UNIVERSIDADE DE SÃO PAULO
FACULDADE DE MEDICINA DE RIBEIRÃO PRETO
DEPARTAMENTO DE FISIOLOGIA

MELINA PIRES DA SILVA

**Modulação nitrérgica de canais para cátions
ativados por hiperpolarização e nucleotídeo cíclico
em neurônios do núcleo supraóptico**

Ribeirão Preto
Fevereiro de 2015

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Tese apresentada à Faculdade de Medicina de
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Orientador: Prof. Dr. Wamberto Antonio Varanda

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da Silva, Melina Pires

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sempre lutou pela minha educação e soube entender
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(Augusto Cury)

Resumo

O núcleo supraóptico (SON), uma das várias áreas hipotalâmicas envolvidas no equilíbrio hidroeletrolítico corporal, é de suma importância na complexa rede de sistemas fisiológicos que atuam na manutenção da homeostase. Este núcleo é constituído por um grupamento de neurônios magnocelulares (MNCs) responsáveis pela síntese e liberação de vasopressina e ocitocina, neuropeptídeos cujos principais efeitos se fazem sobre a excreção de água e sal pelos rins. Alterações da osmolalidade plasmática implicam em mudanças funcionais intrínsecas (propriedades de membrana) e extrínsecas (sinápticas) na excitabilidade desses neurônios, com consequências para a liberação desses neuropeptídeos. Além da osmolalidade, outros fatores são capazes de modular a excitabilidade dos MNCs, dentre elas o óxido nítrico (NO). Vários estudos têm mostrado que o NO desempenha um papel neuromodulador importante nesses neurônios, resultando na inibição da excitabilidade dos MNCs durante condições isotônicas e hipertônicas. Além disso, estas respostas são independentes das conexões sinápticas e envolvem a modulação de canais para cátions ativados por hiperpolarização e nucleotídeo cíclico (HCN). Entretanto, ainda é desconhecido à origem deste mensageiro neural, o envolvimento de outros tipos celulares nesta resposta, os mecanismos de sinalização utilizados pelo NO na modulação dos canais HCN, se esta modulação também ocorre em situações de aumentos de osmolaridade plasmática, e se há diferenças na modulação nitrérgica entre os fenótipos celulares. Assim, dada a importância deste núcleo para a manutenção do meio interno, elucidar os mecanismos pelo qual o NO modula os neurônios magnocelulares do SON torna-se essencial para o entendimento do controle da sua excitabilidade elétrica. Desta forma, considerando que: 1) os neurônios magnocelulares expressam a enzima para a síntese do NO em condições isotônicas e hipertônicas; 2) que os efeitos do NO parecem envolver, principalmente, a guanilato ciclase solúvel com consequente produção de cGMP; e 3) que os canais HCN são modulados por este

segundo mensageiro, nós hipotetizamos que os MNCs são capazes de produzir NO independente de outros tipos celulares e do fenótipo celular, e que a modulação nitrérgica dos canais HCN envolve a produção de cGMP. Frente a isso, este projeto teve como objetivo: 1) investigar o envolvimento das células gliais na modulação nitrérgica observada nos MNCs; 2) desvendar a origem do NO; 3) estudar as vias de sinalização da modulação nitrérgica sobre os canais HCN 4) avaliar se os efeitos do NO nos canais HCN também ocorre durante estímulo hiperosmótico agudo, e 5) se há diferença na modulação do NO em relação ao fenótipo celular. Para tanto, utilizamos a eletrofisiologia celular, essencialmente a técnica de “patch clamp” em fatias do SON e neurônios isolados. Experimentos de fluorescência foram realizados para a detecção da atividade intracelular do NO com o intuito de elucidar a origem deste mensageiro neural. Além disso, técnicas de biologia celular e molecular também foram utilizadas para caracterizar o fenótipo da célula que se está registrando, uma vez que 3 tipos já foram descritos neste núcleo e podem responder de maneira diferente ao mesmo estímulo. Até o presente nossos resultados mostraram que: 1) A modulação nitrérgica nos MNCs não envolve células gliais, 2) O NO é produzido pelos neurônios magnocelulares durante estímulo hipertônico, e este processo é dependente das alterações da osmolalidade, mas não da atividade elétrica dos neurônios frente ao estímulo; 3) Esta modulação ocorre por um mecanismo dependente de S-nitrosilação e independentemente da formação de cGMP; 4) a modulação nitrérgica nos canais HCN não difere entre os diferentes tipos de fenótipos encontrados e também ocorre durante aumentos da osmolaridade plasmática. Frente a estes resultados podemos concluir que a excitabilidade dos neurônios magnocelulares do SON é essencialmente determinada por um efeito do NO sobre os canais HNC por um mecanismo dependente de S-nitrosilação. Desta forma, o NO apresenta-se como mais uma molécula nesse complexo mecanismo de manutenção do equilíbrio hidroeletrólítico do organismo.

Palavras Chaves: 1. Canais HCN, 2. Óxido Nítrico, 3. Ocitocina, 4. Vasopressina, 5. S-nitrosilação.

Abstract

The hypothalamic supraoptic nucleus (SON) plays an important role in the complex systems network that operates to maintain the hydroelectrolytic balance of the body. The nucleus is formed by a cluster of neurons (magnocellular neurosecretory cells - MNCs) responsible for the synthesis and secretion of vasopressin and oxytocin, whose main effects are on the excretion of water and salt by the kidneys. Changes in plasma osmolality lead to intrinsic (membrane properties) and extrinsic (synaptic inputs) functional changes in these neurons, with consequences for neuropeptides release. Furthermore, besides osmolality, studies have shown that the electrical excitability of MNCs can be modulated by several factors, including nitric oxide (NO). Previous studies of our group, demonstrated that NO inhibits the excitability of MNCs during isotonic and hypertonic conditions and that this responses are independent of synaptic connections and involve the hyperpolarization-activated cyclic nucleotide-gated channels (HCN). However, some points remained unclear, such as: What is the origin of NO? Are there other cell types involved in this response? What signaling pathway does NO use to modulate HCN channels? Does NO modulate HCN channels during changes in the plasma osmolality? Are there differences in the nitrergic modulation according to cellular phenotypes? Considering that: 1) MNCs express the enzyme for nitric oxide synthesis, in isotonic and hypertonic conditions; 2) the NO effects involve, mainly, activation of guanylate cyclase with consequent increase in the cGMP production, and that 3) HCN channels are modulated by this second messenger, we hypothesize that MNCs are able to produce NO independently of other cells and cellular phenotypes, and that nitrergic modulation of HCN channels involves the guanylate cyclase activation. To answer those questions and to test our hypothesis, we used cellular electrophysiology, essentially Whole cell patch clamp using slices of the SON and isolated neurons. Fluorescence experiments were also used to detect the intracellular activity of NO in order to clarify the

origin of this neuronal messenger. In addition, cellular and molecular biology approaches were also used to characterize the phenotype of recorded cells, since 3 distinct cellular types have been described in this nucleus. Our results demonstrate that: 1) NO is produced by MNCs during hypertonic stimulation, and this process is dependent on changes in osmolality, but not on the electrical activity of MNCs in response to the stimulus; 2) NO modulates the electric excitability of the neurons by acting on HCN channels and it does not differ among cellular phenotypes; and 3) the effect of NO occurs by a mechanism dependent of S-nitrosylation and independently of cGMP production. Considering these results we may conclude that electrical excitability of magnocellular neurons of the supraoptic nucleus is essentially determined by an effect of NO on I_h currents by a mechanism dependent of S-nitrosylation. Acting on HCN channels, NO is another player in the complex set of mechanisms controlling the hydroelectrolytic balance.

Key Words: 1. HCN Channels, 2. Nitric Oxide, 3. Oxytocin, 4. Vasopressin, 5. S-nitrosylation.

Lista de Abreviaturas

ACSF – Líquido Céfalorraquidiano Artificial.

HCN – Canais para Cátions Ativados por Hiperpolarização e Nucleotídeo Cíclico.

I – Corrente.

I-V – Relação Corrente x Voltagem.

I_h – Corrente Ativada por Hiperpolarização.

MNCs – Neurônios Magnocelulares.

NO – Óxido Nítrico.

NOS_c – Sintase Constitutiva do Óxido Nítrico.

NOS_e – Sintase Endotelial do Óxido Nítrico.

NOS_i – Sintase Induzida do Óxido Nítrico.

NOS_n – Sintase Neural do Óxido Nítrico.

OT – Ocitocina.

QO – Quiasma Óptico.

RNA_m – RNA mensageiro.

SON – Núcleo Supraóptico.

PVN – Núcleo Paraventricular.

VP – Vasopressina.

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1. INTRODUÇÃO

1.1 Enunciado do Problema

Localizado bilateralmente ao quiasma óptico, o núcleo supraóptico (SON) integra informações provindas de áreas importantes envolvidas na regulação e manutenção da osmolalidade dos fluidos corporais (123). Tais informações, ou aferências, modulam a excitabilidade dos neurônios magnocelulares (MNCs) que por sua vez, controlam a secreção de ocitocina (OT) e vasopressina (VP) pela hipófise posterior (144). Em condições isotônicas, registros extracelulares dos MNCs, *in vivo*, mostraram que esses neurônios exibem uma baixa frequência de disparo de potenciais de ação (2-3 Hz) resultando em uma liberação basal de VP e OT (118). Entretanto, registros eletrofisiológicos em ratos anestesiados e em preparações *in vitro* tem revelado que a excitabilidade desses neurônios muda em função da osmolalidade plasmática resultando em alterações na liberação dos neuropeptídios (18, 24, 153). Tais evidências comprovam a participação do SON no equilíbrio hidroeletrólítico e evidenciam a importância de entender os mecanismos de controle dos MNCs.

