Fig. 10*C*). By our previous estimates, these sites correspond to the rVRG (Stornetta *et al.* 2003*b*). The fluorescent microbeads extended 270 ± 15 μm on each side of the injection centre and therefore muscimol, which probably diffuses more freely that microbeads, is likely to have also reached the respiratory neurons located in the pre-Bötzinger complex.

One or two RTN neurons were fully characterized in each of the six rats before muscimol injection. Attempts to hold the same neuron before and after muscimol injection into the ventrolateral medulla were not successful because of the mechanical disturbance created by moving the muscimol injection pipette. The example shown in Fig. 9 illustrates an experiment in which an RTN neuron was characterized before muscimol and a second RTN neuron was found just after the second muscimol injection in the immediate vicinity of the first one. As shown in Fig. 9*A*, muscimol eliminated PND but the RTN neuron recorded after muscimol responded to hyperoxic hypercapnia and to peripheral chemoreceptor stimulation in the same way as the cell recorded before muscimol. Consistent with the loss of central respiratory network activity (i.e. PND), the CO_2-activated neuron recorded after muscimol injection had no detectable respiratory-like modulation, in other words the cell discharged much more regularly (Fig. 9*B versus C*).

On average, the properties of RTN neurons recorded before muscimol (9 neurons in 6 rats) were the same as those recorded after muscimol before any recovery of PND occurred (1 neuron per rat in the same 6 rats) (Fig. 10*A* and *B*). Specifically, under hyperoxia, the CO_2 threshold and the CO_2 sensitivity of the cells were the same (Fig. 10*A*) and the responses of the cells to hypoxia or cyanide were also indistinguishable (Fig. 10*B*). The only difference was that every neuron recorded after muscimol lacked respiratory modulation and discharged much more regularly (Table 1).

Bilateral injections of muscimol into RTN eliminate PND at rest and during chemoreceptor stimulation

RTN is believed to contribute an important source of excitatory drive to the respiratory network under anaesthesia. The final experiments were designed to determine whether bilateral inhibition of RTN is capable of suppressing the stimulatory effect of both central and peripheral chemoreceptor activation on PND. Muscimol (1.75 mM, 30 nl) was injected bilaterally under electrophysiological guidance 200 μm below the facial motor nucleus and 200 μm rostral to the caudal end of this nucleus to target the region that contains the highest density of CO_2-sensitive RTN neurons according to our prior experience (Mulkey *et al.* 2004; Guyenet *et al.* 2005*a*). The centre of the injection sites were in the desired location as shown by histological mapping of fluorescent microbeads included in the injectate (Fig. 11*C*). The beads were found to have spread approximately 260 μm on each side of the injection centre.

As illustrated in Fig. 11*A*, muscimol eliminated PND and no activity, even tonic, could be restored by combined central (10% CO_2) and peripheral

during early inspiration and again during post-inspiration. *B*2, peri-event histogram of the discharge of cell 2 at high CO_2. End-expiratory CO_2 served as trigger. *C*1, interspike interval distribution histogram of cell 1 at high CO_2. The histogram is asymmetric and skewed towards longer intervals. *C*2, interspike interval distribution histogram of cell 2 at high CO_2. The histogram is very symmetric due to the loss of entrainment by the respiratory network.

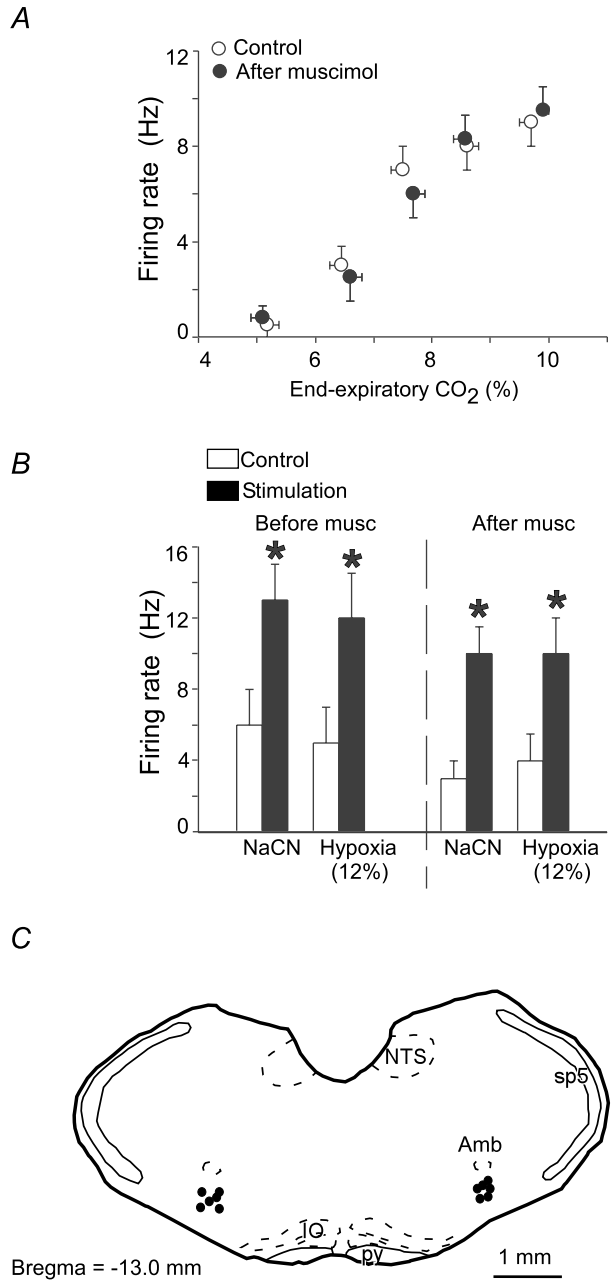

Figure 10. Bilateral injections of muscimol into the ventral respiratory column do not change the response of RTN neurons to central and peripheral chemoreceptor stimulation: group data

A, relationship between firing rate at steady-state and end-expiratory CO_2 measured under hyperoxic conditions. The relationship was the same before (9 cells in 6 rats) and after bilateral injection of muscimol (1.75 mM, 30 nl on each side) into the ventrolateral medulla (6 cells in 6 rats; n.s. by two-way repeated measure ANOVA). *B*, effect of cyanide and transient hypoxia on the discharge rate of RTN neurons before and after muscimol (*$P < 0.05$ from control by unpaired *t* test). *C*, computer-assisted plots of the centre of the muscimol injection sites revealed by the presence of fluorescent microbeads included in the injectate. The centre of all injections were within 200 μm of the coronal level indicated. This level corresponds to the rVRG.

chemoreceptor stimulation (10–13% O_2 or I.V. injection of NaCN). Complete recovery from the effect of muscimol occurred within 2 h (Fig. 11*B*). Muscimol produced the same complete PND inhibition in each of eight rats. These muscimol injections also reduced blood pressure from 121 ± 5 to 97 ± 6 mmHg ($P < 0.01$), consistent with muscimol having inhibited some of the blood pressure-regulating neurons that reside in the ventrolateral medulla at and caudal to RTN level.

Bilateral injections of the same amount of muscimol into the midline raphe at the same coronal plane as RTN (two 30 nl injections; 6 rats) produced no effect on resting mvPND measured at an end-expiratory CO_2 of 7–8% (96 ± 12% of pre-drug mvPND after muscimol; n.s.). Muscimol injection into the raphe had no effect on blood pressure (from 122 ± 5 to 123 ± 5 mmHg; n.s.).

Discussion

The present study indicates that RTN neurons respond both to central P_{CO_2} and to signals from carotid chemoreceptors. The pathway between carotid chemoreceptor afferents and RTN neurons is excitatory and may involve a single intervening glutamatergic neuron located within commNTS (Fig. 12). We conclude that RTN neurons integrate central and peripheral chemoreceptor information and may drive diaphragmatic activity and/or other aspects of the cardiorespiratory network.

Properties of RTN neurons under chloralose–urethane anaesthesia

RTN neurons recorded under chloralose–urethane anaesthesia had similar properties as under halothane, the anaesthetic used in our previous experiments. The sensitivity of RTN neurons to CO_2 under hyperoxic conditions presumably reflects their intrinsic response to extracellular pH (Mulkey *et al.* 2004; Guyenet *et al.* 2005*a*). This CO_2 sensitivity was the same under both anaesthetics (2.2 Hz per 1% change in end-expiratory CO_2). This fact is noteworthy since halothane opens TWIK-related acid-sensitive channels (TASK), which are expressed by many brainstem respiratory neurons and are candidate molecular substrates of central chemosensitivity (Bayliss *et al.* 2001; Washburn *et al.* 2003). The present results do not exclude a contribution of TASK channels to central chemosensitivity but they demonstrate that 1% halothane does not affect the pH sensitivity of RTN neurons any more than chloralose–urethane anaesthesia used at a dose that produces a comparable depth of anaesthesia.

There were a few minor differences between the two anaesthetics, however. The CO_2 threshold of RTN neurons under hyperoxia was slightly higher

Table 1. Interspike interval distribution of RTN neurons before and after injection of muscimol into the ventral respiratory column caudal to RTN

	Inter-spike interval (mean)	Inter-spike interval (S.D.)	Number of neurons
Before muscimol	0.115 ± 0.009	0.052 ± 0.08	9
After muscimol	0.096 ± 0.008	0.022 ± 0.03	6
P (paired *t* test)	n.s.	< 0.05	

Interspike interval distribution histograms (ISH) of RTN neurons were made during periods when the rats were exposed to high levels of end-expiratory CO_2 (9–10%) as shown in Fig. 9C1 and 2. The table shows the mean interspike interval and the standard deviation of these ISH distributions. The average discharge rate of the cells was no different after muscimol was injected bilaterally into the rVRG but the standard deviation of the ISH was significantly smaller indicating that the discharge of the neurons was on average more regular after muscimol injection.

under chloralose–urethane (5% *versus* 4.2%) and the relationship between RTN neuron activity and CO_2 exhibited a somewhat less pronounced saturation at high levels of hypercapnia. PND had a similar CO_2 threshold as under halothane but, like RTN neurons, PND also exhibited a less pronounced saturation at high levels of hypercapnia. As shown previously, the intrinsic response of RTN neurons to P_{CO_2} is linear and the saturation of their discharge rate at high levels of CO_2 is caused by inhibitory inputs from the CPG that increase in intensity along with the central inspiratory drive (Guyenet *et al.* 2005*a*). This input is somewhat weaker under chloralose–urethane as suggested by a generally more modest central respiratory modulation of RTN neurons than under halothane. A weaker input should produce a more linear relationship between RTN activity and CO_2, as was observed.

RTN neurons receive oligo-synaptic excitatory inputs from carotid chemoreceptors

All RTN neurons were vigorously activated by brief periods of hypoxia and by intravenous cyanide. Since these responses were observed only in rats with intact carotid chemoreceptors, they depended entirely on afferent inputs from these organs. These results are congruent with prior

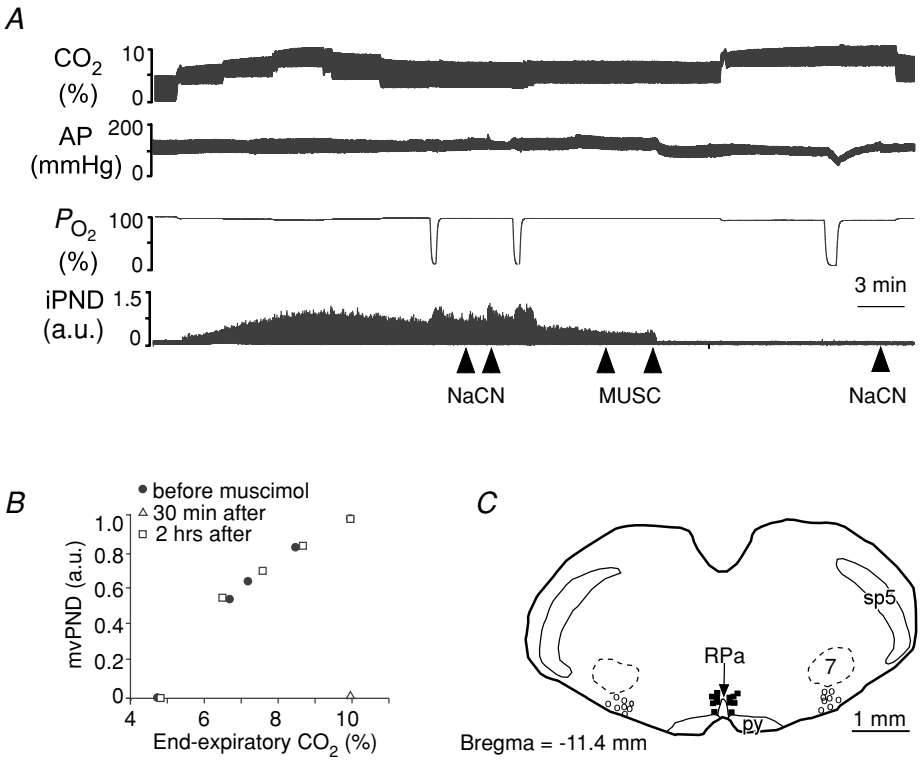

Figure 11. Bilateral injections of muscimol into RTN eliminate PND
A, effect of bilateral injections of muscimol (30 nl, 52 pmol) placed under the caudal end of the facial motor nucleus using antidromic field potentials as guide for pipette placement. PND disappeared and could not be restored by extreme chemoreceptor stimulation (10% end-expiratory CO_2 + hypoxia). *B*, relationship between mvPND and end-expiratory CO_2 at steady-state before muscimol, 30 min after and 2 h after injection (full recovery). Data are from the case shown in *A* (recovery period not shown in *A*). PND was normalized to the maximum value observed before muscimol. *C*, computer-assisted plots of the muscimol injection sites targeted to RTN projected on a single coronal section located 200 μm rostral to the caudal end of the facial motor nucleus (open circles; 8 rats). The plot also shows the location of muscimol injections that were placed in the midline raphe at the same level as RTN in 6 rats. These injections produced no effect on resting PND or blood pressure.

experimentation in rats and cats (Bodineau *et al.* 2000*b*; Mulkey *et al.* 2004).

Carotid chemoreceptor afferents primarily innervate commNTS (Blessing *et al.* 1999). Most of the commNTS neurons that are activated by carotid body stimulation are not respiratory modulated and therefore are probably not part of the respiratory pattern generator (Koshiya & Guyenet, 1996; Paton *et al.* 2001). Many of these carotid body-responsive neurons project to the ventrolateral medulla where one of their targets has long been assumed to be the blood pressure-regulating C1 neurons (Aicher *et al.* 1996; Koshiya & Guyenet, 1996; Paton *et al.* 2001). According to the present study, the commNTS neurons that are activated by carotid body stimulation innervate the RTN neurons and this projection is predominantly glutamatergic. These NTS neurons could also innervate other ventrolateral medullary neurons including the blood pressure-regulating C1 neurons (Aicher *et al.* 1996; Sun & Reis, 1996) but this issue is not addressed by the present experiments.

The presumption that commNTS neurons innervate the CO_2-sensitive neurons is based on the observation that many BDA-labelled axonal varicosities were found in the marginal layer of RTN where the CO_2-sensitive cells have extensive dendrites (Mulkey *et al.* 2004; Guyenet *et al.* 2005*a*). The Fos-expression experiments also indicated that the projection from commNTS to RTN contains many neurons that are activated by hypoxia. We assume that the vast majority of the NTS neurons that express Fos following hypoxia respond to activation of the carotid bodies rather than to secondary effects of hypoxia on blood pressure. In any event, the present results are consistent with prior electrophysiological evidence that commNTS cells with carotid chemoreceptor input innervate the rostral ventrolateral medulla in rats and the RTN region of cats (Koshiya & Guyenet, 1996; Bodineau *et al.* 2000*a*).

In addition, the present study shows that the hypoxia-activated pathway between commNTS and RTN is predominantly glutamatergic since an average of 92% of these neurons contained detectable levels of VGLUT2 mRNA. A smaller percentage of BDA-labelled varicosities were found to contain VGLUT2 immunoreactivity in the anterograde tracing experiments (51%) but this method may underestimate the proportion of terminals that are glutamatergic for two reasons. First, dual labelling for BDA and VGLUT2 may not reveal each marker equally, especially in the depth of the tissue because of incomplete antibody penetration. It is also conceivable that a portion of the BDA-labelled structures that look like axonal varicosities may lack VGLUT2 immunoreactivity simply because they are not synapses. This second interpretation is less likely because, where investigated, the vast majority of BDA axonal varicosities seen at the light microscopic level correspond to synaptic sites seen by electron microscopy of thin sections (Kincaid *et al.* 1998). Therefore, it is more instructive to contrast the high percentage of putative VGLUT2-immunoreactive synapses identified in the projection from commNTS to RTN (around 51% of the BDA-labelled varicosities) with the low percentage of terminals identified as

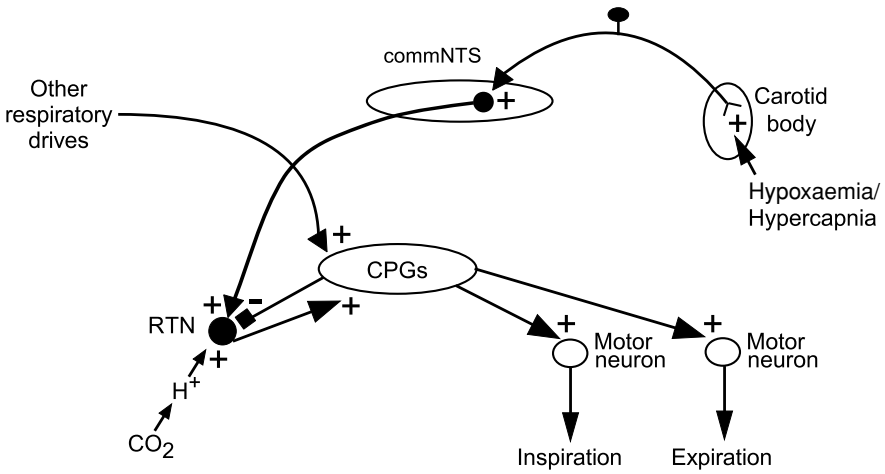

Figure 12. Possible role of RTN in chemosensory integration
We propose that RTN neurons function as integrators of central and peripheral chemoreceptor information. The exact targets of RTN neurons are still undefined and probably include elements of the various respiratory pattern generators located within the ventrolateral medulla (CPGs), possibly premotor neurons (Dobbins & Feldman, 1994). Other potential targets of RTN neurons include the ventrolateral medullary neurons that drive sympathetic cardiovascular efferents (not represented). RTN neurons are subject to inhibition from the CPG (Guyenet *et al*. 2005*a*), a mechanism that could conceivably be related to the need to down-regulate this structure when the pattern generators are activated by other inputs. commNTS, commissural nucleus of the solitary tract; CPG, respiratory pattern generator; RTN, retrotrapezoid nucleus.

GAD67-immunoreactive (5%). In summary, the vast majority of hypoxia-sensitive commNTS neurons with RTN projections are glutamatergic but a small fraction of this pathway may be inhibitory since around 12% of neurons containing Fos and CTB also contained GAD67 mRNA in rats exposed to hypoxia. This conclusion is also compatible with the fact that RTN contained a small number of BDA-labelled terminals that were immunoreactive for GAD67 after injection of the tracer into commNTS. This inhibitory projection may not target the CO_2-activated neurons of RTN but may contact more medially located neurons that regulate sympathetic tone to the skin and the brown adipose tissue, outflows that are inhibited by hypoxia (Madden & Morrison, 2005).

The present results do not prove that the hypoxia-activated glutamatergic neurons of commNTS that innervate RTN are second-order neurons, i.e. receive monosynaptic inputs from carotid chemoreceptor afferents. However, this possibility is likely given that, in slices, low-jitter, presumably monosynaptic, EPSCs can be elicited by stimulating the solitary tract in most of the NTS neurons that project towards the ventrolateral medulla (Bailey *et al.* 2004).

In summary, the present study demonstrates that the RTN region receives glutamatergic inputs from commNTS neurons that are activated by peripheral chemoreceptor stimulation. These glutamatergic neurons could well be the sole central neurons interposed between carotid chemoreceptor afferents and the chemosensitive neurons of RTN though the existence of interneurons within the NTS or the RTN is not ruled out by the present evidence. In any case, the main pathway between carotid chemoreceptor afferents and RTN neurons almost certainly bypasses the respiratory rhythm and pattern-generating network since injections of muscimol into the ventral respiratory column caudal to RTN eliminated PND but did not affect the response of RTN neurons to hypoxia or cyanide.

RTN neurons respond both to brain extracellular fluid P_{CO_2} and to blood gas composition as detected by peripheral chemoreceptors

At present, the theory that a pH-sensitive resting potassium conductance underlies the chemosensitivity of RTN neurons accounts satisfactorily for prior observations in slices (Mulkey *et al.* 2004; Putnam *et al.* 2004). However, ATP release, possibly from nearby non-neuronal cells, may also contribute to the CO_2 responsiveness of RTN neurons or other nearby chemoreceptors *in vivo* (Gourine *et al.* 2005). Whether it is intrinsic, paracrine or both, the response of RTN neurons to brain P_{CO_2} *in vivo* does not seem secondary to the activation of the respiratory network, at least under anaesthesia. This notion is supported by prior evidence that the response of RTN neurons to hyperoxic hypercapnia is unaffected by high concentrations of a glutamate receptor antagonist *in vivo* and that RTN neurons have a comparable response to pH in coronal slices (Mulkey *et al.* 2004; Guyenet *et al.* 2005*a*). Consistent with this interpretation, in the present study the response of RTN neurons to hyperoxic hypercapnia was unaffected by inhibiting the ventral respiratory column with muscimol at sites caudal to the RTN. The sole effect of muscimol was to regularize the discharge of RTN neurons as expected from the loss of the respiratory-phasic inputs that these neurons receive from the central respiratory pattern generator (Guyenet *et al.* 2005*a*).

Steady-state peripheral chemoreceptor stimulation increased the discharge rate of RTN neurons by a fixed amount regardless of the level of end-expiratory P_{CO_2}. In other words, extracellular fluid pH and peripheral chemoreceptor stimulation seem to have roughly additive effects on RTN neuron activity, at least at the mild level of hypoxia that could be reliably investigated without causing hypotension (15% O_2). Interestingly, the effect of peripheral chemoreceptor stimulation and the central action of CO_2 on PND are also typically additive under anaesthesia (Nattie *et al.* 1991). At RTN level, this summation had the effect of lowering the end-expiratory CO_2 threshold of the neurons, i.e. the CO_2 level at which they started to discharge. If the molecular substrate of central chemosensitivity is nothing more than a set of pH-sensitive neuronal conductances, it is logical to expect that the CO_2 response of central chemoreceptor neurons would vary according to the synaptic inputs that they receive. However, to our knowledge, RTN neurons are the first documented example where this convergence involves an excitatory input from peripheral chemoreceptors.

In summary, under anaesthesia, RTN neurons detect brain extracellular fluid pH, probably directly, and they encode this variable in a manner that depends on the strength of the input that they simultaneously receive from carotid chemoreceptors. The CO_2 threshold of the cells is therefore not fixed but dependent on the synaptic inputs that these cells receive.

RTN and the chemical drive to breathe

Injections of muscimol into RTN eliminated PND at rest and during activation of central and peripheral chemoreceptors. These injections were centred below the caudal pole of the facial motor nucleus where the presumed RTN chemoreceptor neurons are most concentrated based on present and prior recordings (Mulkey *et al.* 2004; Guyenet *et al.* 2005*a*,*b*). At face value, these results are congruent with many other lines of evidence which suggest that RTN is a chemosensitive region that drives inspiration (Nattie *et al.* 1991; Feldman *et al.* 2003; Onimaru & Homma, 2003)

and with anatomical evidence that some RTN neurons may be directly antecedent to phrenic premotor neurons (Dobbins & Feldman, 1994). The contribution of the RTN region to breathing is not limited to the anaesthetized state. Even unilateral lesion of this bilateral structure produces notable chronic deficits in the hypercapnic ventilatory response of awake rats (−39%; Akilesh *et al.* 1997). The fact that muscimol also eliminated the effect of carotid chemoreceptor stimulation on PND could also be viewed as evidence that RTN encodes the chemical drive to breathe, at least under anaesthesia.

Although the macrophysiological data (RTN lesions, stimulations, etc.) are generally consistent with the electrophysiological characteristics and projection pattern of RTN neurons, both lines of supporting evidence have inherent limitations. First, regarding the electrophysiological evidence, the discharge and projection pattern of RTN neurons provide necessary but not sufficient evidence of their role in respiratory control since the exact targets of these neurons are still unknown. Second, the exact boundaries of the tissue affected by procedures like muscimol injection, ventral medullary surface cooling, and chemical or electrolytic lesions are difficult to assess accurately (Millhorn, 1986; Fukuda *et al.* 1993; Nattie & Li, 1994; Forster *et al.* 1995).

The fluorescent microbeads that were co-injected with muscimol migrated up to 300 μm from the injection centre. Respiratory and other ventrolateral medullary formation neurons in rat typically have dendrites that spread no farther than 200 μm from their cell bodies in the rostrocaudal direction but exceptions (up to 500 μm spread) are not uncommon (Pilowsky *et al.* 1990; Schreihofer & Guyenet, 1997). Therefore it is conceivable that neurons located from 500 μm up to 800 μm caudal to the centre of the injection sites (3–600 μm caudal to the posterior edge of the facial motor nucleus) could have been inhibited to various degrees by injecting muscimol into RTN. Our estimates of the effective spread of muscimol are much smaller than the 1.7–2 mm chemical spread measured autoradiographically by Edeline *et al.* (2002). This difference can be explained in part by the fact that we administered smaller volumes and eight times less drug than the minimum dose administered by these authors (30 *versus* 50 nl, 50 *versus* 440 pmol). In addition, chemical spread and effective spread are loosely related since the first depends on the sensitivity of the detection method whereas the second depends on a threshold concentration being achieved for meaningful receptor occupancy. Our estimate of the effective spread (at most 800 μm from the centre and probably much less) is consistent with the fact that injections into the raphe 1.5 mm lateral to the centre of RTN produced no respiratory effect at all. They are also consistent with the fact that muscimol injection into the rVRG did not decrease blood pressure. Decreases of 50–60 mmHg would have been expected if the drug had inhibited a significant fraction of the blood-pressure regulating neurons whose cell bodies are largely confined to the Bötzinger level of the ventral respiratory column therefore well within 1 mm of the rVRG (Kanjhan *et al.* 1995; Schreihofer *et al.* 1999*b*). Judging the spread of muscimol from these physiological criteria, injections placed into RTN could have inhibited respiratory neurons located at the Bötzinger level of the VRC but most probably not caudal to that level. The Bötzinger region contains expiratory augmenting neurons and other respiratory neurons (including pre-inspiratory neurons identified *in vitro*) that could play an essential role in generating inspiratory activity (Onimaru *et al.* 1992; Kanjhan *et al.* 1995; Sun & Reis, 1996; Schreihofer *et al.* 1999*a*; Mellen *et al.* 2003). In short, we cannot exclude that muscimol injection into RTN could have eliminated PND in part or even in totality by silencing neurons located caudal to the CO_2-activated neurons that are the focus of the present study. Consequently, alternate functions of RTN neurons should also be considered, particularly the possibility that these neurons could be regulating selected autonomic efferents or respiratory outflows other than to the diaphragm.

Some experiments suggest that the RTN region may preferentially regulate the activity of expiratory muscles (Forster *et al.* 1995; Janczewski & Feldman, 2006). The theory that RTN neurons are part of an expiratory rhythm generator (Janczewski & Feldman, 2006) is not at odds with the present observations. Since the activity of expiratory muscles is increased by central and peripheral chemoreceptor stimulation, RTN neurons could be a, or the, source of chemical drive for this part of the respiratory network. The small respiratory modulation of RTN neurons that we observed could be a consequence of the anaesthesia. On the other hand, the greater sensitivity of expiratory than inspiratory muscle activity to RTN inhibition (Forster *et al.* 1995; Onimaru & Homma, 2003) does not exclude the possibility that RTN could drive both outflows; the differential susceptibility of these outflows to RTN inhibition could simply be due to a higher threshold for activation of the downstream expiratory network. This interpretation is consistent with the fact that inspiratory activity is much more strongly inhibited by RTN lesions in models in which inspiratory drive is reduced by cold or anaesthesia (Nattie & Li, 2000; and present results) than in awake animals in which synaptic activity would certainly be more robust (Li & Nattie, 1997). It is also consistent with the fact that RTN acidification, which is less likely to affect neurons distant from RTN than muscimol, does increase inspiratory activity (Li & Nattie, 1997). Finally, the possibility that RTN neurons drive numerous targets is also consistent with the variety of central respiratory patterns exhibited by these neurons (Guyenet *et al.* 2005*a*). In fact, based on their location, anatomical connections or central respiratory discharge pattern, RTN neurons could also be

regulating other respiratory efferents (airway muscles) or a selection of sympathetic (e.g. to heart, kidney or muscles) or parasympathetic outflows (e.g. to tracheal muscles) (Millhorn & Eldridge, 1986; Perez Fontan & Velloff, 1997; Guyenet *et al.* 2005*a*).

Raphe and breathing

The midline raphe contains serotonergic neurons and perhaps other neurons that regulate breathing and change the response of the respiratory network to CO_2 (Hodges *et al.* 2004; Nattie *et al.* 2004; Richerson *et al.* 2005). Under our experimental conditions, muscimol injection into the midline raphe at the same rostrocaudal level as RTN produced no effect on either PND or blood pressure. Interestingly, muscimol injection into approximately the same region of the medullary raphe in awake rats does not reduce the effect of hypercapnia on ventilation either (Taylor *et al.* 2005). Although this portion of the medullary raphe contains very large numbers of serotonergic neurons (Stornetta *et al.* 2005), these particular serotonergic neurons may not regulate breathing. Alternatively, the raphe neurons that are relevant to breathing could have been inactive in our experiments because of the anaesthesia, or the fraction of the raphe that was impaired by muscimol could have been too small to produce detectable effects on breathing (Hodges *et al.* 2004). The first alternative is more likely because muscimol injection into the rostral medullary raphe of awake rats does produce effects on respiration (Taylor *et al.* 2005). However, these effects are difficult to reconcile with the notion that the rostral medullary raphe contains respiratory chemoreceptors since muscimol potentiates the increase in ventilation produced by hypercapnia (Taylor *et al.* 2005). Furthermore, injection of DL-homocysteic acid into the same region of the raphe produces apnoea and powerful inhibition of the PND under anaesthesia (Verner *et al.* 2004), which also suggests that this region of the raphe inhibits breathing when it is activated. In any event, our data indicate that the midline raphe at RVL level does not contribute detectably to central chemosensitivity under our experimental conditions.

Conclusion: RTN as common central chemoreceptor pathway

Millhorn & Eldridge (1986) have suggested the possibility that afferents from carotid and medullary chemoreceptors may converge on neurons situated upstream of the respiratory centre pattern generators. The dysfunction of a medullary centre that integrates central and peripheral chemoreceptor inputs provides the most plausible explanation of the spectrum of respiratory deficits experienced by central congenital hypoventilation syndrome (CCHS) patients (Shea *et al.* 1993). The present experiments indicate that RTN neurons have the physiological properties and anatomical location that are required of such a chemoreceptor integrating centre. Based on our prior work, we postulate that the so-called central chemoreceptor input to RTN neurons may be nothing more than their intrinsic sensitivity to pH. The present work does not exclude the possibility that integration between intrinsic chemosensitivity and inputs from peripheral chemoreceptors could also be occurring at other brainstem sites, including in regions that are capable of influencing the respiratory network.

References

Aicher SA, Saravay RH, Cravo S, Jeske I, Morrison SF, Reis DJ & Milner TA (1996). Monosynaptic projections from the nucleus tractus solitarii to C1 adrenergic neurons in the rostral ventrolateral medulla: Comparison with input from the caudal ventrolateral medulla. *J Comp Neurol* **373**, 62–75.

Akilesh MR, Kamper M, Li A & Nattie EE (1997). Effects of unilateral lesions of retrotrapezoid nucleus on breathing in awake rats. *J Appl Physiol* **82**, 469–479.

Bailey TW, Aicher SA & Andresen MC (2004). Properties of direct and indirect pathways from the nucleus tractus solitarius (NTS) to caudal ventrolateral medulla (CVLM) and paraventricular hypothalamic nucleus (PVN) – combined tracer and synaptic transmission studies. *Faseb J* **18**, A665.

Bayliss DA, Talley EM, Sirois JE & Lei QB (2001). TASK-1 is a highly modulated pH-sensitive 'leak' K^+ channel expressed in brainstem respiratory neurons. *Respir Physiol* **129**, 159–174.

Bin-Jaliah I, Maskell PD & Kumar P (2004). Indirect sensing of insulin-induced hypoglycaemia by the carotid body in the rat. *J Physiol* **556**, 255–266.

Blessing WW, Yu YH & Nalivaiko E (1999). Medullary projections of rabbit carotid sinus nerve. *Brain Res* **816**, 405–410.

Bodineau L, Frugière A, Marlot D & Wallois F (2000*a*). Connections between retrotrapezoid nucleus and nucleus tractus solitarii in cat. *Neurosci Lett* **280**, 111–114.

Bodineau L, Frugiere A, Marlot D & Wallois F (2000*b*). Effect of hypoxia on the activity of respiratory and non-respiratory modulated retrotrapezoid neurons of the cat. *Auton Neurosci* **86**, 70–77.

Brown DL & Guyenet PG (1985). Electrophysiological study of cardiovascular neurons in the rostral ventrolateral medulla in rats. *Circ Res* **56**, 359–369.

Cream C, Li A & Nattie E (2002). The retrotrapezoid nucleus (RTN): local cytoarchitecture and afferent connections. *Respir Physiol Neurobiol* **130**, 121–137.

Dean JB, Lawing WL & Millhorn DE (1989). CO_2 decreases membrane conductance and depolarizes neurons in the nucleus tractus solitarii. *Exp Brain Res* **76**, 656–661.

Dobbins EG & Feldman JL (1994). Brainstem network controlling descending drive to phrenic motoneurons in rat. *J Comp Neurol* **347**, 64–86.

Edeline JM, Hars B, Hennevin E & Cotillon N (2002). Muscimol diffusion after intracerebral microinjections: a reevaluation based on electrophysiological and autoradiographic quantifications. *Neurobiol Learn Mem* **78**, 100–124.

Ellenberger HH & Feldman JL (1990). Brainstem connections of the rostral ventral respiratory group of the rat. *Brain Res* **513**, 35–42.

Erlichman JS, Li A & Nattie EE (1998). Ventilatory effects of glial dysfunction in a rat brain stem chemoreceptor region. *J Appl Physiol* **85**, 1599–1604.

Feldman JL & McCrimmon DR (1999). Neural control of breathing. In *Fundamental Neuroscience*, ed. Zigmond MJ, Bloom FE, Landis SC, Roberts JL & Squire LR, pp. 1063–1090. Academic Press, San Diego.

Feldman JL, Mitchell GS & Nattie EE (2003). Breathing: rhythmicity, plasticity, chemosensitivity. *Annu Rev Neurosci* **26**, 239–266.

Forster HV, Lowry TF, Ohtake PJ, Pan LG, Korducki MJ & Forster AL (1995). Differential effect of ventrolateral medullary cooling on respiratory muscles of goats. *J Appl Physiol* **78**, 1859–1867.

Fukuda Y, Tojima H, Tanaka K & Chiba T (1993). Respiratory suppression by focal cooling of ventral medullary surface in anesthetized rats – functional and neuroanatomical correlate. *Neurosci Lett* **153**, 177–180.

Gaultier C & Gallego J (2005). Development of respiratory control: evolving concepts and perspectives. *Respir Physiol Neurobiol* **149**, 3–15.

Gourine AV, Llaudet E, Dale N & Spyer KM (2005). ATP is a mediator of chemosensory transduction in the central nervous system. *Nature* **436**, 108–111.

Guyenet PG, Darnall RA & Riley TA (1990). Rostral ventrolateral medulla and sympathorespiratory integration in rats. *Am J Physiol* **259**, R1063–R1074.

Guyenet PG, Mulkey DK, Stornetta RL & Bayliss DA (2005*a*). Regulation of ventral surface chemoreceptors by the central respiratory pattern generator. *J Neurosci* **25**, 8938–8947.

Guyenet PG, Stornetta RL, Bayliss DA & Mulkey DK (2005*b*). Retrotrapezoid nucleus: a litmus test for the identification of central chemoreceptors. *Exp Physiol* **90**, 247–253.

Hodges MR, Martino P, Davis S, Opansky C, Pan LG & Forster HV (2004). Effects on breathing of focal acidosis at multiple medullary raphe sites in awake goats. *J Appl Physiol* **97**, 2303–2309.

Janczewski WA & Feldman JL (2006). Distinct rhythm generators for inspiration and expiration in the juvenile rat. *J Physiol* **570**, 407–420.

Kanjhan R, Lipski J, Kruszewska B & Rong WF (1995). A comparative study of pre-sympathetic and Botzinger neurons in the rostral ventrolateral medulla (RVLM) of the rat. *Brain Res* **699**, 19–32.

Kaufman DL, Houser CR & Tobin AJ (1991). Two forms of the gamma-aminobutyric acid synthetic enzyme glutamate decarboxylase have distinct intraneuronal distributions and cofactor interactions. *J Neurochem* **56**, 720–723.

Kawai A, Ballantyne D, Muckenhoff K & Scheid P (1996). Chemosensitive medullary neurones in the brainstem – spinal cord preparation of the neonatal rat. *J Physiol* **492**, 277–292.

Kincaid AE, Zheng T & Wilson CJ (1998). Connectivity and convergence of single corticostriatal axons. *J Neurosci* **18**, 4722–4731.

Koshiya N & Guyenet PG (1996). NTS neurons with carotid chemoreceptor inputs arborize in the rostral ventrolateral medulla. *Am J Physiol* **270**, R1273–R1278.

Li A & Nattie EE (1997). Focal central chemoreceptor sensitivity in the RTN studied with a CO_2 diffusion pipette in vivo. *J Appl Physiol* **83**, 420–428.

Loeschcke HH (1982). Central chemosensitivity and the reaction theory. *J Physiol* **332**, 1–24.

Madden CJ & Morrison SF (2005). Hypoxic activation of arterial chemoreceptors inhibits sympathetic outflow to brown adipose tissue in rats. *J Physiol* **566**, 559–573.

Mellen NM, Janczewski WA, Bocchiaro CM & Feldman JL (2003). Opioid-induced quantal slowing reveals dual networks for respiratory rhythm generation. *Neuron* **37**, 821–826.

Millhorn DE (1986). Neural respiratory and circulatory interaction during chemoreceptor stimulation and cooling of ventral medulla in cats. *J Physiol* **370**, 217–231.

Millhorn DE & Eldridge FL (1986). Role of ventrolateral medulla in regulation of respiratory and cardiovascular systems. *J Appl Physiol* **61**, 1249–1263.

Mulkey DK, Stornetta RL, Weston MC, Simmons JR, Parker A, Bayliss DA & Guyenet PG (2004). Respiratory control by ventral surface chemoreceptor neurons in rats. *Nat Neurosci* **7**, 1360–1369.

Nattie EE (2001). Central chemosensitivity, sleep, and wakefulness. *Respir Physiol* **129**, 257–268.

Nattie EE & Li A (1994). Retrotrapezoid nucleus lesions decrease phrenic activity and CO_2 sensitivity in rats. *Respir Physiol* **97**, 63–77.

Nattie E & Li A (2000). Muscimol dialysis in the retrotrapezoid nucleus region inhibits breathing in the awake rat. *J Appl Physiol* **89**, 153–162.

Nattie EE, Li A, Richerson G & Lappi DA (2004). Medullary serotonergic neurones and adjacent neurones that express neurokinin-1 receptors are both involved in chemoreception *in vivo*. *J Physiol* **556**, 235–253.

Nattie EE, Li A & St John WM (1991). Lesion in retrotrapezoid nucleus decrease ventilatory output in anesthetized or decerabrate cats. *J Appl Physiol* **71**, 1364–1375.

Onimaru H & Homma I (2003). A novel functional neuron group for respiratory rhythm generation in the ventral medulla. *J Neurosci* **23**, 1478–1486.

Onimaru H, Homma I & Iwatsuki K (1992). Excitation of inspiratory neurons by preinspiratory neurons in rat medulla in vitro. *Brain Res Bull* **29**, 879–882.

Paton JF, Deuchars J, Li YW & Kasparov S (2001). Properties of solitary tract neurones responding to peripheral arterial chemoreceptors. *Neurosci* **105**, 231–248.

Paxinos G & Watson C (1998). *The Rat Brain in Stereotaxic Coordinates*, 4th edn. Academic Press, San Diego.

Perez Fontan JJ & Velloff CR (1997). Neuroanatomic organization of the parasympathetic bronchomotor system in developing sheep. *Am J Physiol* **273**, R121–R133.

Pilowsky PM, Jiang C & Lipski J (1990). An intracellular study of respiratory neurons in the rostral ventrolateral medulla of the rat and their relationship to catecholamine-containing neurons. *J Comp Neurol* **301**, 604–617.

Prabhakar NR & Peng YJ (2004). Peripheral chemoreceptors in health and disease. *J Appl Physiol* **96**, 359–366.

Putnam RW, Filosa JA & Ritucci NA (2004). Cellular mechanisms involved in CO_2 and acid signaling in chemosensitive neurons. *Am J Physiol Cell Physiol* **287**, C1493–C1526.

Richerson GB (2004). Serotonergic neurons as carbon dioxide sensors that maintain pH homeostasis. *Nat Rev Neurosci* **5**, 449–461.

Richerson GB, Wang W, Hodges MR, Dohle CI & Diez-Sampedro A (2005). Homing in on the specific phenotype(s) of central respiratory chemoreceptors. *Exp Physiol* **90**, 259–266.

Richerson GB, Wang WG, Tiwari J & Bradley SR (2001). Chemo sensitivity of serotonergic neurons in the rostral ventral medulla. *Respir Physiol* **129**, 175–189.

Ritucci NA, Erlichman JS, Leiter JC & Putnam RW (2005). Response of membrane potential and intracellular pH to hypercapnia in neurons and astrocytes from rat retrotrapezoid nucleus. *Am J Physiol Regul Integr Comp Physiol* **289**, R851–R861.

Scheid P (ed) (guest ed Putnam RW, Dean JB & Ballantyne D) (2001). Special issue: Central chemosensitivity. *Respir Physiolo Neurobiol* **129** (1–2), 1–278.

Schreihofer AM & Guyenet PG (1997). Identification of C1 presympathetic neurons in rat rostral ventrolateral medulla by juxtacellular labeling in vivo. *J Comp Neurol* **387**, 524–536.

Schreihofer AM, Stornetta RL & Guyenet PG (1999*a*). Evidence for glycinergic respiratory neurons: Bötzinger neurons express mRNA for glycinergic transporter 2. *J Comp Neurol* **407**, 583–597.

Schreihofer AM, Stornetta RL & Guyenet PG (1999*b*). Regulation of sympathetic tone and arterial pressure by rostral ventrolateral medulla after depletion of C1 cells in rats. *J Physiol* **529**, 221–236.

Shea SA, Andres LP, Shannon DC, Guz A & Banzett RB (1993). Respiratory sensations in subjects who lack a ventilatory response to CO_2. *Resp Physiol* **93**, 203–219.

Smith JC, Morrison DE, Ellenberger HH, Otto MR & Feldman JL (1989). Brainstem projections to the major respiratory neuron populations in the medulla of the cat. *J Comp Neurol* **281**, 69–96.

Spengler CM, Gozal D & Shea SA (2001). Chemoreceptive mechanisms elucidated by studies of congenital central hypoventilation syndrome. *Respir Physiol* **129**, 247–255.

Stocker SD, Simmons JR, Stornetta RL, Toney GM & Guyenet PG (2006). Water deprivation activates a glutamatergic projection from the hypothalamic paraventricular nucleus to the rostral ventrolateral medulla. *J Comp Neurol* **494**, 673–685.

Stornetta RL & Guyenet PG (1999). Distribution of glutamic acid decarboxylase mRNA-containing neurons in rat medulla projecting to thoracic spinal cord in relation to monoaminergic brainstem neurons. *J Comp Neurol* **407**, 367–380.

Stornetta RL, Rosin DL, Simmons JR, McQuiston TJ, Vujovic N, Weston MC & Guyenet PG (2005). Coexpression of vesicular glutamate transporter-3 and gamma-aminobutyric acidergic markers in rat rostral medullary raphe and intermediolateral cell column. *J Comp Neurol* **492**, 477–494.

Stornetta RL, Rosin DL, Wang H, Sevigny CP, Weston MC & Guyenet PG (2003*a*). A group of glutamatergic interneurons expressing high levels of both neurokinin-1 receptors and somatostatin identifies the region of the pre-Botzinger complex. *J Comp Neurol* **455**, 499–512.

Stornetta RL, Sevigny CP & Guyenet PG (2002). Vesicular glutamate transporter DNPI/VGLUT2 mRNA is present in C1 and several other groups of brainstem catecholaminergic neurons. *J Comp Neurol* **444**, 191–206.

Stornetta RL, Sevigny CP & Guyenet PG (2003*b*). Inspiratory augmenting bulbospinal neurons express both Glutamatergic and enkephalinergic phenotypes. *J Comp Neurol* **455**, 113–124.

Sun MK & Reis DJ (1996). Excitatory amino acid-mediated chemoreflex excitation of respiratory neurones in rostral ventrolateral medulla in rats. *J Physiol* **492**, 559–571.

Taylor NC, Li A & Nattie EE (2005). Ventilatory effects of muscimol microdialysis into the rostral medullary raphe region of conscious rats. *Respir Physiol Neurobiol* (in press).

Verner TA, Goodchild AK & Pilowsky PM (2004). A mapping of cardiorespiraory responses to chemical stimulation of the midline medulla oblongata in ventilated and freely breathing rats. *Am J Physiol Integr Comp Physiol* **287**, R411–R421.

Washburn CP, Bayliss DA & Guyenet PG (2003). Cardiorespiratory neurons of the rat ventrolateral medulla contain TASK-1 and TASK-3 channel mRNA. *Respir Physiol Neurobiol* **138**, 19–35.

Weston MC, Stornetta RL & Guyenet PG (2004). Glutamatergic neuronal projections from the marginal layer of the rostral ventral medulla to the respiratory centers in rats. *J Comp Neurol* **473**, 73–85.

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Peripheral chemoreceptor inputs to retrotrapezoid nucleus (RTN) CO2-sensitive neurons in rats

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Central chemoreceptors and sympathetic vasomotor outflow

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Central chemoreceptors and sympathetic vasomotor outflow

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The present study explores how elevations in brain P_{CO_2} increase the sympathetic nerve discharge (SND). SND, phrenic nerve discharge (PND) and putative sympathoexcitatory vasomotor neurons of the rostral ventrolateral medulla (RVLM) were recorded in anaesthetized sino-aortic denervated and vagotomized rats. Hypercapnia (end-expiratory CO_2 from 5% to 10%) increased SND (97 ± 6%) and the activity of RVLM neurons (67 ± 4%). Injection of kynurenic acid (Kyn, ionotropic glutamate receptor antagonist) into RVLM or the retrotrapezoid nucleus (RTN) eliminated or reduced PND, respectively, but did not change the effect of CO_2 on SND. Bilateral injection of Kyn or muscimol into the rostral ventral respiratory group (rVRG-pre-Bötzinger region, also called CVLM) eliminated PND while increasing the stimulatory effect of CO_2 on SND. Muscimol injection into commissural part of the solitary tract nucleus (commNTS) had no effect on PND or SND activation by CO_2. As expected, injection of Kyn into RVLM or muscimol into commNTS virtually blocked the effect of carotid body stimulation on SND in rats with intact carotid sinus nerves. In conclusion, CO_2 increases SND by activating RVLM sympathoexcitatory neurons. The relevant central chemoreceptors are probably located within or close to RVLM and not in the NTS or in the rVRG-pre-Bötzinger/CVLM region. RVLM sympathoexcitatory neurons may be intrinsically pH-sensitive and/or receive excitatory synaptic inputs from RTN chemoreceptors. Activation of the central respiratory network reduces the overall sympathetic response to CO_2, presumably by activating barosensitive CVLM neurons and inhibiting RTN chemoreceptors.

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In the absence of input from carotid bodies and cardiopulmonary receptors hypercapnia markedly increases sympathetic nerve discharge (SND) to the heart and blood vessels (Hanna *et al.* 1988). The effect of CO_2 is generally attributed to the following chain of events: brain extracellular fluid acidification stimulates central 'respiratory' chemoreceptors, these chemoreceptors activate the respiratory pattern generator (CPG) and the CPG ultimately drives the sympathetic generating network by phasically exciting the sympathoexcitatory neurons of the rostral ventrolateral medulla (RVLM) (Millhorn & Eldridge, 1986; Richter & Spyer, 1990; Guyenet & Koshiya, 1992). This theory relies on the following evidence. SND activation by central chemoreceptors occurs in bursts that are synchronized with the central respiratory cycle (Millhorn, 1986; Millhorn & Eldridge, 1986; Guyenet *et al.* 1990; Habler *et al.* 1994). Damage to the ventrolateral medullary surface via cooling, lesions or chemicals typically attenuates PND and the respiratory oscillations of SND in roughly proportional manner (Hanna *et al.* 1979; Millhorn, 1986; Millhorn & Eldridge, 1986). During central chemoreceptor stimulation, RVLM sympathoexcitatory neurons exhibit patterns of central respiratory-related activity that are similar to those of barosensitive sympathetic ganglionic neurons (McAllen, 1987; Haselton & Guyenet, 1989; Darnall & Guyenet, 1990; Guyenet *et al.* 1990; Miyawaki *et al.* 1995).

However, a number of assumptions underlying the currently accepted view on how CO_2 affects SND have not been tested. For example, the proposed circuit presumes that the sympathetic generating network does not contain pH-responsive elements. Already challenged 25 years ago (Trzebski & Kubin, 1981), this premise is even less persuasive at present given recent evidence that the pontomedullary region may contain multiple sites for respiratory chemoreception (nucleus of the solitary

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tract (NTS), retrotrapezoid nucleus (RTN), pre-Bötzinger complex, raphe) (Nattie & Li, 1996; Feldman *et al.* 2003; Hodges *et al.* 2004; Mulkey *et al.* 2004; Putnam *et al.* 2004; Richerson *et al.* 2005). Noradrenergic neurons are pH-sensitive (Pineda & Aghajanian, 1997) and RVLM sympathoexcitatory neurons may also have this property given that they express high levels of TASK channels, have close relationships with capillaries and possess dendrites that reach the ventral medullary surface (Milner *et al.* 1987, 1989; Washburn *et al.* 2003). Second, the observation that RVLM vasomotor neurons are respiratory rhythmic is insufficient to conclude that these cells cause the increased SND associated with central chemoreceptor stimulation. Only one study has examined whether the overall activity of these neurons is actually increased by central chemoreceptor stimulation (Haselton & Guyenet, 1989) and none has examined whether their degree of activation is commensurate with the rise in sympathetic vasomotor tone. Third, the fact that SND or RVLM neurons are activated in bursts during central chemoreceptor stimulation does not mean that such bursts are caused by respiratory rhythmic excitatory volleys to the RVLM neurons. Respiratory synchronous bursts can be sculpted by periodic inhibitory inputs from a background of tonic excitation. Many ventral respiratory group (VRG) expiratory neurons and the chemosensitive neurons of RTN derive their respiratory modulation in this fashion during central chemoreceptor stimulation (Sun *et al.* 2001; Guyenet *et al.* 2005*a*). The same type of synaptic mechanism could also account for the central respiratory modulation of RVLM neurons since their principal tonically active GABAergic input from caudal ventrolateral medulla (CVLM) neurons is strongly respiratory modulated (Schreihofer & Guyenet, 2003; Mandel & Schreihofer, 2006).

The goal of the present experiments is to test several of the unproven assumptions that underlie our present understanding of how central chemoreceptors activate vasomotor SND. The main objectives are to test rigorously the role of RVLM neurons in this reflex, to determine whether the activation of these cells relies on glutamate-mediated excitatory inputs and to circumscribe the region of the medulla oblongata which contains the pH-sensitive elements that are primarily responsible for elevating sympathetic vasomotor tone.