A modulação da excitabilidade dos neurônios do SON é resultante da interação de múltiplas entradas sensoriais que incluem: osmorreceptores periféricos, osmorreceptores centrais, bem como a osmossensibilidade intrínseca dos MNCs per se (18). Os osmorreceptores periféricos encontram-se principalmente na vasculatura mesentérica do intestino delgado superior e na veia porta hepática (9, 29). Já em relação aos centrais, estes se localizam em núcleos constituídos por neurônios designados como osmorreceptores que se localizam fora da barreira hematoencefálica (órgãos circunventriculares – órgão vascular da lamina terminal, órgão subfornical e pré-óptica medial) e enviam projeções para os neurônios do SON e PVN (5, 17, 65). Entretanto, embora estas conexões sinápticas sejam importantes para o controle da atividade dos neurônios do SON (65), estudos têm demonstrado que na sua

ausência os MNCs são capazes de alterar sua excitabilidade em resposta ao estresse osmótico (107). Esta sensibilidade intrínseca, frente a alterações na osmolalidade, é resultado de uma despolarização (estímulo hipertônico) ou hiperpolarização (estímulo hipotônico) do potencial de membrana celular mediado por canais mecanossensíveis denominados de canais para cátions inativados por estiramento, cuja atividade é inversamente proporcional ao grau de distensão da membrana durante as alterações de volume celular (15, 105, 108). Frente a estes resultados, os autores denominaram os neurônios do SON como osmosensíveis, ou seja, neurônios sensíveis a alterações de osmolalidade capazes de induzir respostas osmorregulatórias (91, 107). Todavia, a osmorregulação no sistema hipotálamo-neurohipofiseal é um processo integrado, no qual a lesão de osmorreceptores centrais ou inativação dos canais mecanossensíveis nos MNCs resultam em respostas efectoras ineficientes. Assim, a convergência de múltiplas entradas sensoriais é responsável pelas respostas altamente elaboradas que resultam no reestabelecimento do equilíbrio hidroeletrolítico.

Embora a osmolalidade plasmática seja um dos principais fatores que promovem a ativação desses neurônios, vários neurotransmissores e neuropeptídeos podem, direta ou indiretamente, controlá-los (66, 133), e o óxido nítrico tem sido foco de estudo nos últimos anos (129, 141, 147).

1.2 Papel do Óxido Nítrico no Núcleo Supraóptico

Durante as últimas décadas, o óxido nítrico (NO) tem sido apontado como uma molécula de sinalização fisiológica crítica nos tecidos dos mamíferos, em particular no sistema nervoso central (49). Sua descoberta como um neurotransmissor alterou radicalmente a maneira de se pensar a respeito da transmissão sináptica. O NO é um gás, cuja formação ocorre por meio da catálise enzimática promovida pela sintase do óxido nítrico (NOS). Esta

enzima existe sob duas formas: constitutiva (NOSc) e induzida (NOSi). A NOSc é encontrada em duas isoformas, a neuronal (NOSn) e a endotelial (NOSe). A NOSn e NOSe, requerem a formação do complexo cálcio-calmodulina para sua ativação (75), enquanto que a NOSi, expressa em macrófagos, exerce sua atividade de maneira independente do cálcio mas estimulada por outras moléculas como citocinas (44). No entanto, todas as isoformas utilizam a L-arginina como substrato, que através de sua oxidação forma NO e citrulina (Figura 1), sendo este último reciclado em L-arginina para a formação de uma nova molécula de NO (27, 98) Em relação à expressão das enzimas que catalisam a formação do NO, a NOSn é abundantemente encontrada em várias regiões do SNC, tais como córtex, bulbo olfatório, núcleo acumbens, amígdala, hipocampo, tálamo, corpo trapezóide, magno da rafe, núcleo do trato solitário e cerebelo (21, 28). No hipotálamo estão presentes todas as isoformas, sendo a ordem de expressão NOSn > NOSe > NOSi (12). No SON a principal isoforma é a NOSn (150), sendo sua expressão gênica modulada por alterações na osmolalidade plasmática, ou seja, aumentos na osmolalidade induzem aumentos equivalentes na expressão gênica da enzima (69, 146).

Figura 1. Síntese do Óxido Nítrico. Na presença de NADPH, oxigênio e alguns cofatores (tetrahydrobiopterina (BH₄), Mononucleotídeo de Flavina (FMN), flavina adenina dinucleotídeo (FAD)) a sintase do óxido nítrico juntamente com o cálcio catalisa a reação transformando L-arginina em NO e citrulina. O NO uma vez formado, produzirá efeitos autócrinos e/ou parácrinos, bem como *feedback* negativo sobre a enzima NOS. Já a citrulina será convertida em arginina succinato, e L-arginina pela ação da arginina succinato sintase e liase. (33).

Vários estudos têm demonstrado que o NO age como um neuromodulador da excitabilidade dos neurônios magnocelulares do SON, tanto em condições basais como durante alterações da tonicidade do meio extracelular (34, 35, 47, 67, 77). Até o momento, alguns mecanismos foram propostos na tentativa de explicar esta modulação. Dentre eles, estão a potenciação dos impulsos sinápticos GABAérgicos (32, 111, 141), modulação de canais para potássio ativados por cálcio em condições isotônicas (2, 68) e modulação sobre canais ativados por hiperpolarização e nucleotídeo cíclico (HCN) (130). O NO também pode modular a atividade dos neurônios de modo independente de cGMP, seu principal segundo mensageiro (2).

Apesar de esses estudos terem demonstrado uma modulação nitrérgica nos neurônios magnocelulares do SON, a origem celular deste neuromodulador também não está clara. Recentemente vários trabalhos têm sugerido ainda, a participação das células gliais do

hipotálamo no controle da osmolalidade (104, 109, 112, 116, 131). Neurônios e astrócitos do SON estão em contato, e durante situações de hipertonicidade mostrou-se que os astrócitos são ativados minutos antes que os neurônios, evidenciando a capacidade dessas células de iniciarem processos osmorregulatórios no sistema nervoso central, em particular no núcleo supraóptico (85, 163). Além disso, como os astrócitos possuem toda maquinaria necessária para a produção de NO (36, 164), seria razoável pensar em uma modulação glial, de origem nitrérgica, sobre os neurônios desse núcleo.

1.3 Óxido Nítrico e Vias de Sinalização: cGMP e S-Nitrosilação

Já em relação às vias de sinalização utilizadas pelo NO, em algumas regiões do sistema nervoso central, a ativação da guanilato ciclase com consequente formação de cGMP está bem definida (49). Entretanto o uso desta via na modulação nitrérgica no SON ainda é controversa. Atualmente, essa visão clássica do cGMP como mediador exclusivo dos efeitos do NO tem sido revista, dada a capacidade deste mensageiro de interagir com proteínas por meio de ligações químicas. Essas interações influenciam vários processos celulares por alterarem a função de proteínas por meio de ligações reversíveis com grupamentos tióis de resíduos de cisteínas (60). A combinação do NO com a cisteína resulta em complexo mais estável, o S-nitrosothiol, que prolonga a atividade biológica do NO (3). Esse mecanismo é denominado de S-nitrosilação (Figura 2).

Figura 2. Esquema de s-nitrosilação de proteínas. Após ser sintetizado, o NO se liga a resíduos de cisteína da proteína resultando em um composto nitrosilado. Extraído de da Silva et al, 2014 (33).

A princípio, qualquer proteína que possua um grupamento tiol pode ser nitrosilada, porém estudos tem demonstrado certa especificidade. O NO não irá nitrosilar grupamentos tióis de baixo peso molecular, tais como cisteína e glutathione na ausência de oxigênio (O_2). Isso porque, a interação do NO com O_2 e/ou ânions superóxido (O_2^-) resulta na produção de espécies reativas de oxigênio e nitrogênio, tais como, dióxido de nitrogênio (NO_2), trióxido de nitrogênio (N_2O_3), e peroxinitrito ($ONOO^-$). O NO_2 e $ONOO^-$ são potentes oxidantes e podem atuar sobre resíduos de proteínas e lipídeos (121). Enquanto que o N_2O_3 é um oxidante mais leve e de preferência nitrosila amins e tióis (157). Tem sido demonstrado que o NO pode utilizar esta via para modular proteínas de membrana incluindo canais iônicos (3, 25, 30, 79). Além disso, Wenker e colaboradores (155) demonstraram que os canais HCN são alvos da S-nitrosilação endógena em motoneurônios, sugerindo uma via alternativa de sinalização do NO no SON.