Methods

Animals

The experiments were performed on 68 male Sprague-Dawley rats (Taconic; Germantown, NY, USA) weighing 270–350 g. Procedures were in accordance with NIH Animal Care and Use Guidelines and were approved by the University of Virginia Animal Care and Use Committee.

Surgery and anaesthesia

General anaesthesia was induced with 5% halothane in 100% oxygen. The rats received a tracheostomy and surgery was done under artificial ventilation with 1.4–1.5% halothane in 100% oxygen. All rats were subjected to the following previously described surgical procedures: femoral artery cannulation for arterial pressure (AP) measurement, femoral vein cannulation for administration of fluids and drugs, removal of the occipital plate for insertion of a pipette for microinjection or insertion of a recording electrode into the medulla oblongata via a dorsal transcerebellar approach, and skin incision over the lower jaw for placement of a bipolar stimulating electrode next to the mandibular branch of the facial nerve (Guyenet *et al.* 2005*a*). The phrenic nerve was accessed by a dorsolateral approach after retraction of the right shoulder blade. All animals were bilaterally vagotomized to prevent entrainment of the phrenic nerve discharge to the ventilator. In most rats a complete baro- and peripheral chemoreceptor deafferentation was performed by sectioning the vagosympathetic trunks, the superior laryngeal nerves and the glossopharyngeal nerves (proximal to the junction with the carotid sinus nerves). In the others (baro- and chemo-receptor intact group), the vagus nerves were carefully separated from the vagosympathetic trunk and selectively transected bilaterally. We presume that this procedure left the aortic depressor nerves intact but these very fine nerves were not identified.

Splanchnic sympathetic nerve discharge (sSND) was recorded as previously described (Schreihofer & Guyenet, 2000*a*,*b*; Schreihofer *et al.* 2000). The right splanchnic nerve was isolated via a retroperitoneal approach, and the segment distal to the suprarenal ganglion was placed on a pair of Teflon-coated silver wires that had been bared at the tip (250 μm bare diameter; A-M Systems, www.a-msystems.com). In some experiments, lumbar sympathetic nerve discharge (lSND) was also recorded as previously described (Haselton & Guyenet, 1989; Guyenet *et al.* 1990). In this case a ventral approach to the sympathetic chain was used. The nerves and wires were embedded in dental impression material (polyvinylsiloxane; Darby.Spencer.Mead Dental Supply, www.darbyspencermead.com), and the incision site was closed around the exiting recording wires.

Upon completion of surgical procedures, halothane concentration was adjusted (0.9–1%) for each animal to a level sufficient to abolish the corneal reflex and the retraction of distal phalanges to strong nociceptive stimulation of the hindpaw. Since halothane has a marked depressant effect on carotid body function, urethane was used in experiments requiring preservation of the peripheral chemoreflex. Thus, in the rats with intact baro- and chemoreceptors, after completion of surgical procedures, halothane was replaced by urethane

(1.2–1.5 g kg^{-1}) administered slowly I.V. All rats were ventilated with 100% oxygen throughout the experiment. Rectal temperature (maintained at 37°C) and end tidal-CO_2 were monitored throughout the experiment with a capnometer (Columbus Instruments, Ohio, USA) that was calibrated twice per experiment against a calibrated CO_2–N_2 mix. This instrument provided a reading of $< 0.1\%$ CO_2 during inspiration in animals breathing 100% oxygen and an asymptotic, nearly horizontal reading during expiration. We previously showed that the capnometer readings closely approximate arterial P_{CO_2} (Guyenet *et al.* 2005*a*). Regardless of the anaesthetic used, the adequacy of anaesthesia was monitored during a 20 min stabilization period by testing for absence of withdrawal response, lack of AP change and lack of change in PND rate or amplitude to firm toe pinch. After these criteria were satisfied, the neuromuscular blocker pancuronium was administered at the initial dose of 1 mg kg^{-1} I.V. and the adequacy of anaesthesia was thereafter gauged solely by the lack of increase in AP and PND rate or amplitude to firm toe pinch. No adjustment of the halothane concentration was needed. However, approximately hourly supplements of one-third of the initial dose of urethane were needed to satisfy these criteria during the course of the recording period (4 h).

In vivo recordings of physiological variables and neuronal activity

Arterial pressure (AP), the mass discharge of the phrenic (PND), splanchnic (sSND) and lumbar (lSND) nerves, tracheal CO_2 and single units were recorded as previously described (Guyenet *et al.* 2005*a*).

As in prior work, the caudal and ventral boundaries of the facial motor nucleus were identified in each rat by the large (up to 5 mV) negative antidromic field potential generated in the facial motor nucleus by stimulating the mandibular branch of the facial nerve (for details see Brown & Guyenet, 1985). Before starting the experiments, ventilation was adjusted to lower end-expiratory CO_2 – 4% at steady state (60–80 cycles s^{-1}; tidal volume 1–1.2 ml (100 g)$^{-1}$). These conditions were selected because 4% end-expiratory CO_2 was below the threshold of the PND. Variable amounts of pure CO_2 were then added to the breathing mixture to adjust end-expiratory CO_2 to the desired level.

All analog data (end-expiratory CO_2, sSND, lSND, PND, AP and unit activity) were stored on a microcomputer via a micro1401 digitizer from Cambridge Electronics Design (CED, Cambridge, UK) and were processed off-line using version 5 of the Spike 2 software (CED). Processing included action potential discrimination and binning, neuronal discharge rate measurement, and PND 'integration' (iPND) consisting of rectification and smoothing (τ, 0.015 s). Neural minute × volume (mvPND, a measure of the total phrenic nerve discharge per unit of time) was determined by averaging iPND over 50 s and normalizing the result by assigning a value of 0 to the dependent variable recorded at low levels of end-expiratory CO_2 (below threshold) and a value of 1 at the highest level of P_{CO_2} investigated (between 9.5 and 10%). The CED software was also used for acquisition of peri-event histograms of neuronal activity and peri-event averages of iPND. The peri-event histograms of neuronal single-unit activity were triggered on iPND trace and represented the summation of at least 100 respiratory cycles (350–800 action potentials per histogram).

The steady-state relationship between RVLM neuronal activity and end-expiratory CO_2 was obtained by stepping the inspired CO_2 level to various values for a minimum of 3 min and up to 5 min. The mean discharge rate of the neuron was measured during the last 30 s of each step at which time end-expiratory CO_2 and the discharge of the neuron appeared to have reached equilibrium. End-expiratory CO_2 was measured by averaging the maximum values recorded from 10 consecutive breaths at the midpoint of the time interval sampled.

Stimulation of carotid chemoreceptors was done with bolus injections of sodium cyanide (NaCN) (50 μg kg^{-1}, I.V.). Evidence that the stimulus activated brainstem neurons via stimulation of carotid chemoreceptors was obtained by demonstrating that denervation of these receptors eliminated the excitatory effect of the stimulus on PND.

Extracellular recording and juxtacellular labelling of neurons in the RVLM

Single-unit recording experiments were performed in seven baroreceptor-denervated halothane-anaesthetized rats, artificially ventilated rats. A concentric bipolar stimulating electrode was placed in the dorsolateral funiculus of the spinal cord at T2 for antidromic activation of RVLM neurons (Brown & Guyenet, 1985). The exposed surface of the spinal cord was immersed in warm mineral oil. The discharges of barosensitive neurons in the RVLM were recorded extracellularly as previously described (Schreihofer & Guyenet, 1997) using glass electrodes filled with 1.5% biotinamide (Molecular Probes, www.molecularprobes.com) in 0.5 M sodium acetate. Optimal electrode resistance for recording and labelling cells was 20–40 MΩ measured *in vivo*. Recordings were made with an intracellular amplifier in bridge mode (Axoclamp 2A, Axon Instruments, www.axon.com) to allow monitoring of action potentials during injection of current through the electrode. The RVLM was located using stereotaxic coordinates: 0–500 μm caudal to the caudal end of the facial nucleus (identified by the antidromic field potential), 1.6–2.0 mm lateral to the

midline, and 0–200 μm below the ventral extent of the facial field potential. The RVLM neurons selected for labelling had the following characteristics: proper location relative to the facial motor nucleus, spontaneous activity (18–20 Hz at rest) and antidromic activation from the spinal cord. As RVLM neurons generally exhibit a high level of spontaneous activity and are frequently subjected to medium-latency orthodromic excitation (> 8–10 ms), spontaneous collisions prevented identification of spinal-projecting axons except in neurons with fast-conducting spinal axons (latency < 8 ms). After electrophysiological characterization, RVLM bulbospinal neurons were filled with biotinamide using the previously described juxtacellular labelling method (200 ms pulses of 1.0–4.0 nA at 2.5 Hz for 1–3 min) (Schreihofer & Guyenet, 1997).

Intraparenchymal injections

Muscimol (Sigma; 1.75 mM in sterile saline pH 7.4) or kynurenic acid (Kyn) (Sigma; 50, 100 or 200 mM first dissolved in 1 N NaOH and then diluted in phosphate-buffered saline pH 7.4) was pressure injected (30–50 nl in 5 s) bilaterally through single-barrel glass pipettes (20 μm tip diameter). The solutions contained a 5% dilution of fluorescent latex microbeads (Lumafluor, New City, NY, USA) for later histological identification of the injection sites (Guyenet *et al.* 1990). The glass pipettes containing the drug–microbeads mixture also allowed recordings of field potentials and multiunit brain activity, properties that were used to help direct the electrode tip to the desired sites. Injections into RVLM were thus guided by recording the facial field potential and were placed 200 μm below the level corresponding to the lower edge of the field, 0–400 μm caudal to the caudal end of the field and 1.6–2.0 mm lateral to the midline. Injections into the RTN were also guided by recording the facial field potential and were placed 200 μm below the lower edge of the field, 1.6–1.9 mm lateral to the midline and 200–400 μm rostral to the caudal end of the field. Injections into the rostral ventral respiratory group (rVRG) were guided by locating inspiratory-related multiunit activity. The target region was found 500 μm rostral to the calamus scriptorius, 1.8 mm lateral to midline and 1.9–2.2 mm below the dorsal surface of the brainstem using electrodes angled 20 deg forward. The electrophysiological recordings were made on one side only and the second injection was placed 1–2 min later in the symmetric brain location (3.6 mm away) based on the stereotaxic coordinates of the first one. The classification of respiratory neurons was based on the timing of their discharge in relation to that of the phrenic nerve using accepted nomenclature (Feldman & McCrimmon, 1999). Injections into the commissural part of the nucleus of the solitary tract (commNTS) were made 400 mm caudal to the calamus scriptorius, in the midline and 0.3–0.5 mm below the dorsal surface of the brainstem.

Histology

At the end of the experiment the rat was deeply anaesthetized with halothane and perfused transcardially with PBS (pH 7.4) followed by paraformaldehyde (4% in 0.1 M phosphate buffer, pH 7.4). The brain was removed and stored in fixative for 24 h at 4°C. Using a vibrating microtome (Leica VT 1000S, Nussloch, Germany), 30 μm coronal sections were cut through the medulla and stored in a cryoprotectant solution at 20°C (Schreihofer & Guyenet, 1997). All histochemical procedures were done using free-floating sections according to previously described protocols. The sections were later processed for visualization of biotinamide and phenylethanolamine *N*-methyl transferase (PNMT) (Schreihofer & Guyenet, 1997). Biotinamide was revealed by incubating the sections in streptavidin conjugated with Alexa 488 (1 : 200; 3 h; Jackson Immunoresearch Laboratories, West Grove, PA, USA). This method was combined with immunofluorescent detection of PNMT using an already described rabbit polyclonal antibody (provided by M. C. Bohn) used at 1 : 2000 dilution and revealed with a Cy3-tagged anti-rabbit IgG (1 : 200; Jackson). Finally, the sections were mounted in sequential rostrocaudal order onto slides, dehydrated through a graded series of alcohol and xylene and covered with Krystalon (EMD Chemicals Inc, NJ, USA).

A conventional Zeiss Axioskop 2 multifunction microscope (brightfield, darkfield and epifluorescence) was used for all observations. Injection sites (fluorescent microbeads) and biotinamide-labelled neurons were plotted using a previously described computer-assisted mapping technique based on the use of a motor-driven microscope stage controlled by the Neurolucida software (Stornetta & Guyenet, 1999). The Neurolucida files were exported to the NeuroExplorer software (MicroBrightfield, Colchester, VT, USA) to count the various types of neuronal profiles within a defined area of the reticular formation.

Section alignment between brains was done relative to a reference section. To align sections around RVLM or RTN level, the most caudal section containing an identifiable cluster of facial motor neurons was identified in each brain and assigned the level 11.6 mm caudal to Bregma (Bregma −11.6 mm) according to the atlas of Paxinos & Watson (1998). Levels rostral or caudal to this reference section were determined by adding a distance corresponding to the interval between sections multiplied by the number of intervening sections. The same method was also used to identify the Bregma level of the muscimol injections targeted to the rVRG. When the object of the experiment was to locate injection sites at commissural NTS level, the

reference section used to align all others was the one closest to mid-area postrema level (Bregma −13.8 mm).

The Neurolucida files were exported to the Canvas 9 software drawing program for final modifications. Images were captured with a SensiCam QE 12-bit CCD camera (resolution 1376 × 1040 pixels, Cooke Corp., Auburn Hills, MI, USA). The neuroanatomical nomenclature is after Paxinos & Watson (1998).

Statistics

Statistical analysis was done with Sigma Stat version 3.0 (Jandel Corporation, Point Richmond, CA, USA). Data are reported as means ± standard error of the mean (S.E.M.). *t* test, paired *t* test and one-way parametric ANOVA followed by the Newman-Keul multiple comparisons test were used as appropriate. Significance was set at $P < 0.05$.

Results

Effect of central chemoreceptor stimulation on SND and PND

These experiments were performed in vagotomized, baro- and chemo-denervated rats (henceforth called completely denervated rats). PND and splanchnic SND (sSND) increased as a function of end-expiratory CO_2 and steady-state values were reached after about 3 min (Fig. 1*A*). Splanchnic SND exhibited its characteristic early inspiratory modulation (inset in Fig. 1*A*). The relationship between sSND and end-expiratory CO_2 at steady state was curvilinear suggesting the probable existence of a threshold around 4% CO_2 (Fig. 1*B*). The sSND response to CO_2 was anaesthetic-dependent and somewhat larger under halothane than urethane anaesthesia (Fig. 1*B*; steady-state SND increase from

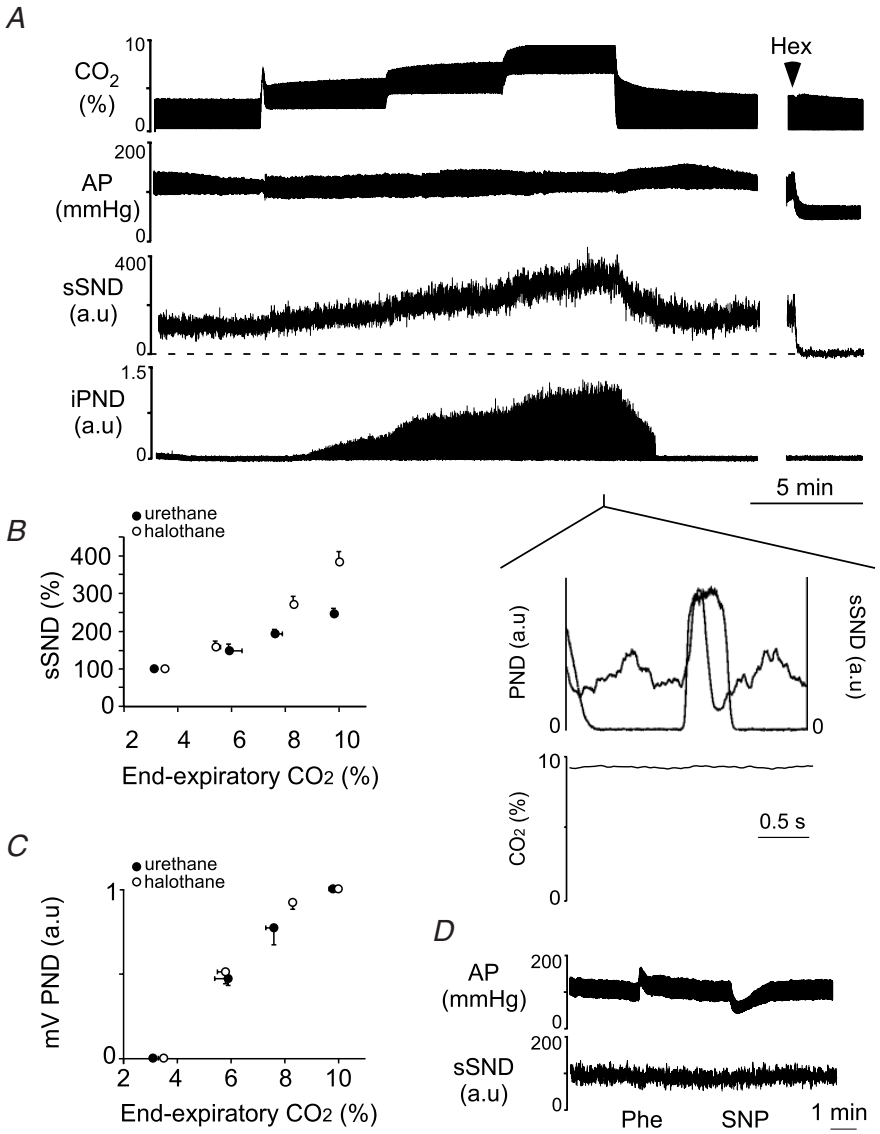

Figure 1. Effect of hypercapnia on SND and PND in vago-sino-aortic denervated rats
A, effect produced by step-wise increases in end-expiratory CO_2 on splanchnic SND (sSND) and PND in one rat. The ganglionic blocker hexamethonium (Hex) was used to define baseline SND. Inset shows the peri-event average of sSND triggered on the ascending phase of iPND. *B*, steady-state relationship between sSND and end-expiratory CO_2 under halothane (11 rats) or urethane anaesthesia (8 rats). sSND recorded at 3.3–3.8% CO_2 was defined as 100 units. The noise (0 unit) was defined by the hexamethonium method. *C*, steady-state relationship between mvPND (1 normalized unit equals the maximum value of the signal) and end-expiratory CO_2 for the rats shown in *B*. *D*, evidence that the animals were completely barodenervated (Phe, phenylephrine, 5 μg kg^{-1} I.V.; SNP, sodium nitroprusside 30 μg kg^{-1} I.V.).

Table 1. Effect of Kyn injection into RVLM on lumbar SND

	Low CO_2 (5%)	High CO_2 (10%)	Number of animals
Before Kyn	100	191 ± 3*	4
After Kyn	102 ± 4	193 ± 3*	4
Recovery	101 ± 2	189 ± 5*	4

Values are mean ± S.E.M. *Significantly different from rest (low CO_2). Kyn, kynurenic acid; SND, sympathetic nerve discharge.

3–3.5% to 9.5–10% CO_2: 388 ± 4% under halothane *versus* 250 ± 2% under urethane; $P < 0.05$). After normalization, the PND response to CO_2 was the same under both anaesthetics (Fig. 1*C*). The lack of response in sSND to acute changes in AP (Fig. 1*D*) and the absence of pulse synchrony in the sSND (data not shown) demonstrated that the preparation was baro-denervated. All animals showed complete denervation as indicated by one or both of these tests.

Lumbar SND (lSND) was recorded instead of splanchnic SND in four halothane-anaesthetized denervated rats. As expected, the respiratory modulation of lSND consisted of an early inspiratory depression, followed by a post-inspiratory peak (not illustrated; Haselton & Guyenet, 1989). Increasing end-expired CO_2 from 5% to 10% caused a 93% rise in lSND (Table 1). This increase was the same as that of the sSND when the latter was also measured while stepping end-expiratory CO_2 between 5 to 10% (97 ± 6%).

Effect of central chemoreceptor stimulation on the discharge rate of RVLM vasomotor neurons

Under anaesthesia, the baro-regulated class of sympathetic efferents is mainly controlled through sympathoexcitatory neurons located in the RVLM (Guyenet, 2006). The next experiments were designed to determine whether the

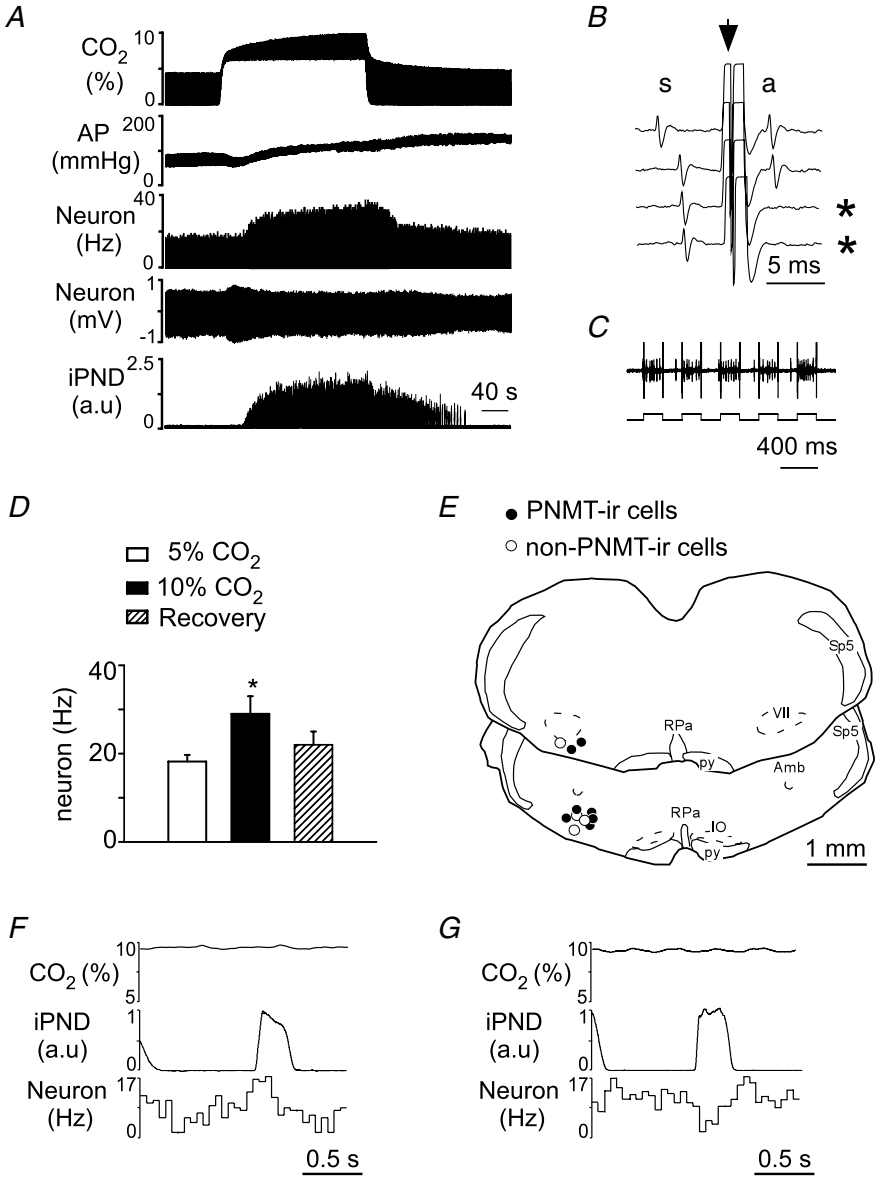

Figure 2. Effect of hypercapnia on the discharge rate of putative bulbospinal vasomotor neurons of the rostral ventrolateral medulla (RVLM) in vagotomized and sino-aortic deafferented rats
A, effect produced by stepping end-expiratory CO_2 from 5 to 10% on the discharge of a putative bulbospinal vasomotor neuron. *B*, time-controlled collision test showing that the neuron shown in *A* sends an axon to or through spinal segment T3 (* indicates two sweeps when collision occurred. Collisions (absence of the antidromic spike, a, were caused by decreasing the interval between a spontaneous spike, s, and the stimulus delivered to the spinal cord (arrow). *C*, procedure used to label the cell juxtacellularly with biotinamide (upper trace: unit; lower trace current monitor; 3.4 nA). *D*, mean effect of stepping CO_2 from 5 to 10% on 11 bulbospinal RVLM neurons (*$P < 0.05$ from both control and recovery values). *E*, location of the biotinamide-labelled neurons. The two coronal sections represent from top to bottom Bregma −11.6 and −11.8 mm after Paxinos & Watson (1998). *F*, PND-triggered activity histogram of the RVLM bulbospinal neuron shown in *A–C* reveals a respiratory pattern reminiscent of that of the splanchnic nerve. *G*, different RVLM neuron exhibiting a respiratory pattern reminiscent of that of the lumbar nerve. Amb, ambiguus nucleus; IO, inferior olive; py, pyramidal tract; RPa, raphe pallidus; sp5, spinal tract of trigeminal nerve; VII, facial motor nucleus.

sympathoexcitatory response to CO_2 is also associated with an increased activity of these RVLM neurons. Since the experiments had to be conducted in completely denervated rats, the identification of the bulbospinal sympathoexcitatory neurons located in the RVLM relied on criteria other than their barosensitivity namely location, spontaneous activity, axonal projection to the thoracic cord and respiratory modulation typical of lumbar or splanchnic SND. Cell location and cell phenotype (adrenergic or not) was determined following juxtacellular labelling (3.4 ± 0.2 nA for an average of 2 min). Eleven units with these characteristics were identified, labelled with biotinamide and recovered histologically (1–2 neurons per rat in 7 rats). Complete barodenervation was demonstrated by the lack of effects of raising or lowering AP on unit activity and the absence of a pulse modulation in the discharge of the cells (data not shown). Figure 2*A* shows the slowly developing activation of a putative sympathoexcitatory neuron. The collision test used to demonstrate that this cell had an axon at thoracic level is shown in Fig. 2*B*. The entrainment of the cell by extracellularly applied current (3.4 nA), which produces the uptake of biotinamide by the stimulated cell, is shown in Fig. 2*C* (upper trace, neuron; lower trace, juxtacellularly applied current). Every bulbospinal RVLM neuron tested was activated by hypercapnia. The average antidromic latency was 3.8 ± 0.6 ms (range: 3.1–7.8 ms). On average, increasing end-expired CO_2 from 5 to 10% raised the discharge rate of these cells from 18.2 ± 1.5 to 30 ± 3 spikes s^{-1} (67% increase; $P < 0.05$; Fig. 2*D*; range 20–130%). The increase in mean discharge rate was associated with an increase in AP of 25 ± 5 mmHg ($P < 0.05$; comparison between mean AP at rest and at the end of the hypercapnic episode). The unit shown in Fig. 2*A* had a respiratory modulation that was of central origin as indicated by the fact that ventilation and PND were asynchronous (flat PND-triggered end-expiratory CO_2 average, Fig. 2*F*). The peri-event activity histogram of this unit had an early inspiratory peak characteristic of splanchnic SND (Fig. 2*F*; compare with Fig. 1*A*, inset). This pattern was found in 4 of 11 units. Five units had the typical lumbar SND pattern (decreased firing probability during early inspiration and increased discharge during post-inspiration; Fig. 2*G*). The remaining two units had no obvious respiratory modulation. The location of the 11 biotinamide-labelled cells is shown in Fig. 2*E*. Seven neurons were PNMT-immunoreactive (Figs 2*E*, and 3*A* and *B*) and four were not (Figs 2*E*, and 3*C* and *D*). All the cells were found within the cluster of PNMT-ir neurons that defines the pressor region of the RVLM.