1.4 Perguntas

Dados do nosso laboratório têm demonstrado que o NO é capaz de modular a excitabilidade dos neurônios magnocelulares tanto em condições basais (147), quanto durante estímulos hipertônicos agudos (34) levando a uma inibição da atividade dessas células. Além disso, estas respostas ocorreram independentes das conexões sinápticas e envolveram a modulação de canais HCN (130). Entretanto, ainda é desconhecido a origem deste mensageiro neural, o envolvimento de outros tipos celulares nesta resposta, os mecanismos de sinalização utilizados pelo NO na modulação dos canais HCN, se esta modulação difere em relação ao fenótipo celular e se ocorre em situações de estresse osmótico. Desta forma, considerando que: 1) os neurônios magnocelulares expressam a enzima para a síntese do NO em condições isotônicas e hipertônicas; 2) que os efeitos do NO parecem envolver, principalmente, a guanilato ciclase solúvel com consequente produção de cGMP; e 3) que os canais HCN possuem sítio de ligação para cGMP, nós hipotetizamos que os neurônios magnocelulares são capazes de produzir NO para gerar seu efeito inibitório na excitabilidade dos neurônios do SON não envolvendo outros tipos celulares neste processo, e que a modulação nitrérgica nos canais HCN envolve a produção de cGMP.

Diante do detalhado acima, as perguntas que surgiram foram: 1) Qual a origem do NO?; 2) Qual (is) molécula (s) poderia (m) estar atuando como segundos mensageiros na sinalização nitrérgica nos MNCs?; 3) Há envolvimento de outros tipos celulares, presentes no SON, nesta resposta?; 4) Como ocorre a modulação nitrérgica sobre os canais HCN? 5) O NO modula os canais HCN durante alterações da osmolalidade? 5) Há diferenças na modulação nitrérgica entre os fenótipos celulares?

2. OBJETIVOS

Para responder as perguntas destacadas acima os objetivos deste trabalho foram:

- 1) Investigar o envolvimento das células gliais na modulação nitrérgica observada nos MNCs.
- 2) Desvendar a origem do NO.
- 3) Estudar as vias de sinalização da modulação nitrérgica sobre os canais HCN.
- 4) Avaliar mecanismos de s-nitrosilação.
- 5) Avaliar se NO modula os canais HCN durante estímulo hiperosmótico agudo.
- 6) Avaliar se há diferença na modulação nitrérgica nos MNCs em relação ao fenótipo celular.

Para tanto, realizamos experimentos de eletrofisiologia, para medidas de correntes iônicas e atividade neuronal intrínseca, e fluorescência para detecção da produção de NO desvendando assim sua origem, bem como experimentos de imunofluorescência para caracterização dos fenótipos celulares.

3. METODOLOGIA

3.1 Animais

No presente estudo foram utilizados ratos Wistar, com idade entre 25 e 30 dias, provenientes do biotério central da Faculdade de Medicina de Ribeirão Preto/USP. Um dia antes dos experimentos os animais foram mantidos no Biotério do Departamento de Fisiologia, em caixas coletivas (3 animais por caixa), em temperatura de 23 ± 2 °C, sob regime de luz com ciclo claro-escuro de 12h (Luz 7:00 – 19:00h). Todos os animais receberam água e ração à vontade, exceto para experimentos onde este parâmetro necessitou ser manipulado (ver abaixo).

Duas horas antes do experimento os animais foram levados ao laboratório e mantidos lá até a hora do início do mesmo, sem manipulação, a fim de minimizar o estresse. Todos os protocolos utilizados foram aprovados pelo comitê de ética de experimentação animal da Faculdade de Medicina de Ribeirão Preto – USP (023/2012).

3.2 Eletrofisiologia

Experimentos eletrofisiológicos tiveram como meta central avaliar a contribuição dos canais HCN para a excitabilidade dos MNCs, a participação das células gliais na modulação nitrérgica desses neurônios e dos canais HCN, e os mecanismos de modulação do NO sobre estes canais. Para tanto, duas preparações distintas foram adotadas: fatias do SON e neurônios isolados em cultura.

3.2.1 Fatias do SON

Para obtenção das fatias hipotalâmicas contendo o SON procedeu-se como descrito abaixo. Após a decapitação dos animais com o auxílio de uma guilhotina, foi feita

craniotomia na linha média da estrutura dorsal da caixa craniana utilizando-se uma pequena tesoura de pontas finas. O encéfalo foi rapidamente removido e transferido para um recipiente contendo solução de ACSF (Líquido cérebro espinhal artificial, ver soluções) próximo a 0 °C, previamente equilibrado com mistura carbogênica (95% O₂ – 5% CO₂). O tempo deste procedimento não excedeu 2 minutos, a fim de manter a integridade funcional do tecido. O encéfalo permaneceu submerso no ACSF durante 2 minutos. Em seguida, foi seccionado de modo a ter-se um bloco de tecido contendo o hipotálamo cuja base caudal foi colada (com cola de cianoacrilato) em um suporte de ágar a 4 % em ACSF. A superfície dorsal deste bloco fica apoiada na porção vertical do suporte. Este, por sua vez, foi colado na plataforma acessória de um vibrátomo (modelo VT 1200 S, Leica, Bensheim, Alemanha) e o conjunto mergulhado em ACSF resfriado (próximo a 0 °C), contido em câmara própria do aparelho. Após o posicionamento adequado da lâmina do vibrátomo ajustamos a frequência e a velocidade de corte, de modo a se obterem fatias viáveis com 230 µm de espessura. Cada fatia, contendo o núcleo supraóptico, foi transferida a uma câmara contendo ACSF a 35 °C, borbulhado continuamente com mistura carbogênica, conforme descrito por Stuart e Sakmann (142). Três fatias de cada animal contendo os núcleos supraópticos foram mantidas na câmara de incubação durante um período de 30 a 60 minutos, e logo após foram utilizadas para os registros eletrofisiológicos.

3.2.2 Neurônios do SON Isolados

As vantagens de se utilizarem preparações com neurônios isolados são: 1) interromper a interação neurônio–glia e desta forma excluir a influência dessas células sobre a excitabilidade neuronal, e 2) a possibilidade de averiguar com maior precisão as ações do NO sobre a cinética dos canais HCN.

Neste modelo experimental foram utilizados três ratos Wistar (25 – 30 dias) para dissociação mecânica dos neurônios. Após a remoção do encéfalo, (realizado da mesma forma que nos experimentos de eletrofisiologia, descritos acima) fatias de 230 μm de espessura, contendo o SON, foram feitas e em seguida, transferidas para um recipiente com solução de Hank (especificada em soluções) constantemente borbulhada com oxigênio. As fatias permaneceram nesta solução por aproximadamente 60 minutos.

Após o período de incubação, pequenos blocos de tecido contendo a região do núcleo supraóptico foram transferidos para um recipiente com solução de Hank sem Ca^{+2} e Mg^{+} suplementada com tripsina (7 mg/ml - Sigma Chemical Corporation, St. Louis, MO, EUA) por um período de 30 minutos a 37 °C. Após o tempo de digestão, os fragmentos foram lavados 3 vezes com Hank sem Ca^{+2} e Mg^{+} e em seguida incubados em 2 ml da mesma solução acrescida de 0,05 % de inibidor de tripsina (Sigma Chemical Corporation). Após a inibição enzimática, os fragmentos do núcleo supraóptico foram dissociados com o auxílio de três pipetas Pasteur de vidro, com diâmetros decrescentes. A solução foi novamente substituída por meio de cultura Neurobasal acrescido de 2% de B27 (suplemento sem soro – Life Technologies Corporation, Carlsbad, CA, EUA), 0,5 mM de glutamina e centrifugada a 1000 rpm por 5 min. O sobrenadante foi retirado, ressuspendido em 2 ml de meio de cultura neurobasal suplementado com penicilina e estreptomicina (Sigma Chemical Corporation, St. Louis, MO, EUA) e semeado em lamínulas, previamente tratadas com poli L-lisina (Sigma Chemical Corporation,). Após 30 a 40 minutos, tempo necessário para que os neurônios possam aderir na lamínula, foi adicionado 2 ml de meio Neurobasal e as placas foram incubadas em estufa a 37 °C, em atmosfera com 5% de CO_2 e umidificada. Após 24 horas em cultura, 1 ml de meio foi removido e foi adicionado neurobasal contendo 0,1 μM de citosina arabinosídeo (ARA-C) para inibir o crescimento de células gliais. Os registros eletrofisiológicos foram feitos após 48 horas em cultura.

3.2.3 Patch Clamp

A técnica de *patch clamp* no modo *Whole cell*, foi utilizada tanto para as fatias como para as células isoladas. As fatias ou neurônios isolados ficaram numa câmara de registro montada sobre a platina de um microscópio (Nikon E-600FN, Melville, New York). A mesma foi continuamente perfundida com ACSF saturado com mistura carbogênica, a um fluxo de 2 a 3 ml/min. As fatias foram fixadas com uma pequena grade, construída com um aro de platina e fios de nylon para mantê-la imóvel no fundo da câmara e evitar seu deslocamento durante os registros.

Micropipetas com pontas entre 2-3 μm de diâmetro e resistências de 4-6 $\text{M}\Omega$ (quando preenchidas com a solução de pipeta) foram fabricadas a partir de capilares de vidro borosilicato num estirador P-97 (Sutter Instrument Co.). As pontas das micropipetas foram polidas numa microforja (Narishige MF-83) com a intenção de se obter selos mais estáveis. A pipeta, uma vez preenchida com solução apropriada, foi fixada ao “probe” montado em um micromanipulador e conectada ao amplificador de *patch* modelo 200B (Molecular Devices – Foster City, CA, EUA) através de um eletrodo de Ag/AgCl. Outro eletrodo de Ag/AgCl, imerso em solução aquosa de KCl 3M acrescentada de ágar a 2,5% para aumento de viscosidade serviu como ponto de referência de potencial zero, conectando o banho à terra. As saídas de corrente e voltagem do amplificador estavam ligadas a um conversor A/D e D/A (Digidata 1440 - Molecular Devices – Foster City, CA, EUA) controlado por um microcomputador, para coleta e armazenagem dos dados em disco rígido. Os sinais de voltagem ou de corrente foram filtrados a 5 KHz e digitalizados a 10 KHz. Tanto a aquisição quanto a análise dos sinais foram realizadas com auxílio de programas do pacote pClamp 10 (Molecular Devices – Foster City, CA, EUA). A placa digitalizadora também serviu para a

geração e aplicação dos protocolos de pulsos de voltagem e de injeção de correntes inerentes a cada situação experimental.