Effects of kynurenic acid injection into the rostral ventrolateral medulla on respiratory and sympathetic outflows

Most sympathoexcitatory reflexes are severely attenuated by blocking ionotropic excitatory amino acid (EAA) receptors in the RVLM (Guyenet, 2006). In order to test whether the sympathoactivation caused by stimulation of central chemoreceptors conforms to this general rule we injected the broad spectrum ionotropic EAA receptor antagonist kynurenic acid (Kyn) into the RVLM and tested the effect of this treatment on the rise in sSND and PND caused by stepping end-expiratory CO_2 from 5% to 10% for 5 min. Hypercapnia produced an immediate

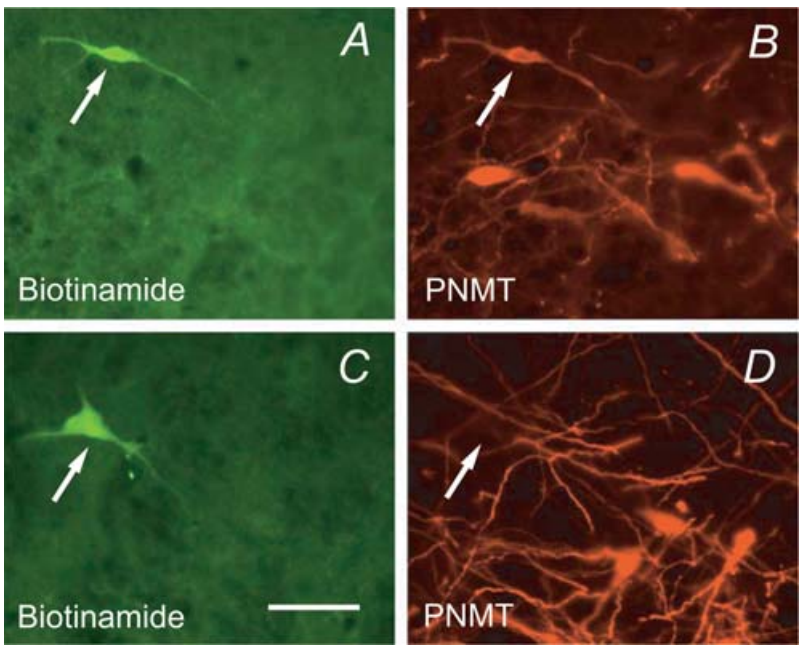

Figure 3. Phenotype of the recorded rostral ventrolateral medulla (RVLM) neurons
A and *B*, example of a biotinamide-labelled neuron that was immunoreactive for phenylethanolamine *N*-methyl transferase (PNMT, C1 adrenergic neuron). *C* and *D*, example of a recorded neuron that was negative for PNMT though located next to a cluster of C1 cells (calibration, 50 μm).

hypotension of -12 ± 8 mmHg followed 30 s later by a gradual return of AP to or slightly above control level. Immediately after return to 5% CO_2, AP increased by 23 ± 2 mmHg and returned to control values after 5 min.

Injections of Kyn (50 nl, concentration varying from 50 to 200 mM) were placed bilaterally in the RVLM of 18 totally denervated animals (50 mM in 6 rats, 100 mM in 5 rats and 200 mM in 7 rats). Kyn produced no effect on resting AP and sSND at any of the three doses. At 50 and 100 mM, Kyn reduced PND amplitude measured at 10% CO_2 by 54 and 72%, respectively, and increased the rate of the phrenic discharge (data not illustrated). At 200 mM, Kyn almost completely eliminated PND (Fig. 4*A* and *D*). Half-recovery time of PND ranged from 20 to 40 min and complete recovery from the effect of Kyn on PND occurred within 60 min (Fig. 4*D*). Kyn did not change the increase in sSND caused by hypercapnia at any of the three concentrations tested. The results produced by the highest dose of Kyn (200 mM) are illustrated in Fig. 4. Note that the drug changed neither the baseline value of sSND nor the level of sSND reached at steady state during hypercapnia (Fig. 4*A* and *B*). The hypercapnia-induced change in SND is replotted in Fig. 4*C* for clarity. The Kyn injection sites at the 200 mM dose ($N = 7$) are shown in Fig. 4*E*. These sites were centred on the region that contains the bulk of the bulbospinal sympathoexcitatory neurons of the RVLM (compare Figs 2*E* and 4*E*).

The surprising lack of effect of even the highest dose of Kyn on sSND response to CO_2 prompted us to verify that identical injections of Kyn could block the effect of peripheral chemoreceptor stimulation on sSND as described before (Koshiya *et al.* 1993; Sun & Reis, 1995). Bilateral injection of Kyn (50 nl, 200 mM into RVLM) in eight vagotomized urethane-anaesthetized rats with intact carotid sinus nerves did not change AP (118 ± 2.5 mmHg *versus* control, 121 ± 3 mmHg) or sSND at rest but these

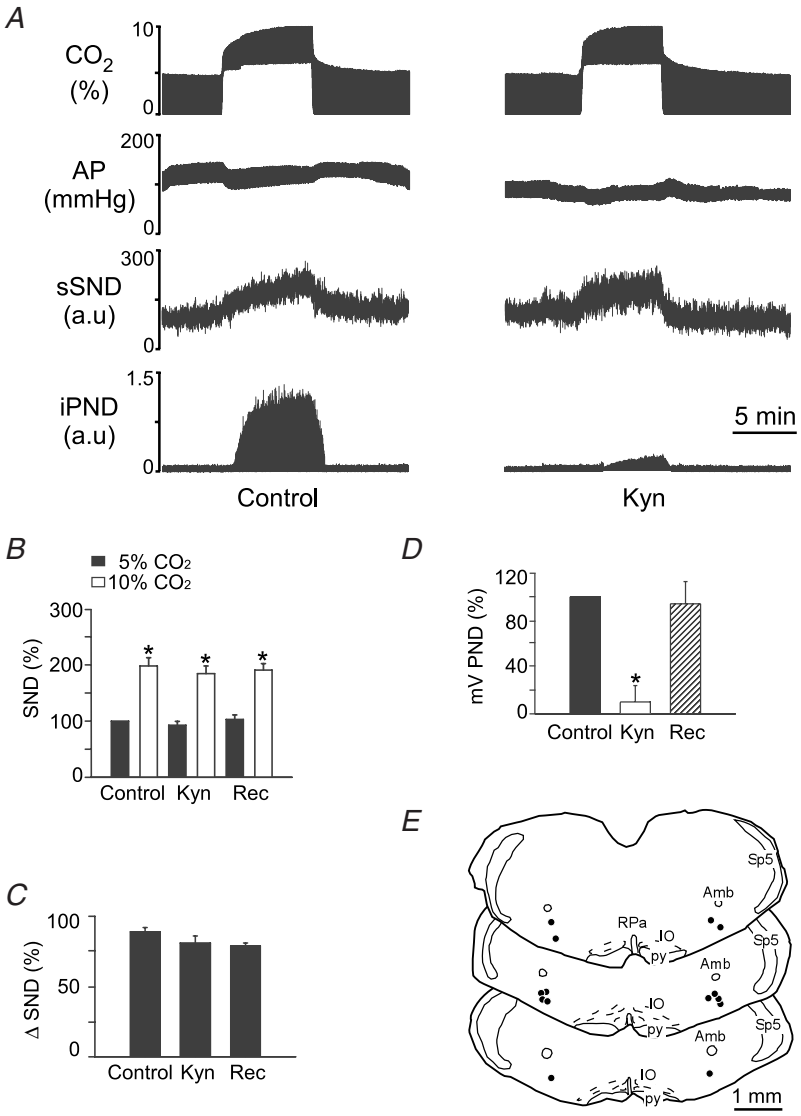

Figure 4. Kynurenic acid (Kyn) injection into the rostral ventrolateral medulla (RVLM) does not change the effect of hypercapnia on sSND in vagotomized sino-aortic deafferented rats
A, effect produced by stepping end-expiratory CO_2 from 5 to 10% on sSND and PND in one rat before and 5 min after bilateral microinjection of Kyn into RVLM (200 mM, 50 nl). *B*, average results from 7 rats. Resting sSND during the control period (5% CO_2) was defined as 100 units (*significant difference between 10 and 5% CO_2). Rec, recovery period (> 60 min after Kyn injection). *C*, effect produced by stepping end-expiratory CO_2 from 5 to 10% on sSND before, during and after Kyn (n.s.). *D*, effect of Kyn on mvPND elicited by stepping end-expiratory CO_2 from 5 to 10% ($^*P < 0.05$). *E*, injection sites. The three coronal sections represent from top to bottom Bregma −11.8, −11.96 and −12.3 mm after Paxinos & Watson (1998).

injections virtually eliminated resting PND and reduced by 86 ± 7% the increase in AP and sSND produced by I.V. injection of NaCN (Fig. 5*A* for representive case; Fig. 5*B* and *C* for average results).

Effects of bilateral injection of kynurenic acid into the retrotrapezoid nucleus on respiratory and sympathetic outflows

The RTN region contains chemosensitive neurons that probably provide an important source of excitatory drive to the respiratory network, especially under anaesthesia (Feldman *et al.* 2003). EAA receptor blockade in the RTN region has also been described as capable of decreasing the activity of the central respiratory pattern generator (Nattie & Li, 1995). The next experiments were therefore designed to determine whether bilateral blockade of EAA receptors in RTN can suppress the stimulatory effect of central chemoreceptor activation on PND and sSND. Kyn (200 mM, 50 nl) was injected bilaterally under electrophysiological guidance 200 μm below the facial motor nucleus and 200 μm rostral to the caudal end of this nucleus to target the region that contains the highest density of CO_2-sensitive RTN neurons according to our prior data (Mulkey *et al.* 2004; Guyenet *et al.* 2005*a*). Kyn reduced PND amplitude during hypercapnia by an average of 49% (Fig. 6*A* and *D*). Kyn also increased PND rate. However, the drug had no effect on the increase in sSND caused by increasing end-expiratory CO_2 from 5 to 10% (Fig. 6*B* and *C*). The effect of Kyn on PND was fully reversible within 60 min (Fig. 6*D*). Kyn injections did not change resting AP (control, 98 ± 4 mmHg; Kyn, 99 ± 5 mmHg). The Kyn injection sites were located below the caudal and medial edge of the facial motor nucleus (Fig. 6*E*). The fluorescent beads used to track the injection sites had spread approximately 240 μm on each side of the injection centre.

In brief, Kyn did not reduce the central stimulatory effect of hypercapnia on sSND, regardless of whether the injections were placed at the very rostral end of the RVLM, the region that includes the RTN (Fig. 6*E*), or slightly more caudally within the classically defined pressor region of RVLM (Fig. 4*E*). However, PND inhibition by Kyn was much greater when the drug was injected in the classically defined RVLM than when it was placed more rostrally into RTN (compare Figs 4*D* and 6*D*). This difference suggests that the VLM site at which Kyn inhibits respiration in our preparation is probably not the RTN but a region located caudal to it. This result is consistent with prior evidence that the discharge of RTN neurons is not affected by Kyn in our preparation (Mulkey *et al.* 2004). The difference between our results and those of Nattie & Li (1995) could be due to the fact that the carotid sinus nerves were cut in our preparation, thereby eliminating the glutamatergic excitatory input to the chemosensitive neurons of RTN (Takakura *et al.* 2006).

Effects of muscimol injection into the commissural portion of the solitary tract nucleus (commNTS) on respiratory and sympathetic chemoreflexes

The presence of acid-responsive neurons in the NTS and the fact that NTS acidification *in vivo* alters respiration has been interpreted as evidence that this structure contains central respiratory chemoreceptors (Nattie & Li, 2002; Feldman *et al.* 2003). The next experiments were designed to test the role played by commNTS in the activation of sSND and PND by increases in brain P_{CO_2}. This was accomplished by measuring the effects produced by microinjecting the $GABA_A$ receptor agonist muscimol (single 30 nl midline injection of 1.75 mM muscimol) into the commNTS of seven completely denervated halothane-anaesthetized rats. In order to verify our underlying assumption that muscimol did inhibit commNTS neurons we also examined the effect of similar injections on peripheral chemoreflexes. The latter experiments were

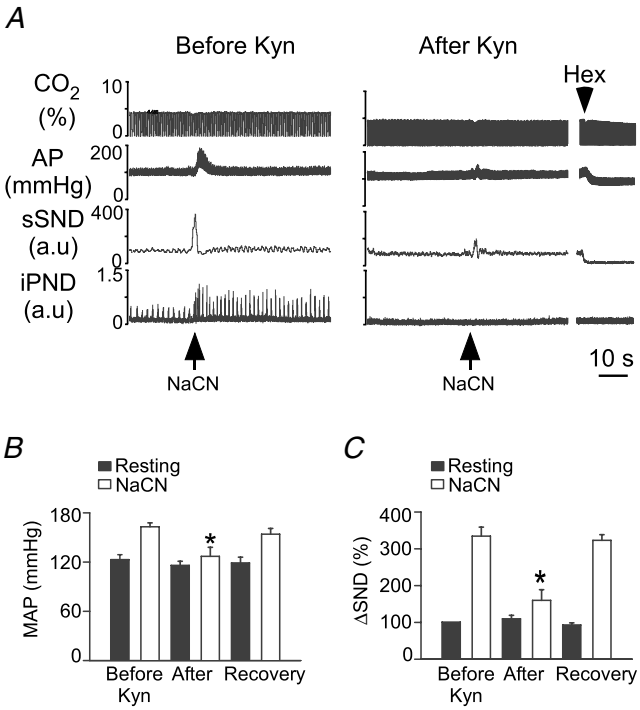

Figure 5. Kynurenic acid (Kyn) injection into rostral ventrolateral medulla (RVLM) greatly attenuate the peripheral chemoreflex in vagotomized rats

A, response of the splanchnic and phrenic nerves to peripheral chemoreceptor stimulation with I.V. cyanide (NaCN: 50 μg kg^{-1}) in a urethane-anaesthetized rat, before (left panel) and 5 min after bilateral injection of Kyn (200 mM, 50 nl: right panel) into RVLM. *B*, effect of Kyn on the cyanide-induced rise in MAP (*$P < 0.05$; 8 rats). *C*, effect of Kyn on the cyanide-induced rise in sSND (*$P < 0.05$; 8 rats).

conducted in eight vagotomized urethane-anaesthetized rats with intact carotid sinus nerves.

Muscimol had no effect on the sympathetic or the phrenic component of the central chemoreflex (representative example in Fig. 7*A*; summary data in Fig. 7*B* and *C*; histologically verified injection sites in Fig. 7*E*).

In vagotomized rats with intact carotid sinus nerves, muscimol injection into commNTS (7 rats) did not change resting PND but eliminated the activation of the sympathetic and respiratory outflows produced by stimulating the carotid bodies with NaCN (representative example in Fig. 8*A*; summary data in Fig. 8*B* and *C*). The muscimol injection sites (not illustrated) were indistinguishable from those shown in Fig. 7*E*.

Role of the caudal ventrolateral medulla in the central sympathetic chemoreflex

The rise in sympathetic vasomotor outflow elicited by central chemoreceptor stimulation is usually considered to be driven synaptically by the activation of the central respiratory pattern generator (Richter & Spyer, 1990). To test the validity of this assumption we examined whether pharmacological blockade of the central respiratory pattern generator alters the sympathetic response to hypercapnia in completely denervated rats. Pharmacological blockade of the respiratory network was performed by injecting drugs into the ventral respiratory columm caudal to the RVLM (also called CVLM). Kyn (200 mM, 50 nl per side) was used in a group of six completely denervated rats and muscimol (1.75 mM,

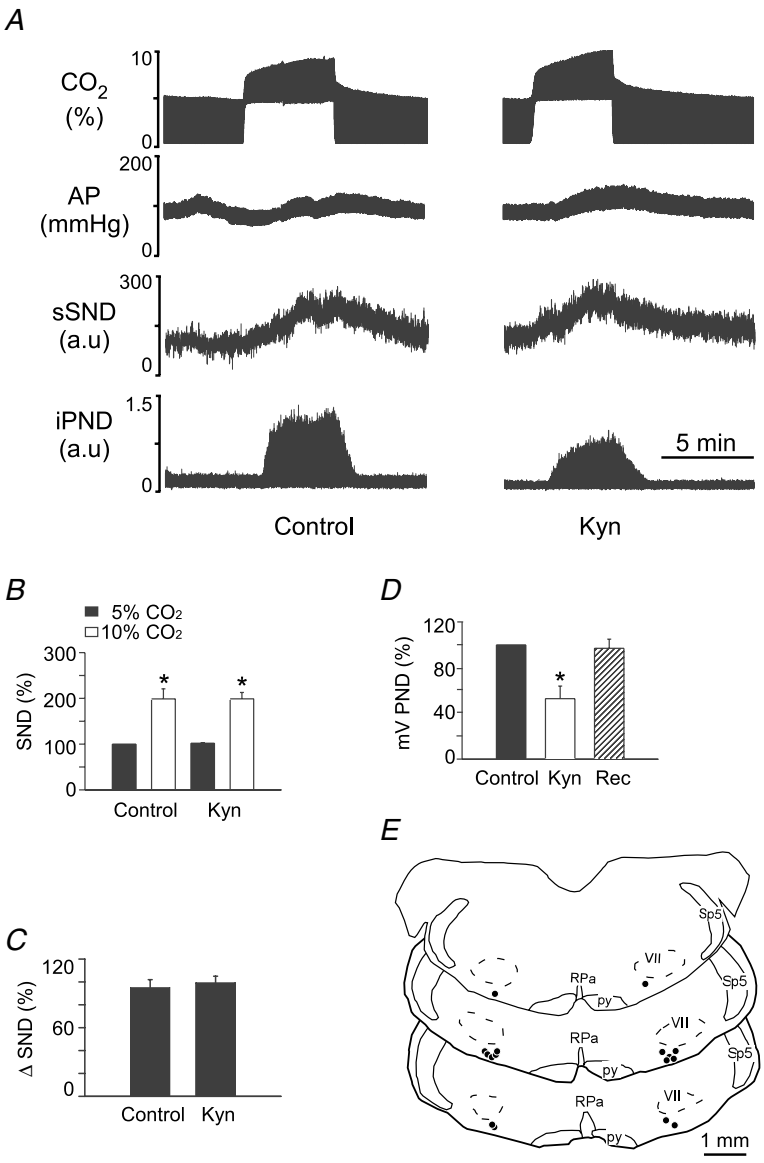

Figure 6. Kynurenic acid (Kyn) injection into the retrotrapezoid nucleus (RTN) does not change the effect of hypercapnia on sSND in vagotomized sino-aortic deafferented rats
A, effect produced by stepping end-expiratory CO_2 from 5 to 10% on sSND and PND in one rat before and after bilateral microinjection of Kyn into RTN (200 mM, 50 nl). *B*, average results from 8 rats. sSND at rest during the control period was defined as 100 units (*$P < 0.05$, significant difference between 10 and 5% CO_2). *C*, effect produced by stepping end-expiratory CO_2 from 5 to 10% on the CO_2-induced change in sSND before and 5 min after injection of Kyn (n.s.). *D*, effect of Kyn on mvPND elicited by stepping end-expiratory CO_2 from 5 to 10% (*$P < 0.05$ relative to control and recovery). *E*, injection sites. The three coronal sections represent from top to bottom Bregma −11.0, −11.3 and −11.6 mm after Paxinos & Watson (1998).

30 nl per side) was used in a second group of rats ($n = 9$). The injections were placed under electrophysiological guidance into a region of the rVRG/CVLM where strong inspiratory-related multiunit activity could be recorded from the injection pipette. The effects of these injections on the respiratory system were gauged by their effect on PND.

Kyn injection into the CVLM eliminated PND for up to 60 min and prevented its activation by CO_2 (Fig. 9*A* and *D*). The injection centres were located close to 1.5 mm posterior to the caudal end of the facial motor nucleus (Fig. 9*E*). By our previous estimates, these sites correspond to the CVLM and the rostral end of the rVRG (Stornetta *et al.* 2003). The fluorescent microbeads extended 250 ± 12 μm on each side of the injection centre and therefore Kyn, which diffuses more freely that microbeads, is likely to have also reached the respiratory neurons located in the pre-Bötzinger complex. Bilateral injection of Kyn increased resting AP from 100 ± 3 to 132 ± 5 mmHg ($P < 0.05$) and resting sSND from 100 to 125% ($P < 0.05$). Kyn also significantly increased the effect of hypercapnia on sSND (Fig. 9*B* and *C*).

Virtually identical results were obtained when muscimol instead of Kyn was injected into CVLM (8 rats; example in Fig. 10*A*; summary data in Fig. 10*B*–*D*; injection sites in Fig. 10*E*). Note that muscimol increased resting AP from 103 ± 2 to 139 ± 5 mmHg ($P < 0.05$), eliminated PND, increased resting sSND to 148 ± 9% of control level ($P < 0.05$) and increased the magnitude of the sympathetic nerve response to central chemoreceptor stimulation (Fig. 10*C*).

Discussion

The present study indicates that the sympathoactivation caused by rises in brain P_{CO_2} is predominantly mediated

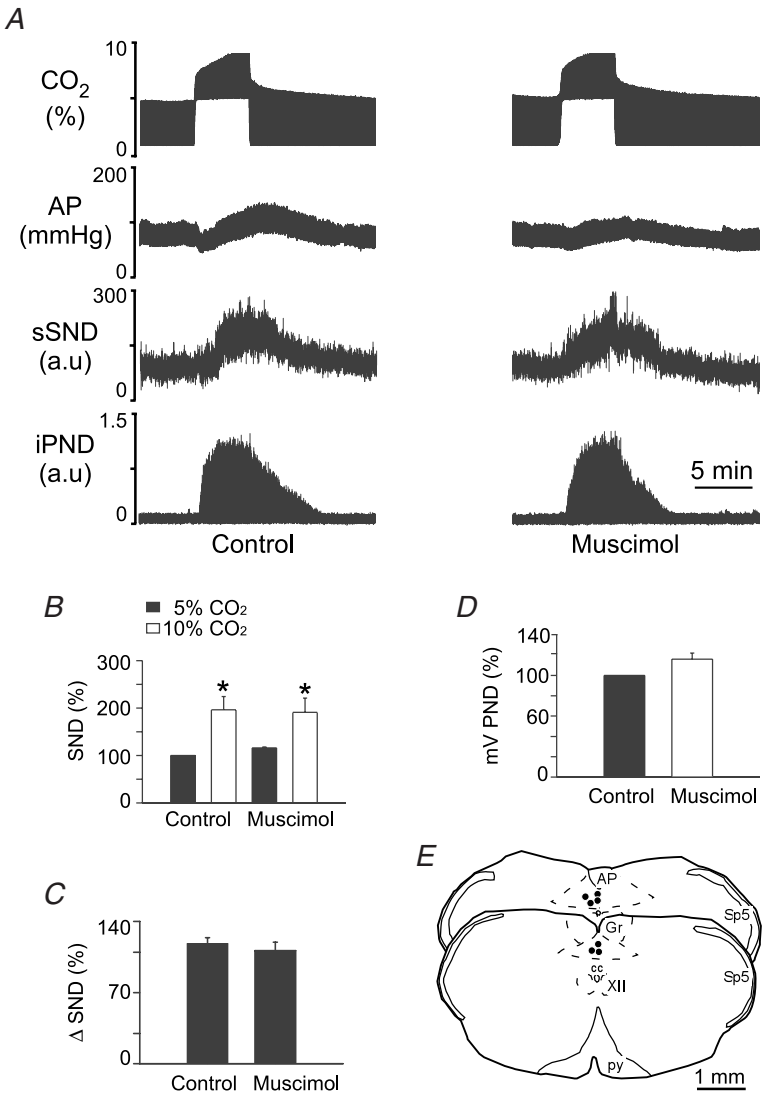

Figure 7. Muscimol injection into the commissural part of the solitary tract nucleus (commNTS) does not change the effect of hypercapnia on sSND or PND in vagotomized sino-aortic denervated rats
A, effect produced by stepping end-expiratory CO_2 from 5 to 10% on sSND and PND in one rat before and after a single midline microinjection of muscimol into commNTS (1.75 mM, 30 nl). *B*, average results from 7 rats. sSND at rest during the control period was defined as 100 units (*$P < 0.05$ between 10 and 5% CO_2). *C*, effect produced of stepping end-expiratory CO_2 from 5 to 10% on the CO_2-induced increase in sSND before and 5 min after injection of muscimol (n.s.). *D*, effect of muscimol on mvPND elicited by stepping end-expiratory CO_2 from 5 to 10% (n.s.). *E*, injection sites. The two coronal sections represent from top to bottom Bregma −14.08 and −14.3 mm after Paxinos & Watson (1998). AP, area postrema; cc, central canal; Gr, gracile nucleus; XII, hypoglossal nucleus; for other abbreviations see Fig. 2.

by the activation of C1 and other sympathoexcitatory neurons of the RVLM. We also show that the activation of these neurons does not rely on chemoreceptors located within the commNTS or the rVRG-pre-Bötzinger region (CVLM). Finally, we also show that hypercapnia activates an inhibitory input to RVLM neurons, which reduces the overall effect of CO_2 on the sympathetic outflow. These results and the pertinent literature are discussed in the context of the largely novel scheme depicted in Fig. 11. An essential aspect of the proposed scheme is that the excitatory effect of CO_2 on RVLM sympathoexcitatory neurons results either from the intrinsic chemosensitivity of these cells or from the activation of the pH-sensitive interneurons located in RTN. The second essential feature of the scheme is that the respiratory modulation of SND is attributed to two processes: active inhibition by GABAergic neurons located in CVLM and disfacilitation of the RTN neurons. The respiratory modulation of the sympathoexcitatory neurons is viewed as attenuating the overall activation of the sympathetic outflow caused by elevations of central nervous system (CNS) P_{CO_2}.