Nas fatias, as células foram visualizadas com o auxílio de uma câmera (PCO Imaging; PCO sensicam qe) e um sistema de contraste de interferência diferencial (DIC). Os neurônios magnocelulares, por sua vez, foram inicialmente identificados de acordo com sua localização anatômica, dorso-lateralmente ao quiasma óptico (115), bem como pelos seus aspectos morfológicos (Figura 3). A micropipeta de vidro foi posicionada no núcleo supraóptico e o selo de alta resistência com a membrana celular foi alcançado em modo *voltage clamp*.

No modo *current clamp* a atividade intrínseca dos neurônios foi avaliada através de registros dos potenciais de membrana e no modo *voltage clamp* foram aplicados protocolos de voltagem previamente determinados e registradas as correntes macroscópicas.

Os registros eletrofisiológicos foram sempre feitos numa condição, controle para em seguida aplicar-se uma determinada droga ou alterarmos a osmolalidade do banho. As soluções perfundiram a câmara de registros por gravidade e todas as drogas foram aplicadas trocando-se a linha de perfusão. A saída do líquido de perfusão da câmara foi feita por meio de uma bomba de sucção. Bloqueadores sinápticos da transmissão glutamatérgica (DL-AP5 e CNQX) e GABAérgica (picrotoxina) foram utilizados durante todo tempo de registro e em todos os experimentos aqui demonstrados.

A ativação dos canais HCN foi feita no modo *voltage clamp* a partir da aplicação de pulsos de voltagem hiperpolarizantes entre -50 mV a -135 mV em passos de -10 mV e duração de 3 segundos cada pulso. Um pré-pulso condicionante foi fixado em -50 mV. Já a análise de excitabilidade dos neurônios foi feita em *current clamp* com injeções de correntes de +50 pA a -40 pA e os registros foram utilizados tanto para a análise da excitabilidade

neuronal (pulsos de corrente despolarizantes) como para análise da resistência de entrada (pulsos de corrente hiperpolarizantes, RI).

Figura 3. Fotomicrografias de fatias contendo o núcleo supraóptico. A) Em A, um esquema adaptado de Paxinos e Watson (115) mostrando a localização anatômica do SON (setas vermelhas), dorso-lateral ao quiasma óptico (och). Em B, uma fotomicrografia de uma fatia hipotalâmica de 230 µm de espessura, a seta em vermelho indica a localização do núcleo. Em C, células visualizadas com o auxílio de um sistema de detecção de luz infravermelha. A micropipeta em contacto com uma célula estabelece a configuração *Whole cell*. SON – núcleo supraóptico.

3.3 Inibição das Células Gliais

Para os experimentos que visaram investigar a participação das células gliais nas respostas nitrérgicas, além de utilizarmos inibidores do metabolismo glial (Fluorocitrato de bário, Sigma Chemical Corporation, St. Louis, MO, EUA), nós também utilizamos a sobrecarga salina (ver detalhes abaixo). Em relação ao fluorocitrato, devido à presença do íon bário na sua composição, um protocolo para purificação foi utilizado seguindo o método

descrito por Paulsen e colaboradores (114). Brevemente, o L- fluorocitrato de bário foi dissolvido em 1 ml de 0,1 M HCl e duas a três gotas de 0,1 M de Na_2SO_4 foram adicionadas para precipitar o íon Ba^{2+} . Em seguida, 2 ml de Na_2PO_4 0,1 M de foi adicionado e a solução foi centrifugada a 1.000 xg por 5 min. O sobrenadante foi diluído em NaCl 0,9 % para obter a concentração final, e o pH foi ajustado para 7,4. Neste protocolo as fatias foram incubadas em ACSF de registro (ver soluções) acrescido de 200 μM de fluorocitrato por um período de 20 minutos antes de iniciar os registros eletrofisiológicos.

Em um segundo grupo de animais, a retração glial foi induzida através da sobrecarga salina que altera a relação morfológica e funcional entre neurônios e células gliais. Utilizada por vários pesquisadores (53, 58, 127), a sobrecarga consistiu em desidratar o animal por 7 dias por meio da ingestão de NaCl 1,8 % o que resulta em uma retração dos processos astrocíticos sobre os neurônios, alterando assim sua interação com os mesmos (126, 127). Neste modelo, os animais tiveram acesso somente ao NaCl 1,8 %, ao invés de água, e ração *ad libitum*. Desta forma foi possível analisar se com a retração glial há alguma alteração na modulação nitrérgica dos neurônios do SON.

3.4 Detecção do NO por Fluorescência

Com a finalidade de avaliar a produção de NO nos neurônios do SON, experimentos de fluorescência foram desenhados, visando detectar as alterações na sua atividade durante estresse osmótico. Os experimentos foram realizados em duas preparações distintas: cultura de neurônios e fatias do núcleo supraóptico. Para neurônios isolados, utilizamos um microscópio confocal multiusuário (TCS SP5, Leica, Heidelberg, Alemanha) da Faculdade de Medicina de Ribeirão Preto. Nos experimentos utilizando fatias do núcleo supraóptico, as alterações de fluorescência foram detectadas por meio de um microscópio epifluorescente (Nikon E-600FN, Melville, New York, EUA). Para ambas as situações, os neurônios

magnocelulares do núcleo supraóptico foram carregados com um indicador fluorescente da produção de óxido nítrico, DAF-FM (Sigma Chemical Corporation, St. Louis, MO, EUA), durante 30 minutos (Figura 4). O indicador foi dissolvido em dimetil sulfóxido (DMSO) e 0,02 % de ácido plurônico F127 em concentração estoque de 5 mM e, então, diluído em solução de registro, a uma concentração final de 30 μ M. A taxa de aquisição das imagens foi definida pela cinética do processo. Para o estresse osmótico foi utilizado solução de Hank (neurônios isolados) ou ACSF (fatias) acrescida de manitol com osmolalidade de 330 mosm/(Kg.H₂O). As imagens foram analisadas com o auxílio do software ImageJ (W.S. Rasband/National Institute of Health, Bethesda, MD, EUA).

Figura 4. Marcação do NO pelo indicador DAF-FM. Imagens representativas de uma fatia do SON incubado com o indicador fluorescente da produção de NO, DAF-FM, visualizado em campo claro (**A**) e durante a excitação do indicador (**B**). Linha tracejada em **B** representa a pipeta de vidro utilizada para registros de *patch clamp*. (DAF-FM: Excitação = 488 nm; Emissão= 515 nm).

3.4.1 Imunofluorescência para Neurônios Isolados

Devido à perda de morfologia dos neurônios magnocelulares em cultura, experimentos de fluorescência foram utilizados para identificação do fenótipo celular. Logo após o

experimento de imunofluorescência para detecção da produção de NO, os neurônios isolados do SON foram fixados com paraformaldeído 4% por 30 minutos em temperatura ambiente, lavados 3x com PBS (tampão fosfato) por 5 min cada lavagem, permeabilizados com Triton X-100 0,25 % e incubados com albumina de soro bovino (BSA 10 %) durante 1 hora em câmara úmida. Após o período de bloqueio, as lamínulas foram incubadas durante a noite a 4 °C com anticorpo primário anti-OT (Rabbit, 1:500, Peninsula Laboratories, San Carlos, CA) e anti-VP (Guinea Pig anti-(Arg⁸) -Vasopressin, 1:500, Peninsula Laboratories, San Carlos, CA) também em câmara umidificada. Em sequência, os neurônios foram incubados com anticorpo secundário Alexa flúor 488 e Cy5, ambos com diluição de 1:500 por 1 hora. As lamíνας foram montadas com prolong (Invitrogen Corporation, Carlsbad, CA, USP) e a fluorescência foi analisada em um microscópio confocal (TCS SP5, Leica, Bensheim, Alemanha). As imagens foram analisadas utilizando o software ImageJ (W.S. Rasband/National Institute of Health, Bethesda, MD, EUA). A colocalização foi observada pela sobreposição das imagens, obtidas a cada 0.8 µm. Como experimento controle, algumas lamínulas foram incubadas somente com anticorpo secundário e um marcador nuclear (DAPI).

3.5 Caracterização Fenotípica dos Neurônios do SON

Como já foi demonstrado que neurônios vasopressinérgicos e ocitocinérgicos do SON podem responder de maneira diferente a uma mesma situação experimental (153), caracterizar os diferentes fenótipos torna-se de suma importância. Assim, para diferenciá-los utilizamos três ferramentas: Eletrofisiologia, técnica de reação da transcriptase reversa em tempo real (PCR) e a imunofluorescência do neurônio registrado.