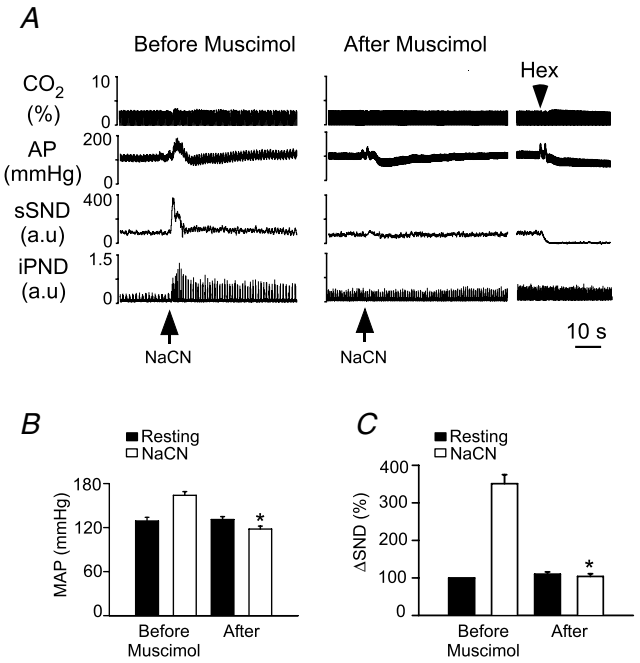

Figure 8. Muscimol injection into commissural part of the solitary tract nucleus (commNTS) blocks peripheral chemoreflexes in vagotomized rats
A, response of the splanchnic and phrenic nerves to peripheral chemoreceptor stimulation with I.V. cyanide (50 μg kg^{-1}) in a urethane-anaesthetized rat before (left panel) and 5 min after a single midline injection of muscimol (1.75 mM, 30 nl: right panel) into commNTS. *B*, effect of muscimol on the cyanide-induced rise in MAP (*$P < 0.05$ when comparing effect of NaCN before and after muscimol; 7 rats). *C*, effect of muscimol on the cyanide-induced rise in sSND (*$P < 0.05$; 7 rats).

RVLM sympathoexcitatory neurons contribute to the sympathoactivation caused by central chemoreceptor stimulation

Under anaesthesia, hypercapnia markedly increases sympathetic efferent activity in vagotomized and sino-aortic denervated preparations (Preiss & Polosa, 1977; Hanna *et al.* 1981; Millhorn & Eldridge, 1986). In agreement with this well-established phenomenon, the mass activity of the splanchnic or lumbar nerves almost doubled at steady state in our preparation when end-expiratory CO_2 was raised from 5 to 10% and SND more than tripled relative to the baseline recorded at 3.5% CO_2. The type of respiratory entrainment observed in these sympathetic nerves during central chemoreceptor stimulation (early inspiratory peak in splanchnic and post-inspiratory peak in lumbar) also conformed to prior observations in rats (Numao *et al.* 1987; Darnall & Guyenet, 1990; Guyenet *et al.* 1990; Pilowsky *et al.* 1996). As shown before, the magnitude of the respiratory modulation of SND (difference between apex and nadir) is less pronounced in rats than in other species and its pattern is also different. In our experimental preparation, virtually all the activity recorded in these nerves is silenced by stimulating arterial baroreceptors (Haselton & Guyenet, 1989) therefore the recorded activity consists overwhelmingly of baroregulated vasoconstrictor efferents (Guyenet, 2006).

In our experiments hypercapnia produced an almost immediate hypotension, which most probably results from a direct (i.e. not sympathetically mediated) effect of CO_2 on the vasculature and, possibly, on cardiac contractility. Blood pressure gradually came back up during hypercapnia to reach about control level at the end of the episode. Then, just after the hypercapnia episode, BP tended to overshoot briefly. We assume that this overshoot reflects the increase in sympathetic activity at a time when the direct depressant effect of CO_2 on the vasculature was rapidly waning.

The effect of central chemoreceptor stimulation on the activity of RVLM vasomotor neurons has rarely been examined before, presumably because of the difficulty in identifying these neurons in a preparation in which arterial baroreceptors have been cut. The only prior attempt (Haselton & Guyenet, 1989) reported a mean increase in discharge of 36 ± 8% between 5 and 10% end-expiratory CO_2. The present result is 67 ± 4%. The difference is probably due to the fact that we measured the effect of CO_2 at steady state (3–5 min equilibration) in the present experiments whereas the cells were exposed to shorter periods of hypercapnia in our prior study. As shown by the kinetics of the phrenic response, several minutes are required for equilibration. This delay is required to establish a steady state in brain pH and medullary blood flow (Sato *et al.* 1992). The difference may

also be related to the selection of neurons. In the present study, our sample consisted exclusively of neurons with fast-conducting axons in which the collision test could be performed. Medium latency (8–25 ms) orthodromic spikes evoked by spinal cord stimulation collide systematically with antidromic spikes in slow-conducting bulbospinal sympathoexcitatory RVLM neurons, preventing the identification of their spinal axon. Seven of the 11 recorded cells were PNMT-immunoreactive, which is consistent with the previously described properties of fast-conducting barosensitive sympathoexcitatory neurons of the RVLM (Schreihofer & Guyenet, 1997). In the present case we do not know with certainty that the presumed sympathoexcitatory neurons that we recorded would have been regulated by arterial baroreceptors but this is very likely because, in our previous experience, all spontaneously active bulbospinal neurons located in the rostral C1 region are inhibited by raising AP in animals with intact baroreceptor afferents. In any event, the essential point in the present context is that the recorded neurons were identified as sympathoexcitatory neurons. This is unquestionably the case for the seven bulbospinal adrenergic cells that were recorded because such C1 cells are known to innervate sympathetic preganglionic neurons and probably the case of the other four bulbospinal neurons although the level of evidence is less persuasive in this case (for review see Guyenet, 2006).

It is of course impossible to be sure that a 67% increase in the activity of RVLM neurons fully accounts for a 97% increase in vasomotor SND. This uncertainty is compounded by the fact that we could not identify neurons with slowly conducting axon velocity in the present study. In other words, the present data do not exclude the possibility that other neurons (e.g. A5) might contribute to the SND increase caused by central chemoreceptor stimulation.

In preparations with intact baroreceptors and carotid bodies, the activation of RVLM vasomotor neurons by hypercapnia is of more modest amplitude (Haselton & Guyenet, 1989). In some cases, no significant change has been reported at the population level (McAllen, 1987; Mulkey *et al.* 2004). Possible explanations include the heterogeneity of the bulbospinal sympathoexcitatory neurons and, again, the fact that the hypercapnic stimulus might have been of too short duration. The more likely explanation perhaps is that the changes in RVLM neuron activity produced by increasing central P_{CO_2} are attenuated by baroreceptor feedback due to the rise in AP, especially

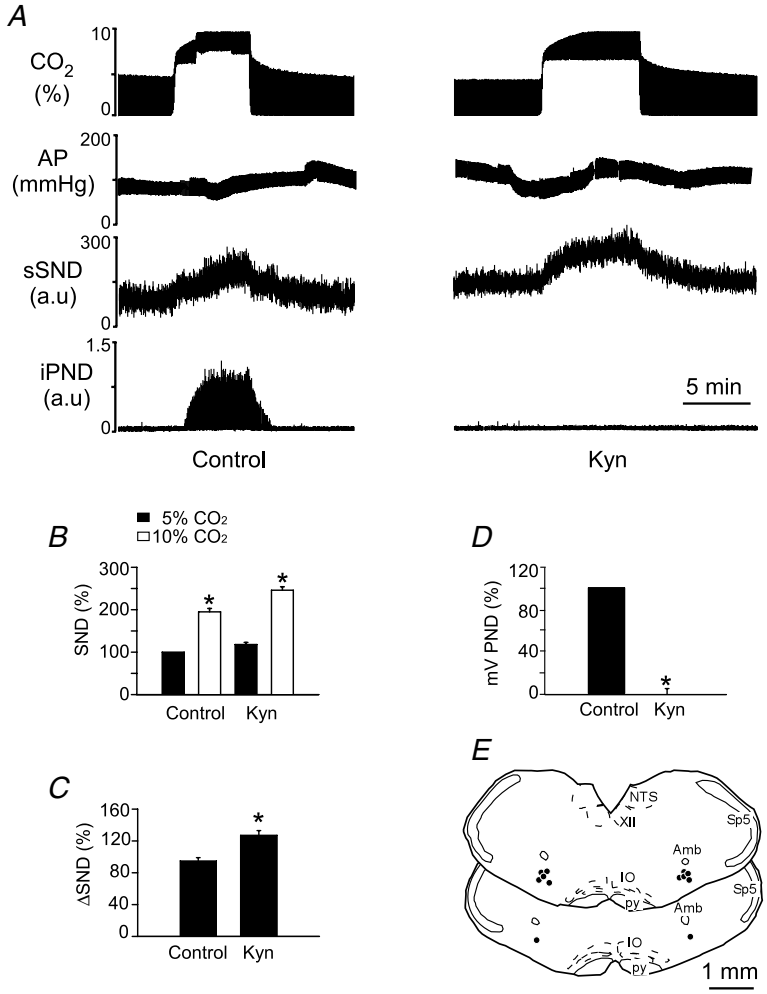

Figure 9. Kynurenic acid (Kyn) injection into the caudal ventrolateral medulla (CVLM) increases the effect of hypercapnia on sSND in vagotomized sino-aortic deafferented rats
A, effect produced by stepping end-expiratory CO_2 from 5 to 10% on sSND and PND in one rat before and after bilateral microinjection of Kyn into CVLM (200 mM, 50 nl). *B*, average results from 6 rats. sSND at rest during the control period was defined as 100 units (*$P < 0.05$ between 10 and 5% CO_2). *C*, effect produced by stepping end-expiratory CO_2 from 5 to 10% on sSND before and after injection of Kyn into CVLM (*$P < 0.05$). *D*, effect of Kyn on mvPND elicited by stepping end-expiratory CO_2 from 5 to 10% (*$P < 0.05$ between control and drug). *E*, injection sites. The two coronal sections represent from top to bottom Bregma −13.3 and −13.6 mm after Paxinos & Watson (1998). NTS, nucleus of the solitary tract; for other abbreviations see Fig. 2.

in those neurons that have the most powerful input from these receptors and are thus the easiest to identify. This attenuation may be further accentuated by the fact that the baroreceptor feedback to the vasomotor SND is more potent under conditions that increase the activity of the central respiratory pattern generator (Chruscielewski *et al.* 1975; Trzebski & Kubin, 1981; Miyawaki *et al.* 1995). This potentiation can probably be explained by the integrative properties of the CVLM neurons that mediate the baroreflex as will be discussed below and is illustrated in Fig. 11.

The chemoreceptors located in the solitary tract and the rVRG-pre-Bötzinger region do not contribute to the sympathoactivation caused by increasing brain P_{CO_2}

Central respiratory chemoreceptors are neurons that directly or indirectly detect changes in CNS interstitial fluid pH and ultimately influence the respiratory network. Such neurons have been tentatively identified in many lower brainstem regions, including the NTS, the medullary raphe and several regions of the ventrolateral medulla including the pre-Bötzinger region and the retrotrapezoid nucleus (Guyenet *et al.* 2005*b*; Nattie & Li, 2006). Whether the same or different chemoreceptors are implicated in the central effect of CO_2 on the sympathetic outflow is an unsettled question. The lack of effect of muscimol injection into commNTS indicates that the caudal cardiorespiratory portion of the NTS plays no role in the sympathoexcitatory response to central chemoreceptor stimulation under the anaesthetic conditions that we selected. The elimination of the peripheral chemoreflex caused by such injections in rats with intact carotid nerves demonstrated how effective muscimol was at inhibiting commNTS neurons. Yet, acidification of commNTS via microdialysis of CO_2-enriched fluid does increase respiration in awake

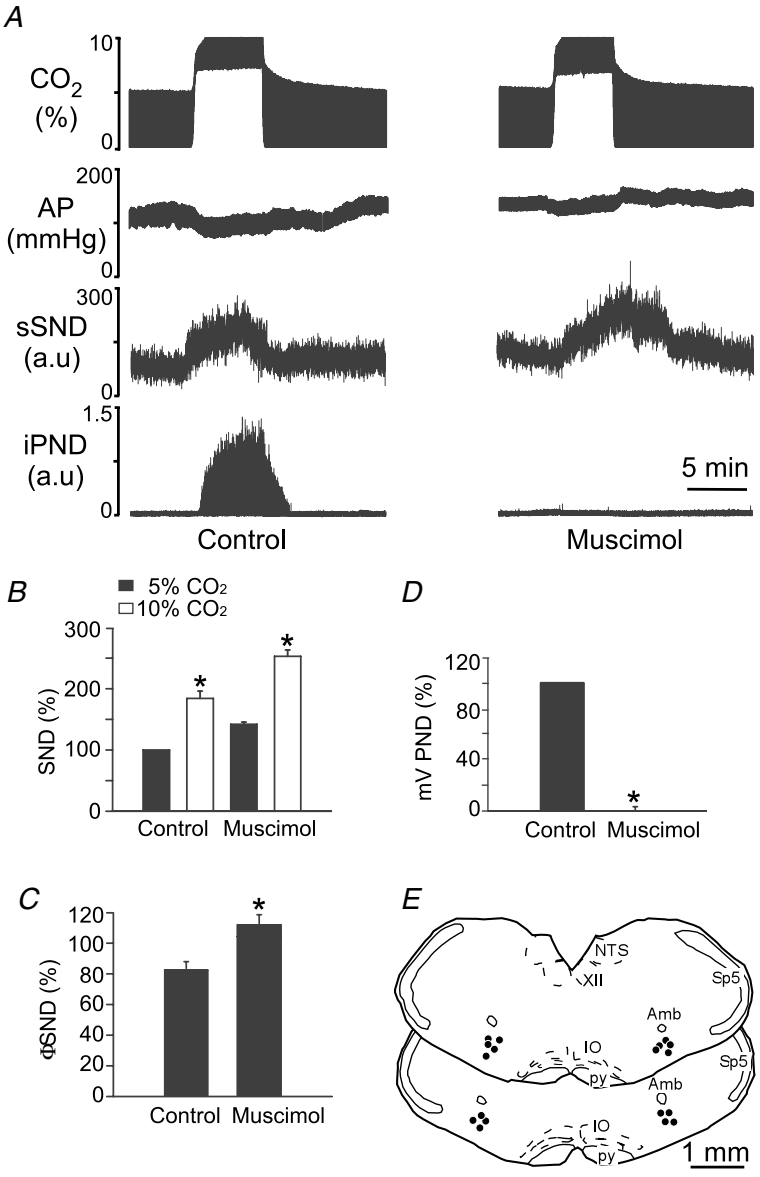

Figure 10. Muscimol injection into the caudal ventrolateral medulla (CVLM) increases the effect of hypercapnia on sSND in vagotomized sino-aortic deafferented rats
A, effect produced by stepping end-expiratory CO_2 from 5 to 10% on sSND and PND in one rat before and after bilateral microinjection of muscimol into CVLM (1.75 mM, 30 nl). *B*, average results from 9 rats. sSND at rest during the control period was defined as 100 units (*$P < 0.05$ for effect of hypercapnia relative to baseline). *C*, effect produced of stepping end-expiratory CO_2 from 5 to 10% on sSND before and 5 min after injection of muscimol into CVLM (*$P < 0.05$ for effect of muscimol). *D*, effect of muscimol on mvPND elicited by stepping end-expiratory CO_2 from 5 to 10% (*$P < 0.05$). *E*, injection sites. The two coronal sections represent from top to bottom Bregma −13.3 and −13.6 mm after Paxinos & Watson (1998).

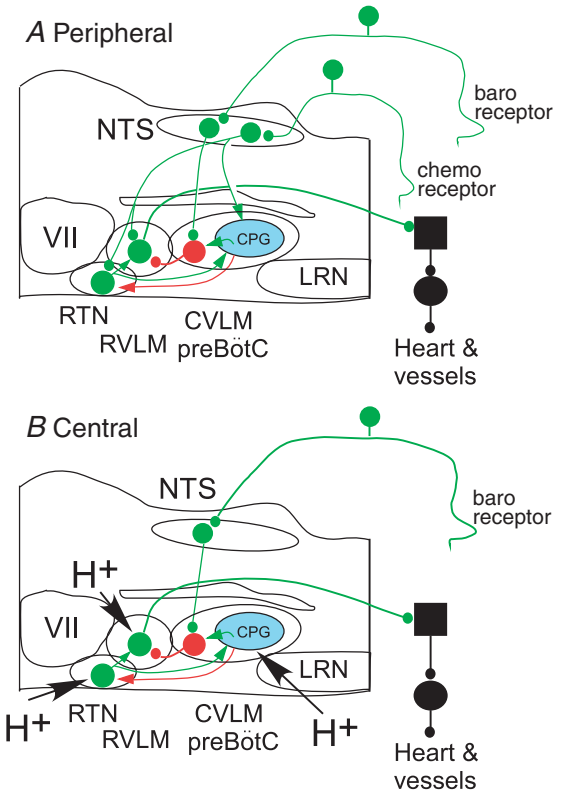

Figure 11. Working model of the sympathetic chemoreflexes
A, peripheral chemoreflex. The increased SND is mediated primarily by activation of the bulbospinal sympathoexcitatory neurons of the RVLM. The excitatory drive of RVLM neurons operates via a direct glutamatergic input from commNTS neurons and via a di-synaptic input that relays via the intrinsically chemosensitive neurons of RTN. The latter pathway is therefore modulated by the degree of central chemoreceptor stimulation. The respiratory modulation of SND and RVLM neurons is seen as deriving from two mechanisms, respiratory-phasic activation by the CPG (respiratory rhythm and pattern-generating network, in blue) of the GABAergic neurons that mediate the sympathetic baroreflex (neuron in red) and respiratory-phasic inhibition of RTN neurons (red arrow, transmitter unidentified). The first mechanism causes an active inhibition of RVLM neurons and the second causes a disfacilitation of RVLM neurons via reduction in RTN discharges. In combination, these two processes attenuate the activation of RVLM neurons during chemoreceptor stimulation and cause the respiratory modulation of SND. *B*, central chemoreflex. The increased SND caused by raising CNS P_{CO_2} is driven via activation of the bulbospinal sympathoexcitatory neurons of the RVLM. The relevant chemoreceptors are located in the RVLM region. The activation of RVLM neurons by extracellular fluid acidification is mediated synaptically via the chemosensitive neurons of RTN and, possibly, by the intrinsic chemosensitivity of the RVLM sympathoexcitatory neurons. The respiratory modulation of RVLM neurons and SND has the same mechanism as in the case of the peripheral chemoreflex (CPG-mediated activation of the CVLM neurons and CPG-mediated inhibition of the RTN). CNS acidification activates the CPG via chemoreceptors located in the RTN and/or the rVRG-pre-Bötzinger region but not in the NTS. The integrative properties of the CVLM GABAergic neurons that relay the sympathetic baroreflex (in red) account for the well-documented potentiation of the baroreflex caused by increasing CPG activity. The scheme only applies to the effect of chemoreceptors on the baroregulated sympathetic efferents that control the heart and resistance vessels. The scheme is not applicable to other sympathetic efferents (e.g. to skin or brown adipose fat). Excitatory neurons in green (large circles, cell bodies; small circles, ionotropic glutamatergic synapses; green arrows, neurochemically uncharacterized excitatory connections). In red, GABAergic CVLM neurons involved in the sympathetic baroreflex. Red arrow, neurochemically unidentified inhibitory input from the CPG to RTN neurons. CPG, central pattern generator; CVLM, caudal ventrolateral medulla; LRN, lateral reticular nucleus; RTN, retrotrapezoid nucleus; RVLM, rostral ventrolateral nucleus; for other abbreviations see Fig. 2.

rats (Nattie & Li, 2002). The discrepancy can be explained three ways. The first explanation, improbable perhaps but impossible to exclude, is that artificial acidification via microdialysis of CO_2 or with acetazolamide does not mimic the effect of systemic hypercapnia. Second, anaesthesia may eliminate the effect produced by acidifying the NTS. Third, the pH-sensitive cells of the NTS (central chemoreceptors) reside in a different location than the second-order cells involved in the peripheral chemoreflex. In support of this view, the NTS region acidified by Nattie & Li (2002) with resulting stimulation of breathing was slightly more rostral and lateral than that which we targeted with muscimol in the present study.

The rVRG-pre-Bötzinger region of the ventral respiratory column also contains putative respiratory chemoreceptors (Solomon *et al.* 2000; Nattie & Li, 2006). These receptors are apparently not involved in the sympathoexcitation caused by hypercapnia since bilateral injection of muscimol or Kyn into this region did not decrease the effect of CO_2 on SND. Kyn, which only blocks EAA transmission, could conceivably have spared the chemoreceptors responsible for activating RVLM neurons but this explanation does not hold in the case of muscimol, a $GABA_A$ receptor agonist that silences all neurons. The neuronal inhibition caused by muscimol was profound given the hours-long disappearance of the PND at rest and during hypercapnia. Inhibition of the rVRG-pre-Bötzinger region with either Kyn or muscimol actually increased the sympathoexcitation caused by hypercapnia, suggesting that this region contains an inhibitory input to RVLM neurons that is activated by CNS acidification.

Putative organization of the medullary network responsible for peripheral and central sympathetic chemoreflexes

The following discussion is an attempt to fit the results of the present study and the pertinent literature into a novel and more comprehensive theory of how the sympathetic vasomotor outflow is regulated by central and peripheral chemoreceptors. In this scheme, the increase in SND caused by central or peripheral chemoreceptor stimulation is seen as resulting from excitatory drives to RVLM neurons. The scheme incorporates the already established notion that RVLM neurons are excited by

peripheral chemoreceptors via a direct glutamatergic input from commNTS (for review and references see Guyenet, 2000). To this notion, we add the concept that this glutamatergic projection also activates RVLM neurons via a di-synaptic excitatory pathway from commNTS to RVLM that relays via the chemosensitive neurons of the retrotrapezoid nucleus (RTN; secondary input) (Fig. 11*A*) (Takakura *et al.* 2006). In the case of the central chemoreflex (Fig. 11*B*) the primary excitatory input to RVLM neurons is seen as originating from the chemosensitive neurons of RTN although the possibility that the RVLM sympathoexcitatory neurons might be intrinsically sensitive to pH is also considered (Fig. 11*B*). The respiratory entrainment of RVLM neurons and SND, which is a feature common to both reflexes, is viewed as being mediated by the following two mechanisms: respiratory synchronous inhibitory inputs to RVLM neurons originating from the GABAergic neurons of the CVLM, that normally mediate the baroreflex, and respiratory-synchronous disfacilitation caused by respiratory-synchronous inhibitory inputs to RTN neurons (Fig. 11*A* and *B*).

The GABAergic cells of the CVLM are respiratory-modulated and many have a phase-spanning respiratory modulation that mirrors that of the sympathoexcitatory neurons of the RVLM (Mandel & Schreihofer, 2006). Such characteristics are therefore consistent with the possibility that these neurons contribute to the respiratory entrainment of RVLM neurons. Inhibition of CVLM neurons by muscimol should thus increase the central sympathetic chemoreflex, as observed in the present experiments. CVLM blockade should also increase the peripheral chemoreflex and eliminate its respiratory oscillations, as observed in prior experiments (Koshiya & Guyenet, 1996).

The proposed scheme is also consistent with prior observation that the RTN chemosensitive neurons have a phase-spanning type of respiratory modulation in rats (Guyenet *et al.* 2005*a*). Like CVLM neurons, RTN cells are heterogeneous with regard to respiratory modulation but cases of respiratory patterns that are identical to those of RVLM sympathoexcitatory neurons (early inspiratory or post-inspiratory peak) have been found, consistent with the possibility that these particular RTN neurons drive RVLM sympathoexcitatory neurons (Guyenet *et al.* 2005*a*). Furthermore, in cats, species in which RVLM neurons and the vasomotor SND are inspiratory modulated, the predominant respiratory pattern of RTN neurons is also inspiratory (Connelly *et al.* 1990). The respiratory entrainment of RTN neurons is most probably due to respiratory-phasic inhibitory inputs (Guyenet *et al.* 2005*a*). Phasic inhibition of these chemosensitive neurons by the central respiratory network should therefore attenuate the chemoreflexes by a process of disfacilitation. We have already demonstrated that respiratory-rhythmic inhibition of RTN neurons disappears after injection of muscimol into the CVLM region whereas the excitatory response of these neurons to central and peripheral chemoreceptor stimulation persists (Guyenet *et al.* 2005*a*). Therefore, by silencing the respiratory pattern generator, muscimol inhibition of the CVLM region should also eliminate the respiratory modulation of RTN neurons and this effect should also contribute to increasing the magnitude of the sympathetic chemoreflex, as was observed.

The present study revealed that the excitatory mechanisms responsible for the central and the peripheral chemoreflex are distinct in one essential respect. Ionotropic glutamatergic receptor blockade in RVLM virtually eliminates SND activation by peripheral chemoreceptors whereas it does not attenuate the central sympathetic chemoreflex (present study). The effect of Kyn on the peripheral chemoreflex is consistent with prior anatomical and electrophysiological evidence that the effect of peripheral chemoreceptor activation is transmitted to RVLM and RTN neurons by a direct glutamatergic projection from commNTS (Sun & Reis, 1995; Aicher *et al.* 1996; Guyenet, 2000; Takakura *et al.* 2006). This fact accounts for the very large attenuation of the peripheral chemoreflex by Kyn injection into RVLM (86% in the present case) since such injections should block the direct activation of the sympathoexcitatory neurons and their indirect activation via the RTN neurons.