3.5.1 Identificação Eletrofisiológica

O fenótipo dos neurônios magnocelulares foi eletrofisiologicamente identificado em função da presença ou ausência de correntes retificadoras. Este protocolo foi previamente descrito por Armstrong e Stern (7, 8, 139) baseado em suas observações de que durante injeções de corrente negativa a relação *Voltagem x Corrente* era linear em neurônios vasopressinérgicos e não linear em neurônios ocitocinérgicos. De acordo com os autores, isto é devido ao fato de que os neurônios OT expressam uma corrente de potássio retificadora sustentada para fora (SOR), a qual é ativada com a aplicação de corrente negativa. Apesar de o protocolo ter sido descrito utilizando microeletrodos intracelulares, recentemente, outros autores validaram o fato de que esta distinção entre os fenótipos pode também ser feita com *patch-clamp* no modo *Voltage Clamp* (61, 165). Estes autores, na verdade, além de correlacionar a presença ou ausência da corrente retificadora para fora sustentada (SOR) com o fenótipo VP ou OT, também atribuíram a neurônios OT a existência de uma retificação para dentro induzida pela hiperpolarização (IR). Assim, no presente trabalho, a distinção eletrofisiológica entre os fenótipos foi feita utilizando-se o protocolo descrito por aqueles autores em *Voltage Clamp*. Este consistiu na aplicação, por 250 ms, de pulsos de voltagem de -40 mV a -135 mV (em passos de -10 mV) a partir de um pulso condicionante no qual a célula é mantida a -40 mV por 100 ms. Por fim, uma relação *I-V* das respostas obtidas foi feita utilizando-se o valor estacionário da corrente nos 10 ms finais dos pulsos. Assim, baseado em Hirasawa e cols. (61) e Zampronio e cols. (165) assumiu-se neste trabalho que neurônios VP apresentam a relação *I-V* linear, ao passo que esta relação para neurônios OT é não linear.

3.5.2 Obtenção de RNA Total de Células Únicas

Com o intuito de validar o protocolo eletrofisiológico para distinção dos fenótipos em *Whole cell*, após realizar o registro, o citoplasma do neurônio foi sugado para o interior da

micropipeta e submetido à análise quantitativa da expressão dos RNAs da vasopressina e ocitocina, para determinar o fenótipo molecular da célula registrada. Como notado por Sucher e Deitcher (143), mesmo em culturas celulares a sucção de uma célula inteira para dentro das pipetas utilizadas em *patch-clamp* é rara. No entanto, a simples difusão do conteúdo citoplasmático da célula registrada, devido à diluição da solução de pipeta durante o registro, já permite que se tenha RNAm suficiente para que o material seja submetido à transcrição reversa. Desta forma, após a sucção do conteúdo celular o material passou pela transcrição reversa, que consiste na síntese de DNA complementar (cDNA) a partir de um molde RNAm. Esta reação foi realizada em um termociclador *Mastercycler Gradient* (Eppendorf, Hamburg, Alemanha) utilizando-se um *kit* próprio para a transcrição reversa com hexanucleotídeos randômicos (High Capacity cDNA Reverse Transcription Kit, Life Technologies Corporation, Carlsbad, EUA), e seguindo o protocolo recomendado pelo fabricante.

3.5.3 Reação de Pré Amplificação

Uma vez que a quantidade de cDNA obtida a partir de uma única célula é muito pequena, fez-se necessário a realização de uma Pré-Amplificação do cDNA obtido a partir da RT. Após a conversão do RNAm para cDNA, o material foi submetido a uma pré-amplificação. Para tal, utilizamos um kit destinado a esta finalidade (TaqMan PreAmp Master Mix Kit, Life Technologies Corporation, Carlsbad, EUA) e as seguintes sondas de hidrólise *TaqMan*: *Rn00564446_g1* (vasopressina), *Rn00566449_m1* (ocitocina) e *NM_031144.2* (β -Actina; gene de referência, controle positivo das reações). A reação foi executada em um termociclador *Mastercycler Gradient* (Eppendorf, Hamburg, Alemanha) seguindo, novamente, as recomendações fornecidas pelo fabricante do *kit* para 14 ciclos de reação. Após a pré-amplificação, foi feita uma quantificação do cDNA para cada amostra, utilizando-se o espectrofotômetro *NanoDrop 1000* (Thermo Scientific, Wilmington, EUA). A concentração de cDNA obtida indicou se havia, na amostra, material suficiente para realização do PCR em

tempo real (qPCR), assim como a pureza da amostra. Para experimentos da análise da expressão de canais HCN no SON as seguintes sondas foram utilizadas: HCN-1 Rn00670384_m1, HCN-2 Rn01408572_mh, HCN3 Rn00586666_m1, HCN4 Rn00572232_m1 e os mesmos procedimentos descritos anteriormente foram seguidos.

3.5.4 PCR em Tempo Real

A reação de qPCR foi realizada em um termociclador com detector de fluorescência modelo *StepOnePlus* (Life Technologies Corporation, Carlsbad, EUA) com suporte a placas de 96 poços de reação. Cada reação foi feita em *singleplex* e em triplicata, sendo utilizadas as mesmas sondas hidrolíticas mencionadas anteriormente e o *kit* para PCR *TaqMan Universal PCR Master Mix* (Life Technologies Corporation, Carlsbad, EUA).

3.5.4.1 Análises dos Resultados RT-qPCR

Para análise dos resultados obtidos pelo RT-qPCR nós utilizamos os mesmos critérios previamente descritos pelo nosso grupo (93). Assim, a quantificação da expressão relativa dos dois genes, OT e VP, respectivamente, foi feita baseada nos seguintes princípios: 1) que a quantidade de amostra em um dado ciclo é uma função da quantidade inicial de material multiplicada pela eficiência de amplificação elevada ao número de ciclos realizados (59):

$$X_{A,n} = X_{A,o} \times (1 + E_A)^n \quad \text{Equação 1,}$$

onde: $X_{A,n}$ é o número de moléculas-alvo no ciclo n ; $X_{A,o}$ é o número inicial de moléculas-alvo; E_A é a eficiência de amplificação do alvo; e n é o número do ciclo; e (2) que a quantidade de moléculas dos genes-alvo é igual ao seu ciclo de quantificação (C_q), ou seja:

$$X_{AVP,Cq} = X_{OT,Cq} \quad \text{Equação 2,}$$

onde: $X_{AVP,Cq}$ e $X_{OT,Cq}$ são a quantidade de moléculas de vasopressina e ocitocina, respectivamente, no ciclo de quantificação Cq .

A partir das equações 2 e 3, tem-se que:

$$\frac{X_{AVP,O}}{X_{OT,O}} = \frac{(1+E_{OT})^{Cq_{OT}}}{(1+E_{AVP})^{Cq_{AVP}}} \quad \text{Equação. 3,}$$

onde a razão $\frac{X_{AVP,O}}{X_{OT,O}}$ expressa a quantidade inicial de cDNA de vasopressina pela quantidade inicial de cDNA de ocitocina.

Assim, neurônios que apresentaram razões maiores que 1 foram considerados como vasopressinérgicos e menores que 1 como ocitocinérgicos. Razões iguais ou próximas a 1 foram consideradas como neurônios com características intermediárias na qual o cDNA para VP e OT são expressos em quantidades similares.

3.5.5 Eletrofisiologia, RT-qPCR e Imunofluorescência em um Único Neurônio.

Para evitar erros sobre a expressão de cDNA e sua correlação com o peptídeo produzido, nós realizamos uma tripla caracterização utilizando a eletrofisiologia, o RT-qPCR e também a imunofluorescência em um único neurônio. Para tanto 0,2 % de biocitina, um traçador intracelular (Life Technologies Corporation, Carlsbad, EUA), foi acrescentado à solução de pipeta, utilizada para os registros eletrofisiológicos. Desta forma, um novo grupo de neurônios foi registrado utilizando o protocolo eletrofisiológico e subsequente a este o citoplasma da célula, diluído na solução de pipeta, foi coletado para procedimentos de transcrição reversa e qPCR (Para maiores detalhes ver seção 3.5.2). Em seguida a fatia foi fixada em paraformaldeído (PFA; 4%) durante 12 horas e desidratadas em sacarose 30 % durante o mesmo período de tempo. Após a desidratação, as fatias foram transferidas para uma solução crioprotetora (0,1 M PBS: 0,3% de etileno glicol, 0,2% de glicerol, v/v) a -20 °C

até serem imunohistoquimicamente processadas. Para o processamento da imunofluorescência, os cortes foram bloqueados e permeabilizados em PBS contendo 10 % de soro de cavalo e 0,1 % de Triton, durante 1 hora à temperatura ambiente. Em seguida, as fatias foram incubadas durante a noite a 4 ° C com anticorpos primários: anti-VP (1:1000 Guinea Pig anti-(Arg⁸) -Vasopressin, Peninsula Laboratories, San Carlos, CA) e anti-OT (1:1000 Rabbit, Peninsula Laboratories, San Carlos, CA). Subsequentemente, as fatias foram lavadas com PBS 0,01 M e incubadas com os anticorpos secundários: anti guinea pig Cy5 para VP e anti rabbit Alexa 488 para OT por 1 hora (1:500). Ao mesmo tempo, os neurônios marcados com biocitina foram revelados por reação com estreptavidina (Alexa Flúor 405 conjugado, Life Technologies Corporation, Carlsbad, CA, EUA), diluída a 1:500. As fatias foram então lavadas três vezes em PBS e montadas em lâminas de vidro com Fluoromount (Sigma Chemical Corporation, St. Louis, MO, EUA). Imagens de fluorescência foram adquiridas sequencialmente (intervalo de 0,3 µm) em um microscópio confocal (TCS SP5, Leica, Bensheim, Alemanha), e as análises foram feitas utilizando o software ImageJ (W.S. Rasband/National Institute of Health, EUA - <http://rsb.info.nih.gov/ij>). Para cada fatia, o neurônio positivo para biocitina foi identificado e projeções em z foram coletadas sequencialmente para analisar a colocalização.

3.6 Alterações do Equilíbrio Hidroeletrólítico e Fenótipo Celular

Com o intuito de investigar se há alterações na proporção de neurônios magnocelulares OT e VP durante desafios fisiológicos, ratos Wistar (25 e 30 dias), foram divididos em três grupos experimentais: controle, restrição hídrica, e ratos submetidos à sobrecarga salina (n = 4 por grupo). No grupo controle, os animais tiveram água *ad libitum* durante 7 dias, enquanto que no modelo de restrição hídrica, os animais tiveram acesso apenas a 30% do volume de água ingerida pelos animais do grupo controle, sendo que a água era oferecida no início da fase escura do ciclo. Já no modelo de sobrecarga salina, os animais tiveram acesso apenas a

solução de NaCl 1,8% *ad libitum* por 7 dias. Todos os grupos tiveram livre acesso a ração. No sétimo dia, os animais foram anestesiados (tribromoetanol: 250 mg.kg⁻¹, ip; Sigma Chemical Corporation, St. Louis, MO, EUA), e perfundidos com PBS 0,1 M, seguido por 4% de paraformaldeído. Fatias contendo o SON (30µm de espessura) foram feitas por auxílio de um criostato HM 505 N (Microm, Charleston - EUA) e armazenadas numa solução crioprotetora (0,1 M PBS: 0,3% de etileno glicol, 0,2% de glicerol, v/v) a -20 °C até serem imunohistoquimicamente processadas. A imunofluorescência foi realizada utilizando-se os mesmos métodos descritos anteriormente (seção 2.5.5) e as imagens foram obtidas sequencialmente (0,3 µm por imagem) a partir de seis secções mediais do SON, de cada rato, em um microscópio confocal (TCS SP5, Leica, Bensheim, Alemanha). Após as aquisições os neurônios VP e OT imunorreativos foram contados bilateralmente sem conhecimento, pelo experimentador, da origem da fatia (se controle, ratos com sobrecarga salina ou restrição hídrica), com o auxílio do software ImageJ - *Cell Counter Plugin*. Os resultados foram representados como média ± epm, e a proporção de cada fenótipo encontrado em cada condição experimental (controle, restrição hídrica, e sobrecarga salina), foram comparados com ratos controle pelo teste estatístico ANOVA One-way e pós-teste de Bonferroni (Prism 5 - GraphPad Software, Inc., La Jolla, CA, EUA). Valores de p menores que 0,05 foram considerados significativos.

3.7 Drogas e Soluções

As drogas utilizadas neste trabalho foram as seguintes: L-NAME (N^ω-nitro-L-arginine methyl éster), inibidor não seletivo da NOS 100 µM; L-arginina, substrato para NOS (500 µM); CNQX (6-cyano-7-nitroquinoxaline-2,3-dione), antagonista competitivo para receptores AMPA (10µM); DL-AP5 (DL-2-Amino-5-phosphonovaleric acid) um antagonista de receptores NMDA (30 µM), picrotoxina um bloqueador de GABA_A (30 µM); 30 µM; ZD7288 um agente bradicárdico que age como bloqueador para I_h (50 µM); tetraetilamonio

(TEA) e 4-aminopiridina (4AP), bloqueadores de canais para K (ambos a 1 mM). ODQ inibidor da guanilato ciclase solúvel (30 μ M), Fluorocitrato de bário, um inibidor do metabolismo glial (200 μ M), SIN 1 doador de NO (100 μ M). Todas as drogas foram obtidas da Sigma Chemical Co (Saint Louis, MO, EUA).

3.7.1 ACSF (Líquido Céfal Raquidiano Artificial - Solução para Corte)

A solução de ACSF utilizada nos procedimentos de obtenção das fatias teve a seguinte composição (em mM): NaCl 121; KCl 3; CaCl₂ 2; MgCl₂ 1,3; NaHCO₃ 26 e glicose 25. A osmolalidade da solução ficou entre 305 – 311 mosm/Kg.H₂O e o pH de 7,4 quando equilibrada com mistura carbogênica (5% CO₂ e 95% de O₂).

3.7.2 ACSF Registro

A solução de ACSF, utilizada nos procedimentos de manutenção e superfusão das fatias, tem a seguinte composição (em mM): NaCl 121; KCl 4; CaCl₂ 2,5, MgCl₂ 1,3, NaHCO₃ 26; e glicose 10. A osmolalidade desta solução ficou entre 295 – 300 mosm/Kg.H₂O e pH de 7,4 quando equilibrada com mistura carbogênica. A solução hipertônica foi obtida adicionando manitol ao ACSF de registro para obter osmolalidade de 330 mosm/Kg.H₂O.

3.7.3 Solução de Hank

A solução utilizada nos experimentos com neurônios isolados teve a seguinte composição (em mM): NaCl 140; KCl 4,6; CaCl₂ 1,3; MgCl₂ 1,6; NaHCO₃ 5; HEPES 10; glicose 10. A osmolalidade ficou entre 300 a 310 mosm/Kg.H₂O e pH de 7,4 quando corrigido com NaOH.

As demais soluções empregadas foram desenhadas a partir da necessidade de cada experimento. O mesmo raciocínio aplica-se as drogas que foram utilizadas, sejam bloqueadores de vias/canais específicos ou indicadores fluorescentes.

3.8 Análises dos Dados

A análise dos dados foi realizada com o auxílio do pacote de programas do pClamp10 (Axon Instruments, Sunnyvale, CA - EUA), Mini Analysis 4.3.2 (Synaptosoft, Fort Lee - NJ), OriginPro 8 (OriginLab Corporation, Northampton, MA – EUA) e Prism 5 (Graphpad software, Inc., La Jolla, CA).

Para isolamento das correntes I_h , foi necessário utilizar um método de subtração, já descrito por outros (51), devido ao fato de que I_h não é a única corrente ativada pelo protocolo aplicado. Assim, foi feita uma subtração da corrente registrada durante o controle daquela registrada com o bloqueador (ZD7288), resultando somente da corrente I_h . O mesmo raciocínio foi utilizado para obtenção da corrente I_h na presença de outros moduladores.

A significância estatística da diferença entre os grupos experimentais foi testada utilizando-se teste t pareado ou não pareado de acordo com cada condição experimental, e valores de $p < 0,05$ foram considerados como significativos. Os valores foram expressos como médias $\pm$ erro padrão das médias.

4. RESULTADOS

4.1 Envolvimento das Células Gliais na Modulação Nitrérgica do SON.

Para avaliarmos a participação das células gliais na modulação nitrérgica observada no SON, nós escolhemos alguns protocolos experimentais que minimizasse a ação destas células na atividade dos neurônios magnocelulares e avaliamos se a modulação nitrérgica ainda estava presente (ver metodologia para maiores detalhes seção 3.2.1 e 3.2.2).

No primeiro utilizamos um inibidor do metabolismo glial (fluorocitrate 200 μ M) por 20 min e em seguida a atividade intrínseca dos neurônios (na presença de bloqueadores glutamatérgicos e GABAérgicos) foi registrada e considerada como condição controle. Após 10 minutos de registro controle o substrato da formação do NO, L-arginina (500 μ M) foi adicionada a solução de banho. Como podemos observar na figura 5, não houve diferença significativa na frequência de disparo entre os grupos de neurônios sugerindo que estas células não interferem na modulação nitrérgica observada no SON.

Figura 5. Efeitos do inibidor do metabolismo glial na modulação nitrérgica dos neurônios magnocelulares do SON. A mostra traçados típicos da atividade intrínseca (registros feitos na presença de bloqueadores sinápticos – picrotoxina, CNQX e DL-AP5), de um dos neurônios do grupo, analisados após a incubação com o bloqueador do metabolismo glial fluorocitrato e com o substrato do NO, L-arginina (500 μ M). Em A1 tem-se as médias da frequência de disparo de potenciais de ação dos neurônios analisados (n=7). Não houve diferença estatística entre os grupos ($5,6 \pm 0,9$ Hz x $5,8 \pm 1,4$ Hz; $p > 0,05$). A análise da frequência foi realizada utilizando 1 min de registro de cada condição experimental.

Devido às críticas ao uso do fluorocitrato e seu efeito em canais iônicos por resíduos de bário que podem permanecer na solução durante o processo de purificação, nós optamos por utilizar sobrecarga salina. Desta forma, após 7 dias a atividade intrínseca dos neurônios foram registradas na presença do substrato para a formação do NO, L-arginina, ou na presença do inibidor da sintase do óxido nítrico, L-NAME.

Como mostrado na figura 6, obtivemos dois resultados distintos na presença de L-arginina. De 11 neurônios registrados 7 apresentaram uma redução na frequência de potenciais de ação ($4,7 \pm 0,6$ Hz x $3,4 \pm 0,4$ Hz; $p < 0,05$) enquanto que os demais apresentaram uma resposta oposta ($4,5 \pm 0,9$ Hz x $6,4 \pm 1,4$ Hz; n=4, $p < 0,05$).