The resistance of the central sympathetic chemoreflex to Kyn indicates that the activation of the RVLM neurons does not involve ionotropic glutamate receptors. This observation suggests two alternative possibilities represented in Fig. 11*B*. The effect of CO_2 on RVLM neurons could result from the intrinsic chemosensitivity of these neurons or it could be caused by synaptic inputs from chemosensitive cells that do not operate via ionotropic glutamate receptors. The notion that RVLM neurons might be intrinsically pH-sensitive still rests on extremely circumstantial evidence outlined in the introduction but it cannot be dismissed *a priori*. The presence within the RVLM region of central chemoreceptors that activate the sympathoexcitatory neurons synaptically is much more plausible however, and the RTN neurons have all the required characteristics. Briefly summarized, RTN neurons are activated by pH, they are located at the rostral end of the RVLM at the ventral surface and they are in close proximity to the sympathoexcitatory neurons (Guyenet *et al.* 2005*b*). Their activation by CO_2 is independent of ionotropic glutamate transmission and their discharge pattern becomes extremely regular after inhibition of the CPG (Mulkey *et al.* 2004; Takakura *et al.* 2006). These cells innervate the region of the RVLM (Rosin *et al.* 2006) and, as mentioned above, a fraction of RTN neurons have the same phase-spanning respiratory modulation (early inspiratory

or post-inspiratory) as the sympathoexcitatory neurons of the RVLM (Guyenet *et al.* 2005*a*). The inability of Kyn to attenuate the central sympathetic chemoreflex (this study) might seem to exclude RTN neurons *a priori* because these cells express VGLUT2 and therefore should release glutamate (Mulkey *et al.* 2004). However, RTN neurons may operate via metabotropic glutamate receptors or they could release other excitatory transmitters. Metabotropic glutamate receptors may be present on RVLM neurons given that agonists of these receptors increase AP substantially when they are injected into RVLM (Tsuchihashi *et al.* 2000). The literature contains many examples of VGLUT2-expressing CNS neurons that release another transmitter that appears to play the dominant role in synaptic transmission, e.g. orexin neurons (Rosin *et al.* 2003).

In summary, the hypothetical scheme presented in Fig. 11 could account satisfactorily for most if not all existing observations. However, its accuracy will have to be subjected to further and far more detailed testing.

References

Aicher SA, Saravay RH, Cravo S, Jeske I, Morrison SF, Reis DJ & Milner TA (1996). Monosynaptic projections from the nucleus tractus solitarii to C1 adrenergic neurons in the rostral ventrolateral medulla: Comparison with input from the caudal ventrolateral medulla. *J Comp Neurol* **373**, 62–75.

Brown DL & Guyenet PG (1985). Electrophysiological study of cardiovascular neurons in the rostral ventrolateral medulla in rats. *Circ Res* **56**, 359–369.

Chruscielewski L, Majcherczyk S & Trzebski A (1975). Influence of combined baro- and chemoreceptor stimulation upon sympathetic activity. *J Physiol* **247**, 28*P*–29*P*.

Connelly CA, Ellenberger HH & Feldman JL (1990). Respiratory activity in retrotrapezoid nucleus in cat. *Am J Physiol Lung Cell Mol Physiol* **258**, L33–L44.

Darnall RA & Guyenet P (1990). Respiratory modulation of pre- and postganglionic lumbar vasomotor sympathetic neurons in the rat. *Neurosci Lett* **119**, 148–152.

Feldman JL & McCrimmon DR (1999). Neural control of breathing. In *Fundamental Neuroscience*, ed.Zigmond MJ, Bloom FE, Landis SC, Roberts JL & Squire LR, pp. 1063–1090. Academic Press, San Diego.

Feldman JL, Mitchell GS & Nattie EE (2003). Breathing: rhythmicity, plasticity, chemosensitivity. *Annu Rev Neurosci* **26**, 239–266.

Guyenet PG (2000). Neural structures that mediate sympatho-excitation during hypoxia. *Respir Physiol* **121**, 147–162.

Guyenet PG (2006). The sympathetic control of blood pressure. *Nat Rev Neurosci* **7**, 335–346.

Guyenet PG, Darnall RA & Riley TA (1990). Rostral ventrolateral medulla and sympathorespiratory integration in rats. *Am J Physiol Regul Integr Comp Physiol* **259**, R1063–R1074.

Guyenet PG & Koshiya N (1992). Respiratory-sympathetic integration in the medulla oblongata. In *Central Neural Mechanisms in Cardiovascular Regulation*, Vol. II, ed. Kunos G & Ciriello J, pp. 226–247. Birkhauser, Boston.

Guyenet PG, Mulkey DK, Stornetta RL & Bayliss DA (2005*a*). Regulation of ventral surface chemoreceptors by the central respiratory pattern generator. *J Neurosci* **25**, 8938–8947.

Guyenet PG, Stornetta RL, Bayliss DA & Mulkey DK (2005*b*). Retrotrapezoid nucleus: a litmus test for the identification of central chemoreceptors. *Exp Physiol* **90**, 247–253.

Habler HJ, Janig W & Michaelis M (1994). Respiratory modulation in the activity of sympathetic neurones. *Prog Neurobiol* **43**, 567–606.

Hanna BD, Lioy F & Polosa C (1979). The effect of cold blockade of the medullary chemoreceptors on the CO_2 modulation of vascular tone and heart rate. *Can J Physiol Pharmacol* **57**, 461–468.

Hanna BD, Lioy F & Polosa C (1981). Role of carotid and central chemoreceptors in the CO_2 response of sympathetic preganglionic neurons. *J Auton Nerv Syst* **3**, 421–435.

Hanna BD, Lioy F & Polosa C (1988). Role of carotid and central chemoreceptors in the CO2 response of sympathetic preganglionic neurons. *J Auton Nerv Syst* **3**, 95–105.

Haselton JR & Guyenet PG (1989). Central respiratory modulation of medullary sympathoexcitatory neurons in rat. *Am J Physiol Regul Integr Comp Physiol* **256**, R739–R750.

Hodges MR, Martino P, Davis S, Opansky C, Pan LG & Forster HV (2004). Effects on breathing of focal acidosis at multiple medullary raphe sites in awake goats. *J Appl Physiol* **97**, 2303–2309.

Koshiya N, Huangfu D & Guyenet PG (1993). Ventrolateral medulla and sympathetic chemoreflex in the rat. *Brain Res 23* **609**, 174–184.

Koshiya N & Guyenet PG (1996). Tonic sympathetic chemoreflex after blockade of respiratory rhythmogenesis in the rat. *J Physiol* **491**, 859–869.

McAllen RM (1987). Central respiratory modulation of subretrofacial bulbospinal neurons in the cat. *J Physiol* **388**, 533–545.

Mandel DA & Schreihofer AM (2006). Central respiratory modulation of barosensitive neurones in rat caudal ventrolateral medulla. *J Physiol* **572**, 881–896.

Millhorn DE (1986). Neural respiratory and circulatory interaction during chemoreceptor stimulation and cooling of ventral medulla in cats. *J Physiol* **370**, 217–231.

Millhorn DE & Eldridge FL (1986). Role of ventrolateral medulla in regulation of respiratory and cardiovascular systems. *J Appl Physiol* **61**, 1249–1263.

Milner TA, Pickel VM, Morrison SF & Reis DJ (1989). Adrenergic neurons in the rostral ventrolateral medulla: ultrastructure and synaptic relations with other transmitter-identified neurons. *Prog Brain Res* **81**, 29–47.

Milner TA, Pickel VM, Park DH, Joh TH & Reis DJ (1987). Phenylethanolamine N-methyltransferase-containing neurons in the rostral ventrolateral medulla of the rat. I. Normal ultrastructure. *Brain Res* **411**, 28–45.

Miyawaki T, Pilowsky P, Sun QJ, Minson J, Suzuki S, Arnolda L, Llewellyn-Smith I & Chalmers J (1995). Central inspiration increases barosensitivity of neurons in rat rostral ventrolateral medulla. *Am J Physiol Regul Integr Comp Physiol* **268**, R909–R918.

Mulkey DK, Stornetta RL, Weston MC, Simmons JR, Parker A, Bayliss DA & Guyenet PG (2004). Respiratory control by ventral surface chemoreceptor neurons in rats. *Nat Neurosci* **7**, 1360–1369.

Nattie EE & Li A (1995). Rat retrotrapezoid nucleus iono- and metabotropic glutamate receptors and the control of breathing. *J Appl Physiol* **78**, 153–163.

Nattie EE & Li A (1996). Central chemoreception in the region of the ventral respiratory group in the rat. *J Appl Physiol* **81**, 1987–1995.

Nattie EE & Li AH (2002). CO_2 dialysis in nucleus tractus solitarius region of rat increases ventilation in sleep and wakefulness. *J Appl Physiol* **92**, 2119–2130.

Nattie E & Li A (2006). Central chemoreception 2005: a brief review. *Auton Neurosci* **126–127**, 332–338.

Numao Y, Koshiya N, Gilbey MP & Spyer KM (1987). Central respiratory drive-related activity in sympathetic nerves of the rat: the regional differences. *Neurosci Lett* **81**, 279–284.

Paxinos G & Watson C (1998). *The Rat Brain in Stereotaxic Coordinates*, 4th edn. Academic Press, San Diego.

Pilowsky P, Arnolda L, Chalmers J, Llewellyn-Smith I, Minson J, Miyawaki T & Sun QJ (1996). Respiratory inputs to central cardiovascular neurons. *Ann NY Acad Sci* **783**, 64–70.

Pineda J & Aghajanian GK (1997). Carbon dioxide regulates the tonic activity of locus coeruleus neurons by modulating a proton- and polyamine-sensitive inward rectifier potassium current. *Neurosci* **77**, 723–743.

Preiss G & Polosa C (1977). The relation between end-tidal CO_2 and discharge patterns of sympathetic preganglionic neurons. *Brain Res* **122**, 255–267.

Putnam RW, Filosa JA & Ritucci NA (2004). Cellular mechanisms involved in CO_2 and acid signaling in chemosensitive neurons. *Am J Physiol Cell Physiol* **287**, C1493–C1526.

Richerson GB, Wang W, Hodges MR, Dohle CI & Diez-Sampedro A (2005). Homing in on the specific phenotype(s) of central respiratory chemoreceptors. *Exp Physiol* **90**, 259–266.

Richter DW & Spyer KM (1990). Cardiorespiratory control. In *Central Regulation of Autonomic Functions*, ed. Loewy AD & Spyer KM, pp. 189–207. Oxford University Press, New York.

Rosin DL, Chang DA & Guyenet PG (2006). Afferent and efferent connections of the rat retrotrapezoid nucleus. *J Comp Neurol* **499**, 64–89.

Rosin DL, Weston MC, Sevigny CP, Stornetta RL & Guyenet PG (2003). Hypothalamic orexin (hypocretin) neurons express vesicular glutamate transporters VGLUT1 or VGLUT2. *J Comp Neurol* **465**, 593–603.

Sato A, Trzebski A & Zhou W (1992). Local cerebral blood flow responses in rats to hypercapnia and hypoxia in the rostral ventrolateral medulla and in the cortex. *J Auton Nerv Syst* **41**, 79–86.

Schreihofer AM & Guyenet PG (1997). Identification of C1 presympathetic neurons in rat rostral ventrolateral medulla by juxtacellular labeling in vivo. *J Comp Neurol* **387**, 524–536.

Schreihofer AM & Guyenet PG (2000*a*). Role of presympathetic C1 neurons in the sympatholytic and hypotensive effects of clonidine in rats. *Am J Physiol Regul Integr Comp Physiol* **279**: R1753–R1762.

Schreihofer AM & Guyenet PG (2000*b*). Sympathetic reflexes after depletion of bulbospinal catecholaminergic neurons with anti-DbetaH-saporin. *Am J Physiol Regul Integr Comp Physiol* **279**, R729–R742.

Schreihofer AM, Stornetta RL & Guyenet PG (2000). Regulation of sympathetic tone and arterial pressure by rostral ventrolateral medulla after depletion of C1 cells in rat. *J Physiol*; **529**, 221–236.

Schreihofer AM & Guyenet PG (2003). Baroactivated neurons with pulse-modulated activity in the rat caudal ventrolateral medulla express GAD67 mRNA. *J Neurophysiol* **89**, 1265–1277.

Solomon IC, Edelman NH & O'Neill MH (2000). CO_2/H^+ chemoreception in the cat pre-Bötzinger complex in vivo. *J Appl Physiol* **88**, 1996–2007.

Stornetta RL & Guyenet PG (1999). Distribution of glutamic acid decarboxylase mRNA-containing neurons in rat medulla projecting to thoracic spinal cord in relation to monoaminergic brainstem neurons. *J Comp Neurol* **407**, 367–380.

Stornetta RL, Sevigny CP & Guyenet PG (2003). Inspiratory augmenting bulbospinal neurons express both glutamatergic and enkephalinergic phenotypes. *J Comp Neurol* **455**, 113–124.

Sun QJ, Goodchild AK & Pilowsky PM (2001). Firing patterns of pre-Botzinger and Botzinger neurons during hypocapnia in the adult rat. *Brain Res* **903**, 198–206.

Sun M-K & Reis DJ (1995). NMDA receptor-mediated sympathetic chemoreflex excitation of RVL-spinal vasomotor neurones in rats. *J Physiol* **482**, 53–68.

Takakura AC, Moreira TS, Colombari E, West GH, Stornetta RL & Guyenet PG (2006). Peripheral chemoreceptor inputs to retrotrapezoid nucleus (RTN) CO_2-sensitive neurons in rats. *J Physio1* **572**, 503–523.

Trzebski A & Kubin L (1981). Is the central inspiratory activity responsible for pCO_2-dependent drive of the sympathetic discharge? *J Auton Nerv Syst* **3**, 401–420.

Tsuchihashi T, Liu Y, Kagiyama S, Matsumura K, Abe I & Fujishima M (2000). Metabotropic glutamate receptor subtypes involved in cardiovascular regulation in the rostral ventrolateral medulla of rats. *Brain Res Bull* **52**, 279–283.

Washburn CP, Bayliss DA & Guyenet PG (2003). Cardiorespiratory neurons of the rat ventrolateral medulla contain TASK-1 and TASK-3 channel mRNA. *Respir Physiol Neurobiol* **138**, 19–35.

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Discussão e Conclusão

Quimiorreceptores centrais e núcleo retrotrapezóide.

Os resultados mostram que os neurônios do NRT respondem tanto para a ativação dos quimiorreceptores centrais quanto para a ativação dos quimiorreceptores periféricos. Esses resultados mostram pela primeira vez a existência de uma via de interação entre os quimiorreceptores periféricos e os neurônios quimiossensíveis do NRT (quimiorreceptores centrais) e que essa possível via envolve os neurônios glutamatérgicos localizados na região comissural do NTS. Com esse trabalho, nós podemos concluir que os neurônios do NRT podem integrar as vias do quimiorreflexo central e periférico e constituir um centro importante para a integração cardio-respiratória.

Os neurônios do NRT apresentam alta sensibilidade a variações de CO_2, mesmo em condições de hiperóxia (100% de O_2), refletindo, portanto, a sua propriedade intrínseca a variações do pH extracelular (Mulkey e cols., 2004; Guyenet e cols., 2005a).

Nesse estudo, foi utilizado o anestésico uretana e alfa-cloralose, pois sabe-se que o anestésico halotana deprime os efeitos da estimulação dos quimiorreceptores periféricos. Observou-se apenas pequenas diferenças entre esse anestésico e o anestésico halotana (anestésico utilizado quando foi demonstrado pela primeira vez as características das células quimiossensíveis do NRT) na atividade das células do NRT (Mulkey e cols., 2004). Aparentemente, a única diferença observada foi que o limiar para a ativação dos neurônios do NRT foi um pouco mais elevado (5% vs. 4.2%) sob o efeito do anestésico uretana e alfa cloralose. Por outro lado, o limiar para disparo do nervo frênico foi o mesmo para os dois anestésicos. A resposta intrínseca dos neurônios do NRT para variações de CO_2 é linear e a sua saturação, em altos níveis de CO_2, parece ser causado por mecanismos inibitórios originados da região geradora do padrão respiratório (mais especificamente na região Pré-Botzinger, região que contém os neurônios responsáveis pela geração do ritmo respiratório). Nessa situação anestésica, esse mecanismo inibitório parece ser mais modesto, pois a modulação respiratória nos neurônios do NRT é muito mais reduzida se comparado com a modulação

respiratória dos neurônios do NRT em condições de anestesia com halotana (Mulkey e cols., 2004; Guyente e cols., 2005; Takakura e cols., 2006).

Todos os neurônios do NRT, analisados nesse estudo, foram, intensamente, ativados pelos quimiorreceptores periféricos (períodos de hipóxia (10-15%) e injeções endovenosas de cianeto de sódio). Essas respostas foram observadas apenas em animais que possuíam os quimiorreceptores periféricos intactos, confirmando experimentos anteriores descritos na literatura (Bodineau e cols., 2000a; 2000b; Mulkey e cols., 2004) e comprovando os efeitos de ativação dos neurônios do NRT quando se tem a ativação dos quimiorreceptores periféricos.

Como já descrito anteriormente, os quimiorreceptores periféricos enviam suas aferências para a região do NTScom (Colombari e cols., 1996; Koshiya e Guyenet, 1996; Blessing e cols., 1999; Takakura e cols., 2006). A maioria desses neurônios projetam-se para a região ventrolateral do bulbo, onde uma dessas projeções seria para os neurônios da região do RVL (Aicher e cols., 1996; Koshiya e Guyenet, 1996; Paton e cols, 2001). De acordo com nosso estudo, os neurônios do NTScom, que são ativados pelos quimiorreceptores periféricos, projetam-se para o NRT e essa projeção é glutamatérgica (Trabalho 3: Takakura e cols., 2006).

Para confirmar a evidência eletrofisiológica da possível projeção do NTScom para o NRT, experimentos de anatomia foram utilizados, envolvendo a injeção de traçadores anterógraos e retrógrados. Após a injeção do traçador anterógrado (BDA – amina dextrana biotinilada), muitos terminais sinápticos encontrados na região do NRT foram observados como sendo glutamatérgicos, pois expressavam a vesícula para transporte de glutamato (VGLUT2) (mais de 50%).

Quando se combinou experimentos de expressão da proteína Fos, juntamente com traçadores retrógrados, observamos que a expressão da proteína Fos mostrou, claramente, que a projeção do NTScom para o NRT possui neurônios que são ativados por hipóxia. Portanto, a via entre o NTScom e o NRT é glutamatérgica, uma vez que mais de 90% dos neurônios do NTScom expressam

VGLUT2 mRNA. O marcador VGLUT2 mRNA consiste num seletivo marcador de neurônios glutamatérgicos, pois sua expressão é necessária e suficiente para promover a despolarização e, portanto, mediar a exocitose do glutamato (Stornetta e cols., 2002).

A pequena porcentagem de terminais glutamatérgicos no NRT, comparado com o número de neurônios encontrados no NTScom pode ser explicada por uma simples razão: a dupla marcação com o traçador anterógrado (BDA) e com a vesícula de glutamato (VGLUT2) pode não ser revelado de maneira idêntica, em especial na penetração do anticorpo em diferentes profundidades no tecido cerebral.

Em resumo, podemos concluir dessa primeira etapa que a maioria dos neurônios do NTScom, sensíveis a variações de hipóxia, são glutamatérgicos. Entretanto, uma pequena porção dessa via é inibitória uma vez que 12% dos neurônios do NTScom expressam GAD67 mRNA. Essa conclusão é compatível com o fato de que uma pequena proporção de terminais sinápticos no NRT são imunorreativos para a glutamina ácido descarboxilase (GAD67). A projeção inibitória pode não atingir os neurônios quimiossensíveis do NRT, mas pode estar envolvida nos neurônios que regulam a atividade simpática para o controle de temperatura (no rato a principal fonte de controle de temperatura consiste o tecido adiposo marrom). A literatura mostra que a atividade simpática para tecido adiposo marrom é inibida após a estimulação dos quimiorreceptores periféricos, contribuindo na redução da temperatura corporal e no consumo de oxigênio (Madden e Morrison, 2005).

A ativação dos neurônios do NRT, mediante alterações nas concentrações de CO_2, não é secundário a ativação de vias respiratórias. Essa idéia é evidenciada pelo fato da atividade dos neurônios do NRT para a hipercapnia não ser afetada após a injeção intracerebroventricular de ácido quinurênico (Mulkey e cols., 2004; Guyente e cols., 2005a). Consistente com essa interpretação, o presente estudo mostra que as respostas de ativação dos neurônios do NRT para hipercapnia não são afetadas após a inibição de uma porção da coluna respiratória ventral (inibição da região CVL ou Pré-Botzinger) com muscimol. O

único efeito produzido pelo muscimol foi a regularização do padrão de disparos dos neurônios do NRT, fazendo com que os neurônios quimiossensíveis do NRT perdessem as suas modulações respiratórias.

A estimulação dos quimiorreceptores periféricos produz um aumento nos disparos dos neurônios quimiossensíveis do NRT, independente do nível de CO_2. As alterações de pH e a estimulação dos quimiorreceptores periféricos parece ter um efeito aditivo na atividade dos neurônios quimiossensíveis do NRT, pelo menos em situações de hipóxia moderada (15%). Esse efeito somatório produz uma diminuição no limiar de disparos dos neurônios quimiossensíveis do NRT, isto é, esses neurônios, sob uma situação de hipóxia, começam a iniciar sua atividade com valores menores de CO_2 se comparados a uma situação controle. Da mesma maneira, os efeitos do CO_2 e da estimulação dos quimiorreceptores periféricos sobre a atividade do nervo frênico parece também ter um efeito aditivo (presentes resultados; Nattie e cols., 1991). Se o único mecanismo que os quimiorreceptores centrais podem reagir são as alterações no pH, parece lógico que as respostas dos quimiorreceptores centrais para variações de pH sejam variáveis, isto é depende do tipo de informação que esses neurônios recebem.

Papel do núcleo retrotrapezóide no controle da respiração.

A injeção bilateral de muscimol no NRT produz a eliminação da atividade do nervo frênico em situações basais (controle) e também durante a ativação dos quimiorreceptores centrais (hipercapnia) e periféricos (hipóxia). As injeções de muscimol foram realizadas embaixo da região caudal do núcleo facial, local onde localizam-se a maior concentração de células quimiossensíveis do NRT (Mulkey e cols., 2004; Guyenet e cols., 2005a; 2005b; Takakura e cols., 2006).

A contribuição do NRT para a respiração não está limitada apenas em casos de anestesia. Mesmo após lesões eletrolíticas unilaterias do NRT, pode-se observar uma deficiência de, aproximadamente, 40% nas respostas ventilatórias para a hipercapnia em ratos acordados (Akilesh e cols., 1997). O fato de a injeção de muscimol também ter eliminado os efeitos da ativação dos quimiorreceptores

periféricos sobre o nervo frênico pode ser visto como uma evidência importante de que os neurônios do NRT participam dos padrões respiratórios.

Nós não podemos também excluir a possibilidade de que a injeção de muscimol produziu a eliminação do nervo frênico por atuar em regiões mais caudais ao NRT, afetando os neurônios controladores do ritmo respiratório (como por exemplo os neurônios da coluna respiratória ventral, incluíndo os neurônios da região Pré-Botzinger). A presença de microesferas fluorescentes nas nossas injeções de muscimol mostraram que a droga pode ter atingido até 300 μm do centro da injeção. Neurônios localizados no bulbo ventrolateral possuem dendritos que se espalham por até 200 μm do corpo celular na direção rostro-caudal (Pilowsky e cols., 1990; Schreihofer e Guyenet, 1997). Entretanto, pode ser que neurônios localizados entre 300 e 600 μm caudais ao limite posterior ao núcleo facial podem ter sido parcialmente inibidos pela injeção de muscimol no NRT.

Alguns experimentos sugerem que os neurônios do NRT podem regular a atividade dos músculos expiratórios (Forster e cols., 1995; Janczewski e Feldman, 2005). A teoria de que os neurônios do NRT são os responsáveis pela expiração (Janczewski e Feldman, 2005) parece não ser muito oposta com as observações encontradas no presente estudo. Para isso é necessário que a atividade dos músculos expiratórios seja aumentada pela ativação dos quimiorreceptores centrais e periféricos. Diante disso, os neurônios do NRT podem fazer parte da via de ativação desse padrão respiratório.

A possibilidade de que o NRT projeta-se para diferentes regiões da coluna respiratória é consistente com o fato de que os neurônios quimiossensíveis do NRT possuem diferentes padrões respiratórios (Guyenet e cols., 2005a). Baseando-se na localização, conexões anatômicas e padrões respiratórios, os neurônios do NRT podem ser regulados por outras eferências respiratórias, pela atividade simpática (coração, rins ou músculos) ou, ainda, pelas eferências parassimpáticas (músculo da traquéia) (Millhorn e Eldridge, 1986; Perez Fontan e Velloff, 1997; Guyenet e cols., 2005a; Takakura e cols., 2006).

Diante de tudo que foi discutido, podemos concluir que a região do NRT possui propriedades fisiológicas e anatômicas que são requisitos essenciais para

a integração do quimiorreflexo central e periférico. Obviamente que não descartamos a possibilidade de que outras regiões do SNC possam também integrar as vias do quimiorreflexo.

Quimiorreceptores centrais e atividade simpática.

A próxima etapa do nosso estudo foi procurar mostrar que o aumento da atividade simpática causado pelo aumento das concentrações de CO_2 (hipercapnia) parece ser mediado pela ativação de neurônios excitatórios e bulbo-espinais da região do RVL. Nós também mostramos que a ativação desses neurônios não depende de neurônios localizados na região do NTScom e na região do CVL. Finalmente, nós mostramos que durante uma situação de hipercapnia ocorre a ativação de neurônios inibitórios na região do CVL, reduzindo o total de aumento de atividade simpática produzido pelo CO_2. Após a inibição da região do CVL com muscimol, o aumento das concentrações de CO_2 produziram uma aumento ainda maior na atividade simpática eferente. Uma descoberta essencial, importante e inédita desse trabalho é que os efeitos excitatórios de uma situação de hipercapnia parece depender de uma ativação direta do CO_2 nos neurônios do RVL, mostrando uma propriedade quimiossensível intrínseca desses neurônios. Um outro aspecto importante é que a modulação respiratória na atividade simpática parece ser atribuída a dois processos: ativação dos neurônios GABAérgicos localizados no CVL e uma desinibição dos neurônios do NRT. Parece que a modulação respiratória na atividade simpática constitui uma redução do total de aumento de atividade simpática causada por uma situação de hipercapnia.