Figura 6. Modulação nitrérgica durante a retração glial por desidratação crônica. A e B são traçados típicos de voltagem obtidos de um dos neurônios de cada grupo analisados na situação controle (na presença de inibidores sinápticos - picrotoxina, CNQX e DL-AP5), e na presença dos inibidores mais 500 μ M de L- Arginina. Os registros mostram a atividade espontânea do neurônio durante 1 min de registro. Em **A1** e **B1** os gráficos de barras resumem os efeitos obtidos em 11 neurônios com 7 deles apresentando uma redução ($4,6 \pm 0,6$ Hz x $3,4 \pm 0,4$ Hz, Inibidores e Inibidores + L-arginina respectivamente; $p < 0,05$) e os outros 4, um aumento da frequência de potenciais de ação ($4,5 \pm 0,9$ Hz x $6,4 \pm 1,4$ Hz; $p < 0,05$). Teste t paramétrico para ambos os grupos.

Por outro lado, quando os neurônios dos animais desidratados foram expostos a um inibidor da sintase do óxido nítrico, L-NAME (100 μ M), nós observamos que todos os neurônios registrados apresentaram um aumento na frequência de disparo (Figura 7) assim como observado na ausência do fluorocitrato (147).

Figura 7. Inibição da enzima NOS aumenta a frequência de disparos dos neurônios MNCs após a retração glial. A, Traçados representativos da atividade intrínseca de um neurônio do grupo durante o controle, somente na presença de inibidores sinápticos, e após a superfusão com os inibidores mais o inibidor da NOS, L-NAME (100 μ M). A1, o gráfico de barras representa as médias ($\pm$ epm) obtidas de 8 neurônios registrados. Na presença do L-NAME houve um aumento na frequência de disparo quando comparado com os inibidores ($4,5 \pm 0,7$ Hz x $2,9 \pm 0,7$ Hz, respectivamente - $n=8$; $p < 0,05$ – teste t paramétrico).

Devido aos diferentes resultados obtidos com o substrato do NO em 4 neurônios, a determinação do fenótipo celular faz-se essencial para o entendimento da modulação nitrérgica na ausência da interação neurônio-glia. Por esta razão, após o registro eletrofisiológico, o citoplasma das células foi coletado para análise. Assim, procurou-se determinar o fenótipo do neurônio utilizando-se de dois métodos distintos: eletrofisiológico e molecular por meio da RT-qPCR de uma única célula.

Em relação à determinação eletrofisiológica, os neurônios registrados, foram diferenciados a partir da análise de linearidade, ou não, da relação Corrente x Voltagem ($I-V$). A linearidade dos pontos é característica de neurônios vasopressinérgicos, enquanto que seu

desvio caracteriza os neurônios ocitocinérgicos. A não linearidade se deve a presença de uma corrente retificadora para fora (SOR) observada em voltagens mais positivas que -60 mV e/ou em decorrência de uma corrente retificadora para dentro (IR), ativada em potenciais mais hiperpolarizados que -80 mV. Em contrapartida a linearidade presente nos neurônios VP se deve a ausência de ambas as correntes.

Como demonstrado na figura 8, entre os neurônios submetidos ao tratamento com L-arginina 55 % apresentaram uma relação $I-V$ linear caracterizando esta população de neurônios como vasopressinérgicos (n=6, sendo 5 células com redução da frequência, e 1 uma com aumento), e 45 % não linear identificando-os como ocitocinérgicos (n=5, sendo 2 células com redução da frequência e 3 com aumento). Por outro lado, os neurônios registrados na presença do L-NAME apresentaram 50% de cada fenótipo. Tais resultados indicam que as respostas obtidas na presença da L-arginina não se devem a uma diferença fenotípica dos neurônios registrados.

Figura 8. Caracterização fenotípica eletrofisiológica. Em A, registro de um neurônio vasopressinérgico obtido a partir de pulsos hiperpolarizantes de -40 a -135 mV. A1, relação $I-V$ obtida a partir dos pontos delimitados pela linha vermelha tracejada. Note a linearidade da resposta. B e B1, registro de um neurônio ocitocinérgico e relação $I-V$ com retificação em voltagens mais despolarizadas que -60 mV, indicando a presença da corrente retificadora de saída. C e C1 registro de um neurônio ocitocinérgico e relação $I-V$ com retificação de entrada observada em voltagem mais hiperpolarizadas que -80 mV. D e E, distribuição percentual dos fenótipos eletrofisiológicos dos neurônios que foram superfundidos com o substrato do NO (L-arginina) e inibidor da NOS (L-NAME) respectivamente.

Por outro lado, com o PCR para célula única, os neurônios que apresentaram uma redução na frequência de potenciais de ação com a L-arginina ($n=7$), foram identificados como vasopressinérgicos, e os neurônios que responderam com um aumento da frequência de potenciais de ação, foram identificados como ocitocinérgicos ($n=4$). Como podemos observar o fenótipo eletrofisiológico não correspondeu ao molecular.

4.2 Caracterização Eletrofisiológica do Fenótipo dos Neurônios Magnocelulares

Devido às discrepâncias fenotípicas encontradas com o protocolo eletrofisiológico quando o comparamos com o RT-qPCR nos animais desidratados, nós decidimos testar a confiabilidade do protocolo eletrofisiológico antes de dar continuidade aos experimentos e assim decidirmos qual método utilizar para caracterização fenotípica dos neurônios. Para isso, registramos 74 neurônios utilizando o protocolo eletrofisiológico descrito na literatura (61, 165) e a presença de relação $I-V$ não linear (correntes retificadores SOR e/ou IR), foi considerada como critério para identificação de neurônios ocitocinérgicos, enquanto que a ausência de retificação serviu à identificação de neurônios vasopressinérgicos. Em sequência, o citoplasma de cada célula registrada foi coletado para posterior determinação molecular do fenótipo neuronal utilizando o RT-qPCR para célula única.

Como pode ser observado na figura 9, de acordo com os critérios de distinção fenotípica eletrofisiológica, de um total de 74 neurônios registrados, 11 apresentaram relação $I-V$ linear caracterizando-os como vasopressinérgicos, enquanto que 39 apresentaram retificação de entrada (IR), 9 de saída (SOR) e 15 apresentaram ambas (SOR/IR) e foram caracterizados como ocitocinérgicos.

Figura 9. Distinção fenotípica utilizando protocolo eletrofisiológico: A, B e C traçados representativos de neurônios ocitocinérgicos distinguidos pela presença de correntes retificadoras de saída (SOR), entrada (IR) ou ambas (SOR/IR) obtidos em resposta a pulsos de voltagem hiperpolarizantes de -40 mV à -135 mV em passos de -10 mV. D – traçado representativo de um neurônio vasopressinérgico. Os gráficos de barras resumem os diferentes tipos de correntes encontradas (E) e a separação fenotípica de acordo com o protocolo eletrofisiológico (F).

4.3 Correlações do Fenótipo Eletrofisiológico com Molecular

Após cada registro eletrofisiológico, o citoplasma da célula diluído, na solução de pipeta, foi imediatamente coletado para transcrição reversa. Todavia, como a quantidade RNAm coletado de uma única célula é relativamente pequena, procedemos a pré-amplificação do material. Assim sendo, inicialmente nos preocupamos em avaliar se este processo poderia alterar o fenótipo celular durante as análises. Para tanto registramos um novo grupo de 10 células, coletamos o citoplasma e uma primeira pré-amplificação foi feita. Em sequência

realizamos a quantificação pelo PCR e o fenótipo foi determinado pela razão dos cDNAs (métodos, seção 3.5.3). Utilizando as mesmas amostras, uma segunda pré-amplificação foi feita e novamente elas foram quantificadas e o fenótipo determinado. Como podemos observar na tabela 1 a etapa de pré-amplificação das amostras não alterou o fenótipo celular.

Amostra	Fenótipo: 1° Pré-Amp	Fenótipo: 2° Pré-Amp
1	Intermediário	Intermediário
2	VP	VP
3	VP	VP
4	OT	OT
5	VP	VP
6	VP	VP
7	VP	VP
8	VP	VP
9	OT	OT
10	Intermediário	Intermediário

Tabela 1. Controle positivo da pré-amplificação: Neurônios magnocelulares do SON foram pré-amplificados 2 vezes. Como mostrado, o fenótipo foi mantido, demonstrando que este procedimento não altera a proporção de RNAm dos neurônios.

Com a confiabilidade do método de pré-amplificação estabelecida, demos sequência a distinção fenotípica dos neurônios registrados previamente e observamos que dos 74 neurônios eletrofisiologicamente caracterizados, 13 tiveram $\frac{X_{VP,O}}{X_{OT,O}} < 1$ indicando uma maior expressão de RNAm para ocitocina. A média $\pm$ epm para estas células foram de $0,07 \pm 0,02$ variando de 0,002 a 0,3. Quarenta e três neurônios apresentaram $\frac{X_{VP,O}}{X_{OT,O}} > 1$ e foram identificados como vasopressinérgicos (razões variaram de 80,7 a 3097, com média de $554,3 \pm 205$). Além disso, oito neurônios apresentaram $\frac{X_{VP,O}}{X_{OT,O}} \sim 1$ (média = $1,2 \pm 0,1$ variando de 0,7 a 1,7) e foram identificados como neurônios intermediários (Figura 10).

Figura 10. Identificação de neurônios magnocelulares pelo fenótipo molecular: Em um total de 74 neurônios registrados utilizando o protocolo eletrofisiológico, 43 foram caracterizados molecularmente como vasopressinérgicos, 13 como ocitocinérgicos e 18 como intermediários, expressando ambos os RNAm. Para maiores detalhes da análise e caracterização dos fenótipos utilizando PCR em tempo real, ver metodotos 3.5.