Em situações de anestesia, o aumento da concentração de CO_2 promove um aumento da atividade simpática em animais vagotomizados e com desnervação sino-aórtica (Preiss e Polosa, 1977; Hanna e cols., 1981; Millhorn e Eldridge, 1986). Em acordo com os dados da literatura, o aumento de atividade simpática produzido pela hipercapnia (variação de CO_2 de 5% para 10%) é praticamente o triplo de uma situação controle (Moreira e cols., 2006). Alguns

neurônios do RVL apresentam um pico de aumento de atividade simpática durante a fase de inspiração (mesmo pico observado no nervo esplâncnico), enquanto que outros neurônios apresentam um pico de aumento de atividade na fase de pós-inspiração (coincidente com o pico de atividade observado no nervo lombar). Esse tipo de modulação respiratória observada na atividade simpática durante a ativação dos quimiorreceptores centrais confirmam os dados da literatura (Numao e cols., 1987; Darnall e Guyenet, 1990; Guyenet e cols., 1990; Pilowsky e cols., 1996).

Em nossos experimentos, a hipercapnia produziu uma hipotensão, que seria resultado de um efeito direto do CO_2 nos vasos sanguíneos e também, possivelmente, na contratilidade cardíaca. Após a hipotensão, a pressão arterial começa a voltar aos valores normais e, no fim do episódio da hipercapnia, a pressão arterial apresenta uma tendência de aumento. Estamos assumindo que esse aumento seria resultante de uma aumento na atividade simpática, quando o efeito local do CO_2 nos vasos sanguíneos já tenha sido removido.

A estimulação dos quimiorreceptores centrais, em animais com os quimiorreceptores e barorreceptores desnervados, promoveu um aumento de aproximadamente 70% na atividade dos neurônios bulbo-espinais do RVL. Até então, apenas um trabalho da literatura tinha descrito que os neurônios do RVL apresentavam um aumento da atividade durante a ativação dos quimiorreceptores centrais (Haselton e Guyenet, 1989). Esse trabalho mostrou que, em situações de hipercapnia, os neurônios do RVL apresentam uma aumento de atividade de aproximadamente 40% (Haselton e Guyenet, 1989). A diferença desse trabalho com o presente deve-se ao fato do tempo de exposição dos neurônios do RVL a situações de hipercapnia (aproximadamente 2 min no trabalho de Haselton e Guyenet e 5 minutos no presente estudo). É preciso um certo tempo para que ocorra o equilíbrio do pH e do fluxo sanguíneo no SNC (Sato e cols., 1992). Uma outra explicação poderia ser também relacionada a seleção dos neurônios da região do RVL. Nesse estudo, nós selecionamos os neurônios com rápida condução, na qual o teste de colisão foi possível ser realizado. Colisões espontâneas podem ocorrer entre os neurônios que apresentam condução lenta

(projeção antidrômica) com os potênciais de ação gerados pela estimulação da medula espinal (projeção ortodrômica), dificultando a identificação da projeção desses neurônios para a medula espinal. Sete dos onze neurônios registrados apresentavam imunorreatividade para a enzima PNMT, o que é consistente com as propriedades de neurônios do RVL barosenssíveis e com condução rápida (Schreihofer & Guyenet, 1997). Nesse trabalho, não podemos ter absoluta certeza de que os neurônios que nós registramos são regulados pelos barorreceptores, pois os animais estavam completamente desnervados, condição essa, ideal para avaliar os efeitos da ativação dos quimiorreceptores centrais (Toney, 2006). Entretanto, trabalhos da literatura mostram que todos os neurônios bulbo-espinais localizados na região C1 (neurônios adrenérgicos do RVL) são inibidos pelo aumento de pressão arterial (Schreihofer e Guyenet, 1997).

O aumento da atividade simpática produzido pela ativação dos quimiorreceptores centrais corresponde a aproximadamente 100% do valor controle. Não podemos concluir que esse aumento seja apenas mediado pelos neurônios da região do RVL, os quais apresentam um aumento de 70% em sua atividade após a ativação dos quimiorreceptores centrais. Portanto, não excluímos que outras regiões do SNC (região A5) podem contribuir no aumento da atividade simpática produzido pela ativação dos quimiorreceptores centrais.

Quimiorreceptores centrais constituem neurônios que diretamente ou indiretamente detectam mudanças de pH no líquido extracelular e promovem mudaças no ritmo respiratório e cardiovascular. Esses neurônios foram descritos em várias regiões do SNC, incluindo o NTS, o núcleos da rafe, e várias regiões do bulbo ventrolateral, como por exemplo a região do CVL e o NRT (Dean e cols., 1990; Mulkey e col., 2004; Guyenet e cols., 2005; Takakura e col., 2006; Nattie e Li, 2006).

A injeção de muscimol no NTScom não bloqueou a resposta de aumento de atividade simpática produzida pela hipercapnia, sugerindo que a região comissural do NTS não participa das respostas de aumento da atividade simpática durante a ativação dos quimiorreceptores centrais. Por outro lado, a injeção de muscimol na

mesma região foi capaz de eliminar a ativação dos quimiorreceptores periféricos. Resultados da literatura mostram que a acidificação dos neurônios da região do NTScom foi capaz de aumentar a respiração em ratos acordados (Nattie e Li, 2002). A acidificação de uma região via microdiálise não mimetiza exatamente uma situação de hipercapnia sistêmica. Um outro fator que deve-se considerar é que a anestesia pode também influenciar e eliminar os efeitos no NTS. Por fim, os neurônios quimiossensíveis do NTS localizam-se em uma região diferente dos neurônios de segunda ordem envolvidos na via do quimiorreflexo periférico. A região do NTS que sofreu acidificação (Nattie e Li, 2002) foi localizada um pouco mais rostral e lateral a região que fizemos as injeções de muscimol. Portanto, permanece em aberto a questão sobre a participação dos neurônios do NTS nas respostas de ativação do quimiorreflexo central.

A região do CVL também contém neurônios descritos como quimiorreceptores centrais (Solomon e cols., 2000; Nattie e Li, 2006). Esses neurônios parecem não estar envolvidos na resposta excitatória produzida por uma situação de hipercapnia, pois a injeção de muscimol ou ácido quinurênico não reduziu os efeitos do CO_2 na atividade simpática. Por outro lado, a inibição da região do CVL promoveu um aumento ainda maior da atividade simpática produzida pela hipercapnia, sugerindo que essa região contém vias inibitórias para a região do RVL que são ativadas numa situação de hipercapnia. A região do CVL contém neurônios GABAérgicos que possuem modulação respiratória e que essa modulação apresenta-se oposto a modulação observada nos neurônios do RVL (Mandel e Schreihofer, 2006). Essas caracteristícas são consistentes com a possibilidade de que esses neurônios contribuem na modulação respiratória dos neurônios do RVL. A inibição da região do CVL com muscimol produziu um aumento na atividade dos quimiorreceptores centrais. A inibição da região do CVL produz apenas a eliminação da oscilação respiratória da atividade simpática, mas não altera a resposta de aumento de atividade simpática produzido pela ativação dos quimiorreceptores periféricos (Kohiya e Guyenet, 1996b; presente resulatdos).

Um aspecto importante a ser discutido é o fato de que os neurônios do NRT também possuem uma heterogenocidade, como os neurônios do CVL, no que diz

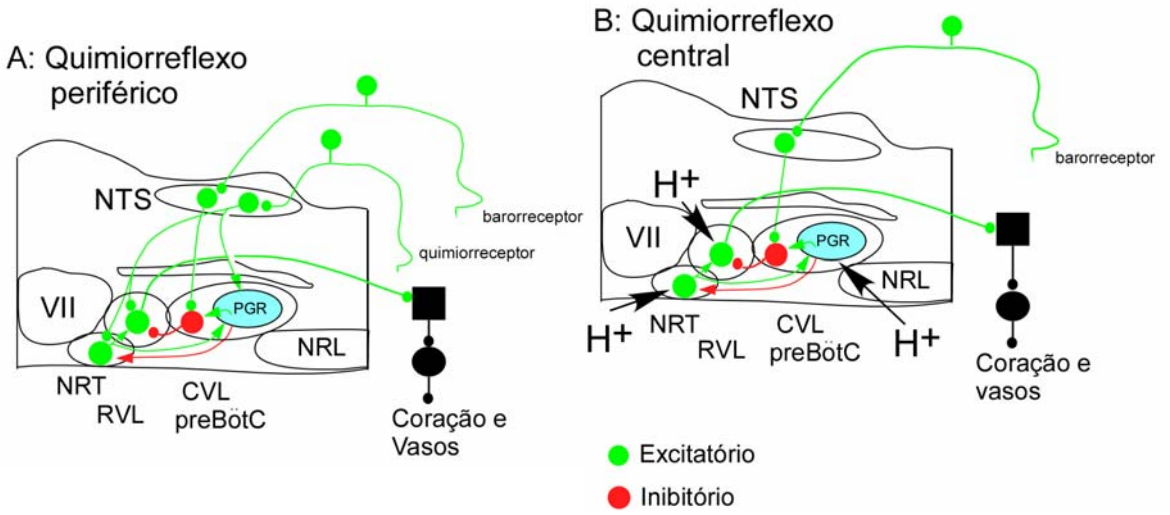
respeito às modulações respiratórias. A única diferença é que os neurônios do NRT apresentam as mesmas modulações respiratórias observadas nos neurônios do RVL (Figura 2). Parece que os neurônios do NRT mantêm a atividade respiratória dos neurônios do RVL (Figura 2). O padrão respiratório predominante dos neurônios do NRT consiste num aumento de atividade na fase de inspiração (Mulkey e cols., 2004; Guyente e cols., 2005a; Takakura e cols., 2006). Os neurônios do NRT recebem projeções inibitórias periódicas da região do CVL, o que deveria atenuar as respostas do quimiorreflexo por um processo de desfacilitação. Resultados anteriores mostraram que a modulação respiratória dos neurônios do NRT desapareceu após a injeção de muscimol na região do CVL, mas as respostas excitatórias da estimulação dos quimiorreflexos central e periférico persistiram (Takakura e cols., 2006). Portanto, a injeção de muscimol na região do CVL produz uma eliminação da modulação respiratória dos neurônios do NRT e esse efeito contribui no aumento da atividade simpática após a estimulação dos quimiorreceptores centrais.

Os mecanismos excitatórios da ativação dos quimiorreceptores periféricos e centrais são distintos em um aspecto essencial. O bloqueio dos receptores glutamatérgicos ionotrópicos, com ácido quinurênico, na região do RVL, promoveu a eliminação de aumento da atividade simpática promovido pela ativação dos quimiorreceptores periféricos, mas não alterou o aumento de atividade simpática promovido pela ativação dos quimiorreceptores centrais. O bloqueio da atividade simpática e respiratória após a estimulação dos quimiorreceptores periféricos em animais com injeção bilateral de ácido quinurênico no RVL é consistente com dados da literatura, mostrando que a via dos quimiorreceptores periféricos é transmitida para o bulbo ventrolateral (RVL e NRT) sendo mediado por projeções glutamatérgicas (Sun e Reis, 1995; Aicher e cols., 1996; Guyenet, 2000; Takakura e cols., 2006). Por outro lado, o não efeito da injeção bilateral de ácido quinurênico na região do RVL sobre as resposta de estimulação do quimiorreflexo central sugere que a ativação desses receptores não envolve receptores glutamatérgicos ionotrópicos. Isso sugere que o aumento da concentração de CO_2 (ativação dos quimiorreceptores centrais) possa ter um efeito quimiossensível intrínseco nos

neurônios do RVL ou que esse efeito não seja mediado sinapticamente por receptores glutamatérgicos ionotrópicos, mas sim por receptores glutamatérgicos metabotrópicos ou até mesmo por outros tipos de receptores. Receptores glutamatérgicos metabotrópicos estão presentes na região do RVL. Dados da literatura mostram que injeções de agonistas glutamatérgicos metabotrópicos no RVL promovem um aumento de pressão arterial (Tsuchihashi e cols., 2000). A literatura contém também exemplos de neurônios que expressam a enzima que produz glutamato, mas liberam outro tipo de neurotransmissor com importante papel na transmissão sináptica, como por exemplo a orexina (Rosin e cols., 2003).

Conclusão

Em resmo, o esquema proposto abaixo tentar explicar e exemplificar a maioria dos mecanismos discutidos nessa segunda parte dessa tese de doutorado, na qual o maior enfoque são as possíveis integrações entre os quimiorreceptores centrais e periféricos, bem como sua relação na manutenção das vias cardiovasculares e respiratórias. Entretanto, novos experimentos são necessários para mostrar mais detalhes e estabelecer o verdadeiro papel das vias excitatórias, bem como da atividade dos quimiorreceptores centrais, localizadas no bulbo, no controle das variáveis cardio-respiratórias. Essa tese de doutorado procurou enfatizar também a aplicação de diferentes técnicas experimentais (registro de pressão arterial, frequência cardíaca, fluxo sanguíneo, atividade de nervos periféricos e atividade de neurônios, imuno-histoquímica e hibridização in-situ) em estudos de neurofisiologia. Um importante aspecto foi procurar relacionar aspectos cardiovasculares e respiratórios, uma vez que essas duas variáveis estão intimamente relacionadas.



A) Quimiorreflexo periférico. O aumento da atividade simpatica é mediado pela ativação dos neurônios simpato-excitatórios da região do RVL. As aferências excitatórias para os neurônios do RVL originam-se de projeções glutamatérgicos diretas do NTScom e também de uma via indireta via NRT (local dos neurônios quimiossensíveis). A modulação respiratória da atividade simpática e dos neurônios do RVL parece ser atribuída à 2 mecanismos. O primeiro mecanismo seria a ativação do padrão gerador do ritmo respiratório (região em azul), que contém os neurônios GABAérgicos da região do CVL e pré-Botzinger (representação do neurônio em vermelho), enquanto que um segundo seria via inibições periódicas da região do NRT (flecha em vermelho). A combinação desses dois mecanismos diminuiria a ativação dos neurôniops do RVL durante a estimulação dos quimiorreceptores periféricos e promoveria a modulação respiratória na atividade simpática. B) Quimiorreflexo central. O aumento da atividade simpática, causado pelo aumento das concentrações de CO_2 parece ser devido a uma direta ativação dos neurônios simpato-excitatórios da região do RVL. Nossa teoria seria que os neurônios do RVL seriam ativados por basicamente dois mecanismos. O primeiro mecanismo seria a ativação de uma sinapse indireta via neurônios quimiossensíveis do NRT, enquanto que o segundo mecanismo seria um próprio aumento da atividade dos neurônios do RVL. Abreviações: PGR, padrão gerador da respiração; CVL, região caudoventrolateral do bulbo; NRL, núcleo reticular lateral; NRT, núcleo retrotrapezóide; NTS, núcleo

do trato solitário; RVL, região rostroventrolateral do bulbo; VII, núcleo facial. Referência: modificado de Moreira e cols., 2006.

Referências

1) Alexander RS. Tonic and reflex functions of medullary sympathetic cardiovascular centers. ***J. Neurophysiol****.* 9: 205-207, 1946.
2) Aicher SA, Saravay RH, Cravo S, Jeske I, Morrison SF, Reis DJ and Milner TA. Monosynaptic projections from the nucleus tractus solitarii to C1 adrenergic neurons in the rostral ventrolateral medulla: Comparison with input from the caudal ventrolateral medulla. ***J. Comp. Neurol****.* 373, 62-75, 1996.
3) Aicher SA, Drake CT. Clonidine evokes vasodepressor responses via alpha2-adrenergic receptors in gigantocellular reticular formation. ***J. Pharmacol. Exp. Ther***. 289(2): 688-94, 1999.
4) Akilesh MR, Kamper M, Li A and Nattie EE. Effects of unilateral lesions of retrotrapezoid nucleus on breathing in awake rats. ***J. Appl. Physiol****.* 82, 469-479, 1997.
5) Barman SM, Gebber GL, Orer HS. Medullary lateral tegmental field: an important source of basal sympathetic nerve discharge in the cat. ***Am. J. Physiol. (Regul. Integr. Comp. Physiol.)*** 278(4): R995-R1004, 2000.
6) Bayliss WM. On the origin from the spinal cord of the vasodilators fibers of the hindlimb, and on the nature of these fibers. ***J. Physiol***. 26:173, 1901.
7) Bin-Jaliah I, Maskell PD and Kumar P. Indirect sensing of insulin-induced hypoglycaemia by the carotid body in the rat. ***J. Physiol****.* 556: 255-266, 2004.
8) Blessing WW. Depressor neurons in rabbit caudal medulla act via GABA receptors in rostral medulla. ***Am. J. Physiol. (Heart Cir. Physiol****.)* 254: H686-H692, 1988.
9) Blessing, WW. Arterial pressure and blood flow to the tissues in *The lower brainstem and bodily homeostasis* 165-268 (Oxford University Press, New York, 1997).
10) Blessing, WW, Yu YH and Nalivaiko E. Medullary projections of rabbit carotid sinus nerve. ***Brain Res****.* 816, 405-410, 1999.

11) Bodineau L, Frugière A, Marlot D and Wallois F. Connections between retrotrapezoid nucleus and nucleus tractus solitarii in cat. ***Neurosci. Lett***. 280, 111-114, 2000a.

12) Bodineau L, Frugiere A, Marlot D and Wallois F. Effect of hypoxia on the activity of respiratory and non-respiratory modulated retrotrapezoid neurons of the cat. ***Auton. Neurosci***. 86, 70-77, 2000b.

13) Braga VA, Machado BH. Chemoreflex sympathoexcitation was not altered by the antagonism of glutamate receptors in the commissural nucleus tractus solitarii in the working heart-brainstem preparation of rats. ***Exp. Physiol.*** 91(3): 551-559, 2006.

14) Brooks VL, Freeman KL and Clow KA. Excitatory amino acids in rostral ventrolateral medulla support blood pressure during water deprivation in rats. ***Am. J. Physiol. (Heart Circ. Physiol.)*** 286, H1642-H1648, 2004.

15) Brown DL and Guyenet PG. Electrophysiological study of cardiovascular neurons in the rostral ventrolateral medulla in rats. ***Circ. Res.*** 56, 359-369, 1985.

16) Campos RR, Possas OS, Cravo SL, Lopes OU, Guertzenstein PG. Putative pathways involved in cardiovascular responses evoked from caudal pressor area. **Braz. J. Med. Biol. Res.**, 27, 2467-2479, 1994.

17) Campos RR, McAllen RM. Tonic drive to sympathetic premotor neurons of rostral ventrolateral medulla from caudal pressor area neurons. ***Am. J. Physiol. (Regul. Integr. Comp. Physiol.)*** 276(4), R1209-R1213, 1999.

18) Cao WH and Morrison SF. Differential chemoreceptor reflex responses of adrenal preganglionic neurons. ***Am. J. Physiol. (Regul. Integr. Comp. Physiol.)*** 281, R1825-R1832, 2001.

19) Chalmers J and Pilowsky P. Brainstem and bulbospinal neurotransmitter system in the control of blood pressure. ***J. Hypertension***, 9: 675-694, 1991.

20) Chan RKW and Sawchenko PE. Organization and transmitter specificity of medullary neurons activated by sustained hypertension: Implications for understanding baroreceptor reflex circuitry. ***J. Neurosci***. 18: 371-387, 1998.

21) Chitravanshi VC, Kachroo A and Sapru HN. A midline area in the nucleus commissuralis of NTS mediates the phrenic nerve responses to carotid chemoreceptor stimulation. ***Brain Res****.* 662 (1-2): 127-33, 1994.

22) Chitravanshi VC and Sapru HN. Chemoreceptor-sensitive neurons in commissural subnucleus of nucleus tractus solitarius of the rat. ***Am. J. Physiol. (Reg. Integr. Comp. Physiol)*** 268: R851-R858, 1995.

23) Ciriello J, Hochstenbach S and Roder S. Central projections of baroreceptor and chemoreceptor afferent fibers in the rat. In: *Nucleus of the Solitary Tract*, edited by Barraco IRA. Boca Raton, FL: CRC, p. 35-50, 1994.

24) Colombari E, Bonagamba LG & Machado BH (1994). Mechanisms of pressor and bradycardic responses to L-glutamate microinjected into the NTS of conscious rats. ***Am. J. Physiol. (Reg. Integr. Comp. Physiol)*** 266: R730-R738.

25) Colombari E, Menani JV and Talman W. Commissural NTS contribute to pressor responses to glutamate injected into the medial NTS of awake rats. **Am. J. Physiol. (Reg. Integr. Comp. Physiol.)** 270: R1220-R1225, 1996.

26) Colombari E, Sato MA, Cravo SL, Bergamaschi CT, Campos RR Jr. and Lopes OU. Role of the medulla oblongata in hypertension. **Hypertension** 38 (3): 549-54, 2001.

27) Cottle MA. Degeneration studies of the primary afferents of the IXth cranial nerves in cat. ***J. Comp. Neurol.*** 122: 329-345, 1964.

28) Cowley,A.W., Jr. Long-term control of arterial blood pressure. ***Physiol. Rev.*** 72, 231-300, 1992.

29) Cravo SL, Morrison SF and Reis D.J. Differentiation of two cardiovascular regions within caudal ventrolateral medulla. ***Am. J. Physiol. (Reg. Integr. Comp. Physiol.)*** 261: R985-R994, 1991.

30) Cream C, Li A and Nattie E. The retrotrapezoid nucleus (RTN): local cytoarchitecture and afferent connections. ***Respir. Physiol. Neurobiol.*** 130: 121-137, 2002.

31) Dampney RA. Functional organization of central pathways regulating the cardiovascular system. ***Physiol. Rev.*** 74(2): 323-64, 1994.

32) Dampney,R.A. *et al.* Medullary and supramedullary mechanisms regulating sympathetic vasomotor tone. ***Acta Physiol Scand.*** 177, 209-218, 2002.

33) Darnall RA and Guyenet P. Respiratory modulation of pre- and postganglionic lumbar vasomotor sympathetic neurons in the rat. ***Neurosci. Lett.*** 119, 148-152, 1990.

34) Dean JB, Lawing WL, & Millhorn DE. CO2 decreases membrane conductance and depolarizes neurons in the nucleus tractus solitarii. ***Exp. Brain Res.*** 76, 656-661, 1989.

35) Ellenberger HH and Feldman JL. Brainstem connections of the rostral ventral respiratory group of the rat. ***Brain Res.*** 513: 35-42, 1990.

36) Erickson JT and Millhorn DE. Fos-like protein is induced in neurons of the medulla oblongata after stimulation of the carotid sinus nerve in awake and anesthetized rats. ***Brain Res.*** 567: 11-24, 1991.

37) Feldberg W and Guertzenstein PG. A vasodepressor effect of pentobarbitone sodium. ***J. Physiol.*** 224: 83-103, 1972.

38) Feldman JL, Mitchell GS and Nattie EE. Breathing: Rhythmicity, Plasticity, Chemosensitivity. ***Ann. Rev. Neurosci.*** 26: 239-266, 2003.

39) Feldman J. L. and Del Negro C. A. Looking for inspiration: new perspectives on respiratory rhythm. ***Nat. Rev. Neurosci.*** 7, 232-242, 2006.

40) Finley JCW and Katz DM. The central organization of carotid afferent projections to the brainstem of the rat. ***Brain Res.*** 572: 108-116, 1992.

41) Forster HV, Lowry TF, Ohtake PJ, Pan LG, Korducki MJ and Forster AL. Differential effect of ventrolateral medullary cooling on respiratory muscles of goats. ***J. Appl. Physiol.*** 78, 1859-1867, 1995.

42) Fussey IF, Kidd C and Whitwam JG. Single unit activity associated with cardiovascular events in the brain stem of the dog. ***J. Physiol.*** 191: 57-58, 1967.

43) Gebber GL. Central determinants of sympathetic nerve discharge. In *Central Regulation of Autonomic Functions*. A.D. Loewy & K.M. Spyer, Eds.:. Oxford University Press. New York, p. 126-144.

44) Gordon FJ and Sved AF. Neurotransmitters in central cardiovascular regulation: glutamate and GABA. ***Clin. Exp. Pharmacol. Physiol.*** 29(5-6):522-4, 2002.

45) Guertzenstein PG. Blood pressure effects obtained by drugs applied to the ventral surface of the brain stem. ***J. Physiol.*** 229(2): 395-408, 1973.

46) Guertzenstein PG and Silver A. Fall in blood pressure produced from discrete regions of the ventral surface of the medulla by glycine and lesions. ***J. Physiol.*** 242: 489-503, 1974.

47) Guertzenstein PG, Hilton SM, Marshall JM and Timms RJ. Experiments on the origin of vasomotor tone. ***J. Physiol.*** 275: 78-91, 1977.

48) Guertzenstein PG and Lopes OU. Cardiovascular responses evoked from the nicotine sensitive area on the ventral surface of the medulla oblongata in the cat. ***J. Physiol.*** 347: 345-360, 1984.

49) Guyenet PG, Filtz TM and Donaldson SR. Role of excitatory amino acids in rat vagal and sympathetic baroreflexes. ***Brain Res.*** 407(2): 272-84, 1987.

50) Guyenet, P.G. Role of the ventral medulla oblongata in blood pressure regulation. In *Central Regulation of Autonomic Functions*. A.D. Loewy & K.M. Spyer, Eds.:. Oxford University Press. New York, p. 145-167, 1990.

51) Guyenet PG, Darnall RA, & Riley TA. Rostral ventrolateral medulla and sympathorespiratory integration in rats. ***Am. J. Physiol. (Regul. Integr. Comp. Physiol.)*** **259**, R1063-R1074, 1990.

52) Guyenet PG. Neural structures that mediate sympathoexcitation during hypoxia. ***Respir. Physiol.*** 121: 147-162, 2000.

53) Guyenet, P.G. and Stornetta, R.L. The presympathetic cells of the rostral ventrolateral medulla (RVLM): anatomy, physiology and role in the control of circulation in *Neural mechanisms of cardiovascular regulation* (eds. Dun,N.J., Machado,B.H. & Pilowsky,P.M.) 187-218 (Kluwer Academic Publishers, Boston, 2004).

54) Guyenet P. G., Mulkey D. K., Stornetta R. L., and Bayliss D. A. Regulation of ventral surface chemoreceptors by the central respiratory pattern generator. ***J. Neurosci.*** 25, 8938-8947, 2005a.

55) Guyenet PG, Stornetta RL, Bayliss DA and Mulkey DK. Retrotrapezoid nucleus: a litmus test for the identification of central chemoreceptors. ***Exp. Physiol.*** 90: 247-253, 2005b.

56) Guyenet PG. The sympathetic control of blood pressure. ***Nat. Rev. Neurosci.*** 7(5), 335-346, 2006.

57) Haibara AS, Colombari E, Chianca Jr, DA, Bonagamba LGH and Machado BH. NMDA receptors in the NTS are involved in bradycardic but not in pressor response of chemoreflex. ***Am. J. Physiol. (Heart. Circ. Physiol.)*** 269: H1421-H1427, 1995.

58) Hanna BD, Lioy F, and Polosa C. The effect of cold blockade of the medullary chemoreceptors on the CO2 modulation of vascular tone and heart rate. ***Can. J. Physiol. Pharmacol.*** 57, 461-468, 1979.

59) Hanna BD, Lioy F, and Polosa C. Role of carotid and central chemoreceptors in the CO2 response of sympathetic preganglionic neurons. ***J. Auton. Nerv. Syst.*** 3, 95-105, 1988.

60) Haselton, J.R. and Guyenet, P.G. Central respiratory modulation of medullary sympathoexcitatory neurons in rat. ***Am. J. Physiol. (Reg. Integr. Comp. Physiol.)*** 256, R739-R750, 1989.

61) Heesch CM, Laiprasert JD, Kvochina L. RVLM glycine receptors mediate GABA(A) and GABA(B) independent sympathoinhibition from CVLM in rats. ***Brain Res.*** 1125(1): 46-59, 2006.

62) Hodges MR, Martino P, Davis S, Opansky C, Pan LG and Forster HV. Effects on breathing of focal acidosis at multiple medullary raphe sites in awake goats. ***J. Appl. Physiol.*** 97, 2303-2309, 2004.

63) Hokfelt,T., Fuxe,K., Goldstein,M., & Johansson,O. Immunohistochemical evidence for the existence of adrenaline neurons in the rat brain. ***Brain Res.*** 66, 235-251, 1974.

64) Horiuchi J and Dampney, R.A. Evidence for tonic disinhibition of RVLM sympathoexcitatory neurons from the caudal pressor area. ***Auton. Neurosci.*** 99(2), 102-110, 2002.

65) Horiuchi J, Killinger S and Dampney RA. Contribution to sympathetic vasomotor tone of tonic glutamatergic inputs to neurons in the RVLM. ***Am J Physiol (Reg. Integr. Comp. Physiol.)*** 287(6): R1335-R1343, 2004.

66) Ito S and Sved AF. Tonic glutamate-mediated control of rostral ventrolateral medulla and sympathetic vasomotor tone. ***Am. J. Physiol. (Reg. Integr. Comp. Physiol.)*** 273 (2): R487-R494, 1997.

67) Ito S, Komatsu K, Tsukamoto K and Sved AF. Excitatory amino acids in the rostral ventrolateral medulla support blood pressure in spontaneously hypertensive rats. **Hypertension** 35: 413-417, 2000.

68) Ito S, Komatsu K, Tsukamoto K and Sved AF. Tonic excitatory input to the rostral ventrolateral medulla in Dahl salt-sensitive rats. **Hypertension** 37: 687-691, 2001.

69) Janczewski WA and Feldman JL. Distinct rhythm generators for inspiration and expiration in the juvenile rat. ***J. Physiol.*** 570: 407-420, 2005.

70) Jordan D and Spyer KM. Studies on the termination of sinus nerve afferents. ***Pflugers Archiv.*** 369: 65-73, 1977.

71) Jordan D and Spyer KM. The excitability of sinus nerve afferent terminals during the respiratory cycle. ***J. Physiol.*** 277: 66, 1978.

72) Kawai A, Ballantyne D, Muckenhoff K and Scheid P. Chemosensitive medullary neurones in the brainstem--spinal cord preparation of the neonatal rat. ***J. Physiol.*** 492: 277-292, 1996.

73) Kiley JP, Eldridge FL and Millhorn DE. The roles of medullary extracellular and cerebrospinal fluid pH in control of respiration. ***Respir. Physiol.*** 59: 117-130, 1985.

74) Koshiya N, Huangfu D and Guyenet PG. Ventrolateral medulla and sympathetic chemoreflex in the rat. ***Brain Res.*** 609 (1-2):174-184, 1993.

75) Koshiya N and Guyenet PG (1994). Role of the pons in the carotid sympathetic chemoreflex. ***Am. J. Physiol. (Reg. Integr. Comp. Physiol.)*** 267 (2): R508-518.

76)Koshiya N and Guyenet PG. NTS neurons with carotid chemoreceptor inputs arborize in the rostral ventrolateral medulla. ***Am. J. Physiol. (Reg. Integr. Comp. Physiol.)*** 270: R1273-R1278, 1996a.

77)Koshiya N and Guyenet PG. Tonic sympathetic chemoreflex after blockade of respiratory rhythmogenesis in the rat. ***J. Physiol.*** 491, 859-869, 1996b.

78)Leone C and Gordon FJ. Is L-glutamate a neurotransmitter of baroreceptor information in the nucleus of the tractus solitarius? ***J. Pharmacol. Exp. Ther.*** 250 (3): 953-962, 1989.

79)Li A and Nattie E. CO2 dialysis in one chemoreceptor site, the RTN: stimulus intensity and sensitivity in the awake rat. ***Respir. Physiol. Neurobiol.*** 133: 11-22, 2002.

80)Loeschcke HH. Central chemosensitivity and the reaction theory. ***J. Physiol.*** 332: 1-24, 1982.

81)Loewy,A.D. & Spyer,K.M. Central regulation of autonomic functions (Oxford University Press, New York, 1990.

82)Machado BH and Bonagamba LG. Microinjection of L-glutamate into the nucleus tractus solitarii increases arterial pressure in conscious rats. ***Brain Res.*** 576: 131-138, 1992.

83)Madden CJ and Morrison SF. Hypoxic activation of arterial chemoreceptors inhibits sympathetic outflow to brown adipose tissue in rats. ***J. Physiol.*** 566, 559-573, 2005.

84)Mandel DA and Schreihofer AM. Central respiratory modulation of barosensitive neurones in rat caudal ventrolateral medulla. ***J. Physiol.*** 572, 881-896, 2006.

85)McAllen RM. Central respiratory modulation of subretrofacial bulbospinal neurons in the cat. ***J. Physiol.*** 388**,** 533-545, 1987.

86)Millhorn D. E. Neural respiratory and circulatory interaction during chemoreceptor stimulation and cooling of ventral medulla in cats. ***J. Physiol.*** 370, 217-231, 1986.

87) Millhorn DE and Eldridge FL. Role of ventrolateral medulla in regulation of respiratory and cardiovascular systems. ***J. Appl. Physiol.*** 61, 1249-1263, 1986.

88) Mitchell RA, Loeschcke HH, Massion WH and Severinghaus JW. Respiratory responses mediated through superficial chemosensitive areas on the medulla. ***J. Appl. Physiol***. 18: 523-533, 1963a.

89) Mitchell RA, Loeschcke HH, Severinghaus JW, Richardson JW and Massion WH. Regions of respiratory chemosensitivity on the surface of the medulla. ***Ann. N.Y. Acad. Sci.*** 109: 661-681, 1963.

90) Miyawaki T, Pilowsky P, Sun QJ, Minson J, Suzuki S, Arnolda L, Llewellyn-Smith I, and Chalmers J. Central inspiration increases barosensitivity of neurons in rat rostral ventrolateral medulla. ***Am. J. Physiol. (Regul. Integr. Comp. Physiol.)*** 268(2): R909-18, 1995.

91) Moreira TS, Sato MA, Takakura AC, Menani JV & Colombari E. Role od pressor mechanisms from the NTS and CVLM in contro of arterial pressure. ***Am. J. Physiol. (Reg. Integr. Comp. Physiol.)*** 289: R1416-R1425, 2005.

92) Moreira T. S., Takakura A. C., Colombari E., and Guyenet P. G. Central chemoreceptors and sympathetic vasomotor outflow. ***J. Physiol.*** 577, 369-386, 2006.

93) Moreira TS, Takakura AC, Menani JV, Sato MA and Colombari E. Glutamatergic mechanisms in the commissural nucleus of the solitary tract are important for pressor response produced by CVLM inhibition. ***Brain Res.***, 2007, submetido.

94) Mulkey D. K., Stornetta R. L., Weston M. C., Simmons J. R., Parker A., Bayliss D. A., and Guyenet P. G. Respiratory control by ventral surface chemoreceptor neurons in rats. ***Nat. Neurosci.*** 7, 1360-1369, 2004.

95) Natarajan, M & Morrison SF. Tonic sympathoinhibition from the caudal ventrolateral medulla. ***Soc. Neurosci. Abstr***. 24: 371, 1998.

96) Natarajan M and Morrison SF. Sympathoexcitatory CVLM neurons mediate responses to caudal pressor area stimulation. *Am****. J. Physiol. (Reg. Integr. Comp. Physiol.)*** 279: R364-R374, 2000.

97) Nattie EE, Li A and St. John WM (1991). Lesion in retrotrapezoid nucleus decrease ventilatory output in anesthetized or decerabrate cats. ***J. Appl. Physiol.*** 71(4), 1364-75.

98) Nattie EE and Li A. Central chemoreception in the region of the ventral respiratory group in the rat. ***J. Appl. Physiol.*** 81, 1987-1995, 1996.

99) Nattie EE. Central chemosensitivity, sleep, and wakefulness. ***Respir. Physiol.*** 129: 257-268, 2001.

100) Nattie EE and Li AH. CO_2 dialysis in nucleus tractus solitarius region of rat increases ventilation in sleep and wakefulness. ***J. Appl. Physiol.*** 92, 2119-2130, 2002.

101) Nattie E. Why do we have both peripheral and central chemoreceptors? ***J. Appl. Physiol.*** 100: 9-10, 2006.

102) Nattie E and Li AH. Central chemoreception: a brief review. ***Auton. Neurosci.*** 126-127: 332-338, 2006.

103) Numao Y, Koshiya N, Gilbey MP and Spyer KM. Central respiratory drive-related activity in sympathetic nerves of the rat: the regional differences. ***Neurosci. Lett.*** 81, 279-284, 1987.

104) Oikawa S, Hirakawa H, Kusakabe T, Nakashima Y and Hayashida, Y. Autonomic cardiovascular responses to hypercapnia in conscious rats: the roles of the chemo- and baroreceptors. ***Auton. Neurosci.*** 117(2): 105-14, 2005.

105) Osborn JW. Hypothesis: set-points and long-term control of arterial pressure. A theoretical argument for a long-term arterial pressure control system in the brain rather than the kidney. ***Clin. Exp. Pharmacol. Physiol.*** 32, 384-393, 2005.

106) Ohta H and Talman WT. Both NMDA and non-NMDA receptors in the NTS participate in the baroreceptor reflex in rats. ***Am. J. Physiol. (Regul. Integr. Comp. Physiol.)*** 267: R1065-R1070, 1994.

107) Palkovits M and Zaborszky L. Neuroanatomy of central cardiovascular control. Nucleus tractus solitarii: afferent and efferent neuronal connections in relation to the baroreceptor reflex arc. ***Prog. Brain Res.*** 47: 9-34, 1977.

108) Paton JFR, Deuchars J, Li YW and Kasparov S. Properties of solitary tract neurones responding to peripheral arterial chemoreceptors. ***Neurosci.*** 105: 231-248, 2001.

109) Perez Fontan JJ and Velloff CR. Neuroanatomic organization of the parasympathetic bronchomotor system in developing sheep. ***Am. J. Physiol. (Regul. Integr. Comp. Physiol.)*** 273, R121-R133, 1997.

110) Pineda J and Aghajanian GK. Carbon dioxide regulates the tonic activity of locus coeruleus neurons by modulating a proton- and polyamine-sensitive inward rectifier potassium current. ***Neurosci***. 77, 723-743, 1997.

111) Pilowsky PM, Jiang C and Lipski J. An intracellular study of respiratory neurons in the rostral ventrolateral medulla of the rat and their relationship to catecholamine-containing neurons. ***J. Comp. Neurol.*** 301, 604-617, 1990.

112) Pilowsky P, Arnolda L, Chalmers J, Llewellyn-Smith I, Minson J, Miyawaki T and Sun QJ. Respiratory inputs to central cardiovascular neurons. ***Ann. N.Y. Acad. Sci.*** 783, 64-70, 1996.

113) Prabhakar NR and Peng YJ. Peripheral chemoreceptors in health and disease. ***J. Appl. Physiol.*** 96: 359-366, 2004.

114) Preiss G and Polosa C. The relation between end-tidal CO2 and discharge patterns of sympathetic preganglionic neurons. ***Brain Res.*** 122, 255-267, 1977.

115) Putnam R. W., Filosa J. A., and Ritucci N. A. Cellular mechanisms involved in CO(2) and acid signaling in chemosensitive neurons. ***Am. J. Physiol. (Cell Physiol.)*** 287, C1493-C1526, 2004.

116) Richerson G. B. Serotonergic neurons as carbon dioxide sensors that maintain ph homeostasis. ***Nat. Rev. Neurosci.*** 5, 449-461, 2004.

117) Richerson G. B., Wang W., Hodges M. R., Dohle C. I., and Diez-Sampedro A. (2005). Homing in on the specific phenotype(s) of central respiratory chemoreceptors. ***Exp. Physiol.*** 90, 259-266, 2005.

118) Richter DW and Spyer KM. Cardiorespiratory control. In *Central Regulation of Autonomic Functions*, eds. Loewy AD & Spyer KM, pp. 189-207. Oxford Univ. Press, New York, 1990.

119) Ritucci N. A., Erlichman J. S., Leiter J. C., and Putnam R.W. Response of membrane potential and intracellular pH to hypercapnia in neurons and astrocytes from rat retrotrapezoid nucleus. ***Am. J. Physiol. (Regul. Integr. Comp. Physiol.)*** 289, R851-R861, 2005.

120) Ritter,S., Bugarith,K., & Dinh,T.T. Immunotoxic destruction of distinct catecholamine subgroups produces selective impairment of glucoregulatory responses and neuronal activation. ***J. Comp. Neurol.*** 432, 197-216, 2001.

121) Rosin DL, Weston MC, Sevigny CP, Stornetta RL and Guyenet PG. Hypothalamic orexin (hypocretin) neurons express vesicular glutamate transporters VGLUT1 or VGLUT2. ***J. Comp. Neurol***. 465, 593-603, 2003.

122) Rosin DL, Chang DA, and Guyenet PG. Afferent and efferent connections of the rat retrotrapezoid nucleus. ***J. Comp. Neurol***. 499: 64-89, 2006.

123) Ross CA, Ruggiero DA and Reis DJ. Projections from the nucleus tractus solitarii to the rostral ventrolateral medulla. ***J. Comp. Neurol.*** 242: 511-534, 1985.

124) Sato A, Trzebski A and Zhou W. Local cerebral blood flow responses in rats to hypercapnia and hypoxia in the rostral ventrolateral medulla and in the cortex. ***J. Auton. Nerv. Syst***. 41, 79-86, 1992.

125) Sato MA, Menani JV, Lopes OU and Colombari, E. Commissural NTS lesions and cardiovascular responses in aortic baroreceptor-denervated rats. ***Hypertension*** 34 (2): 739-743, 1999.

126) Sato MA, Schoorlemmer GH, Menani JV, Lopes OU, Colombari E. Recovery of high blood pressure after chronic lesions of the commissural NTS in SHR. ***Hypertension*** 42(4): 713-718, 2003.

127) Scheid P, guest editors: Putnam RW, Dean JB and Ballantyne D. Special issue: central chemosensitivity. ***Respir. Physiol. Neurobiol.*** 129: 1-278, 2001.

128) Schreihofer,A.M. and Guyenet,P.G. Identification of C1 presympathetic neurons in rat rostral ventrolateral medulla by juxtacellular labeling in vivo. ***J. Comp. Neurol.*** 387, 524-536, 1997.

129) Schreihofer AM and Guyenet PG. Role of presympathetic C1 neurons in the sympatholytic and hypotensive effects of clonidine in rats. ***Am. J. Physiol. (Regul. Integr. Comp. Physiol.)*** 279(5):R1753-62, 2000.

130) Schreihofer, A.M. and Guyenet, P.G. The baroreflex and beyond: Control of sympathetic vasomotor tone by GABAergic neurons in the ventrolateral medulla. ***Clin. Exp. Pharmacol. Physiol****.* 29, 514-521, 2002.

131) Schreihofer,A.M. & Guyenet,P.G. Baroactivated neurons with pulse-modulated activity in the rat caudal ventrolateral medulla express GAD67 mRNA. ***J. Neurophysiol.*** 89, 1265-1277, 2003.

132) Smith JC, Morrison DE, Ellenberger HH, Otto MR and Feldman JL. Brainstem projections to the major respiratory neuron populations in the medulla of the cat. ***J. Comp. Neurol****.* 281: 69-96, 1989.

133) Smith, C. A., Rodman, J. R., Chenuel, B. J., Henderson, K. S., and Dempsey, J. A. Response time and sensitivity of the ventilatory response to CO2 in unanesthetized intact dogs: central vs. peripheral chemoreceptors. ***J. Appl. Physiol.*** 100 13-19, 2006.

134) Solomon IC, Edelman NH and O'Neill MH. CO2/H+ chemoreception in the cat pre-Botzinger complex in vivo. ***J. Appl. Physiol.*** 88, 1996-2007, 2000.

135) Spyer KM. The central nervous organization of reflex circulatory control. In: Central Regulation of Autonomic Functions, edited by Arthur C.Lowey and K.M. Spyer, New York Oxford, 1990.

136) Sun M. K. Central neural organization and control of sympathetic nervous system in mammals. ***Progress in Neurobiology*** 47, 157-233, 1995.

137) Sun M.K. and Reis D. J. NMDA receptor-mediated sympathetic chemoreflex excitation of RVL-spinal vasomotor neurones in rats. ***J. Physiol.*** 482, 53-68, 1995.

138) Sun, M.K. Pharmacology of reticulospinal vasomotor neurons in cardiovascular regulation. ***Pharmacol. Rev.*** 48, 465-494, 1996.

139) Talman WT, Perrone MH and Reis DJ. Evidence for L-glutamate as the neurotransmitter of baroreceptor afferent nerve fibers. ***Science*** 209: 813-815, 1980.

140) Takakura A. C., Moreira T. S., Colombari E., West G. H., Stornetta R. L., and Guyenet P. G. Peripheral chemoreceptor inputs to retrotrapezoid nucleus (RTN) CO2-sensitive neurons in rats. ***J. Physiol.*** 572, 503-523, 2006.

141) Toney GM. Sympathetic activation by the central chemoreceptor 'reflex': new evidence that RVLM vasomotor neurons are involved...but are they enough? ***J. Physiol.*** 577(Pt 1): 3, 2006.

142) Torvik A. Afferent connections to the sensory trigeminal nuclei, the nucleus of the solitary tract and adjacent structures: an experimental study in the rat. ***J.Comp.Neurol.*** 106: 51-139, 1956.

143) Tsuchihashi T, Liu Y, Kagiyama S, Matsumura K, Abe I and Fujishima M. Metabotropic glutamate receptor subtypes involved in cardiovascular regulation in the rostral ventrolateral medulla of rats. ***Brain Res. Bull.*** 52, 279-283, 2000.

144) Urbanski RW and Sapru HN. Evidence for a sympathoexcitatory pathway from the nucleus tractus solitarii to the ventrolateral medullary pressor area. ***J. Auton. Nerv. Syst.*** 23: 161-174, 1988a.

145) Urbanski RW and Sapru HN. Putative neurotransmitters involved in medullary cardiovascular regulation. ***J. Auton. Nerv. Syst.*** 25: 181-193, 1988b.

146) Vardhan A, Kachroo A and Sapru HN. Excitatory amino acid receptors in commissural nucleus of the NTS mediate carotid chemoreceptor responses. ***Am. J. Physiol. (Reg. Integr. Comp. Physiol.)*** 264 (1): R41-R50, 1993.

147) Verberne AJM, Stornetta RL, and Guyenet PG. Properties of C1 and other ventrolateral medullary neurones with hypothalamic projections in the rat. ***J. Physiol.*** 517, 477-494, 1999.

148) Wang SC and Ranson SW. Autonomic responses to electrical stimulation of the lower brain stem. ***J. Comp. Neurol.*** 71: 437-455, 1939.

149) Willette RN, Punnen-Grandy S, Krieger AJ and Sapru HN. Differential regulation of regional vascular resistance by the rostral and caudal ventrolateral medulla in the rat. ***J. Auton. Nerv. Syst.*** 18(2): 143-151, 1987.

150) Zhang W, Mifflin SW. Excitatory amino acid receptors within NTS mediate arterial chemoreceptor reflexes in rats. ***Am. J. Physiol. (Heart and Circ.)*** 265(2): H770-H773, 1993.

Publicações

1) **Moreira TS**, Takakura AC, Renzi A, De Luca Jr. LA, Menani JV. Moxonidine reduces pilocarpine-induced salivation in rats. *Aut. Neurosci. – Basic and Clinical*, 91: 32-36, 2001.
2) **Moreira TS**, Takakura AC, Renzi A, De Luca Jr. LA, Menani JV. Inhibition of pilocarpine-induced salivation in rats by central noradrenaline. *Arc. Oral Biol.*, 47(6): 429-434, 2002.
3) Takakura AC, **Moreira TS**, Renzi A, De Luca Jr. LA, Menani JV. Central α2 adrenergic receptors and cholinergic-induced salivation in rats. *Brain Res. Bull.* 59(5): 383-386, 2003.
4) **Moreira TS**, Takakura AC, Colombari E, Renzi A, De Luca Jr. LA, Menani JV. Central moxonidine on salivary gland blood flow and cardiovascular responses to pilocarpine. *Brain Res.* 987: 155-163, 2003.
5) Takakura AC, **Moreira TS**, Laitano SC, Renzi A, De Luca Jr. LA, Menani JV. Central muscarinic receptors signal pilocarpine-induced salivation. *J. Dental Res.* 82(11): 992-996, 2003.
6) **Moreira TS**, Takakura AC, Menani JV, Sato MA, Colombari E. Central blockade of nitric oxide synthesis reduces moxonidine-induced hypotension. *Br. J. Pharmacol.* 142(4): 765-771, 2004.
7) Takakura AC, **Moreira TS**, De Luca LA Jr, Renzi A, Menani JV, Colombari E. Effects of AV3V lesion on pilocarpine-induced pressor response and salivary gland vasodilation. Brain Res., 1055 (1-2): 111-121, 2005.
8) **Moreira TS**, Sato MA, Takakura AC, Menani JV, Colombari E. Role of pressor mechanisms from the NTS and CVLM in control of arterial pressure. *Am. J. Physiol. (Regul. Integr. Comp. Physiol.)*, 289(5): R1416-R1425, 2005.
9) Takakura AC, **Moreira TS**, Colombari E, West GH, Stornetta RL and Guyenet PG. Peripheral chemoreceptor inputs to retrotrapezoid nucleus (RTN) CO2-sensitive neurons in rats. *J. Physiol.*, 572(2): 503-523, 2006.

10) **Moreira TS**, Takakura AC, Sato MA, Menani JV, Colombari E. Anti-hypertensive effect of central moxonidine: possible role of nitric oxide. *J. Card. Pharmacol.*, 47(6): 780-787, 2006.

11) **Moreira TS**, Takakura AC, Colombari E and Guyenet PG. Central chemoreflex and sympathetic vasomotor outflow. *J. Physiol.*, 577(1): 369-386, 2006.

12) Stornetta RL, **Moreira TS**, Takakura AC, Kang, BJ, Chang DA, West GH, Brunet JF, Mulkey DK, Bayliss DA and Guyenet PG. Selective expression of Phox2b by brainstem neurons involved in chemosensory integration in the adult rat. *J. Neurosci.*, 26(40): 10305-10314, 2006.

13) **Moreira TS**, Takakura AC, Colombari E, West GH and Guyenet PG. Inhibitory input from slowly-adapting lung stretch receptors to retrotrapezoid nucleus (RTN) chemoreceptors. *J. Physiol.*, 580(1): 285-300, 2007.

14) **Moreira TS**, Takakura AC, Menani JV, Colombari E. Involvement of central alpha(1)- and alpha(2)-adrenoceptors on cardiovascular responses to moxonidine. *Eur. J. Pharmacol.* 2007, no prelo.

15) Takakura AC, **Moreira TS**, Menani JV, Campos RR, Colombari E. Commissural nucleus of the solitary tract is important for cardiovascular response to caudal pressor area activation. *Brain Res.* 2007, em revisão.

16) Kang, BJ, Chang DA, Mackay DD, West GH, **Moreira TS**, Takakura AC, Gwilt JM, Guyenet PG and Stornetta RL. "CNS distribution of the transcription factor Phox2b in the adult rat". *J. Comp. Neurol.*, em revisão, 2007.

17) Takakura AC, **Moreira TS**, West GH, Gwilt, JM, Colombari E, Stornetta RL and Guyenet PG. "GABAergic pump cells of solitary tract nucleus innervate retrotrapezoid nucleus chemoreceptors". *J. Neurophysiol.*, submetido, 2007.

18) Takakura AC, **Moreira TS**, Stornetta RL, West GH, Gwilt, JM and Guyenet PG. "Phox2b neurons in the retrotrapezoid nucleus are important for central chemoreception in rats ". *J. Neurosci.*, em preparação, 2007.

19) **Moreira TS,** Takakura AC and Guyenet PG. "Activation of serotonin-3 receptor-expressing C-fiber vagal afferents inhibits retrotrapezoid nucleus (RTN) chemoreceptors". *J. Neurophysiol.*, em preparação, 2007.

20) **Moreira TS**, Takakura AC, Menani JV, Sato MA, Colombari E. "Glutamatergic mechanisms in the commissural nucleus of the solitary tract are important for pressor response produced by CVLM inhibition". *Brain Res.*, em preparação, 2007.