Correlacionando os dois métodos utilizados, podemos observar que dos 11 neurônios identificados como vasopressinérgicos pela eletrofisiologia, sete também foram caracterizados com o mesmo fenótipo pelo RT-qPCR; 1 foi identificado com ocitocinérgico, e 3 como intermediários. A nossa surpresa ficou por conta dos 63 neurônios identificados como ocitocinérgicos pela eletrofisiologia, uma vez que, daqueles, 36 neurônios foram molecularmente identificados como vasopressinérgicos, 15 intermediários e somente 12 foram identificados como sendo neurônios OT. Como podemos observar a análise molecular não corroborou os resultados eletrofisiológicos. Assim, não foi possível estabelecer qualquer relação segura entre a resposta elétrica e a característica molecular. A figura 11 mostra, graficamente, a correlação entre os dois métodos.

Figura 11. Correlação eletrofisiológica molecular. O gráfico de barras compara o fenótipo de 74 neurônios identificados tanto pela eletrofisiologia (barras pretas) quanto pelo RT-qPCR (barras cinza). De 11 neurônios identificados como vasopressinérgico pela eletrofisiologia, 9% (n=1) foi identificado como ocitocinérgicos, 64% (n=7) foram vasopressinérgicos e 27% (n=3) como intermediários pelo RT-qPCR. Por outro lado, de 63 neurônios eletrofisiologicamente identificados como neurônios ocitocinérgicos, 57% (n=36) foram identificados como vasopressinérgicos, 24% (n=15) como intermediários e somente 19% (n=12) como ocitocinérgicos.

Tais resultados colocam em dúvida a confiabilidade do método eletrofisiológico. Todavia, é importante salientar que como a expressão de RNAm não é equivalente à quantidade de neuropeptídeos sintetizados, experimentos capazes de avaliar o fenótipo pela expressão de RNAm e ao mesmo tempo pelo neuropeptídeo sintetizado, são de suma importância para caracterizar o fenótipo dos neurônios com mais precisão. Assim, para resolvermos este problema, nós realizamos experimentos que englobaram: registros eletrofisiológicos, PCR em tempo real, e marcação do neurônio registrado com o traçador biocitina, para posterior análise por imunofluorescência, em um único neurônio. Estes experimentos são importantes para podermos correlacionar a expressão de RNAm com a síntese de peptídeo em cada fenótipo encontrado e compararmos, mais precisamente, com os resultados do protocolo eletrofisiológico.

4.4 Tripla Caracterização de um Único Neurônio

Utilizando a tripla caracterização, 22 neurônios foram eletrofisiologicamente identificados, o citoplasma foi coletado para análise do fenótipo pelo RT-qPCR, e por fim, os neurônios previamente marcados com biocitina foram identificados utilizando a imunofluorescência. Encontramos 11 neurônios imunorreativos para vasopressina e 11 para ocitocina. Inicialmente, quando comparamos esses dados com a biologia molecular, observamos que das células identificadas pela imunofluorescência como vasopressinérgicas, 9 também o foram pelo RT-qPCR, e os dois restantes foram caracterizados como intermediários. Já em relação aos neurônios identificados como ocitocinérgicos pela imunofluorescência, o mesmo fenótipo foi encontrado com o RT-qPCR (Figura 12).

Figura 12. Caracterização fenotípica de neurônios magnocelulares: Fotomicrografias representativas obtidas de dois neurônios imunohistoquimicamente identificados. Os neurônios foram preenchidos com biocitina (0,2 %) durante o registro eletrofisiológico e revelados pela estreptavidina conjugada com Alexa fluor 350 (vermelho –**A1** e **B1**). Em seguida, os neurônios foram marcados para vasopressina (**A2** e **B2**) e ocitocina (**A3** e **B3**) e a identificação do fenótipo foi feita pela colocalização (**A4** e **B4**). Os painéis superiores mostram um neurônio registrado eletrofisiologicamente e imunohistoquimicamente positivo para ocitocina e os inferiores para vasopressina. A barra branca corresponde a 15 μ m. O gráfico de barras representa a distribuição fenotípica dos neurônios de acordo com cada técnica: imunofluorescência, RT-qPCR e com o protocolo eletrofisiológico (**C**). Uma alta correlação entre a imunofluorescência e qPCR pode ser observada, no entanto o mesmo não é verdadeiro quando comparamos estas técnicas com a eletrofisiologia.

Todavia, com a eletrofisiologia, notamos que a maioria das células registradas apresentaram retificações de entrada, saída ou ambas (n=19), e somente 3 não apresentaram

retificações. Quando comparamos estes resultados com os dois métodos anteriores, nós observamos que somente 10 células (1 VP e 9 OT) tiveram correlação com imunofluorescência, e 8 com PCR e imunofluorescência, sendo os outros 2 neurônios caracterizados como intermediários pelo RT-qPCR (Tabela 2, figura 12C).

# Cel	Eletrofisiologia	RT-qPCR Razão/Fenótipo	Imunofluorescência
1	IR (OT)	3,10 (VP)	VP
2	IR (OT)	* (OT)	OT
3	IR (OT)	143,6 (VP)	VP
4	SOR (OT)	* (VP)	VP
5	IR (OT)	0,02 (OT)	OT
6	SOR/IR (OT)	0,01 (OT)	OT
7	LINEAR (VP)	0,01 (OT)	OT
8	IR (OT)	0,01 (OT)	OT
9	SOR (OT)	25,61 (VP)	VP
10	LINEAR (VP)	4,91 (VP)	VP
11	IR (OT)	1,2 (Intermediário)	OT
12	IR (OT)	* (VP)	VP
13	LINEAR (VP)	0,01 (OT)	OT
14	SOR (OT)	* (VP)	VP
15	SOR (OT)	0,03 (OT)	OT
16	IR (OT)	0,01 (OT)	OT
17	IR (OT)	1,4 (Intermediário)	OT
18	IR (OT)	* (VP)	VP
19	IR (OT)	* (VP)	VP
20	IR (OT)	0,05 (OT)	OT
21	IR (OT)	75,69 (VP)	VP
22	IR (OT)	25,31 (VP)	VP

Tabela 2. Tripla caracterização de um único neurônio. A tabela mostra o fenótipo identificado por três métodos distintos: Eletrofisiologia, RT-qPCR e imunofluorescência em um único neurônio. Note a alta correlação entre o RT-qPCR e Imunofluorescência e a baixa correlação de ambos com a eletrofisiologia. * neurônios que expressaram somente um tipo de RNAm.

4.5 Caracterização Fenotípica de Neurônios Magnocelulares do Núcleo Paraventricular

4.5.1 Caracterização Eletrofisiológica

Uma vez que o núcleo paraventricular também é composto por neurônios magnocelulares, nos preocupamos em avaliar a confiabilidade do protocolo eletrofisiológico também para este grupo de neurônios. Os mesmos procedimentos eletrofisiológicos e de RT-qPCR descritos para o SON foram seguidos nesta etapa de experimentos. Levando em consideração que neurônios magnocelulares da porção lateral do PVN são na sua maioria vasopressinérgicos (76, 86, 122), optamos por caracteriza-los e de acordo com o protocolo eletrofisiológico, esperaríamos encontrar um grande numero de neurônios com relações I–V lineares, entretanto, dos 22 neurônios registrados 19 deles apresentaram correntes retificadoras. Assim, como pode ser observado na figura 13 para neurônios magnocelulares da porção lateral do PVN também obtivemos os 4 tipos de respostas, ou seja, das 22 células analisadas, 5 delas apresentaram SOR, 11 IR, 3 ambas as retificações (SOR/IR) e somente 3 apresentaram relações I–V lineares. Portanto, classificando os neurônios de acordo com este protocolo, 19 foram caracterizados como ocitocinérgicos e 3 como vasopressinérgicos (Figura 13).

Figura 13. Identificação fenotípica de neurônios magnocelulares do PVN lateral pela eletrofisiologia. Neurônios magnocelulares do PVN lateral (n=22) foram identificados pelo protocolo eletrofisiológico, seguindo os mesmos critérios descritos na seção 3.3 para neurônios do SON. Quatro relações *I-V* foram encontradas no PVN lateral, sendo três delas correntes retificadoras, e uma linear (A, B, C e D respectivamente). A1, B1, C1 e D1 mostram as médias das relações *I-V* para cada caso encontrado. Dos 22 neurônios registrados, 19 apresentaram correntes retificadoras (5 SOR; 11 IR e 3 SOR/IR) que caracterizaram essas células como ocitocinérgicas, e 3 tiveram uma relação *I-V* linear e foram identificados como vasopressinérgicas. O gráfico de barras resume os dados encontrados em relação às retificações (E) e a classificação fenotípica, de acordo com o protocolo utilizado (F).

4.5.2 Caracterização Fenotípica do PVNmL pelo RT-qPCR

Em relação à caracterização molecular dos 22 neurônios previamente analisados pela eletrofisiologia, 16 deles foram identificados como vasopressinérgicos com $\frac{X_{VP,0}}{X_{OT,0}} > 1$ (variando entre 109,7 a 2296,3, com média = $4433,4 \pm 2849,8$) sendo que 6 dos 16

expressaram somente RNAm para vasopressina. Quatro neurônios tiveram $\frac{X_{VP,O}}{X_{OT,O}} < 1$ (0,0007 a 0,04, com media= $0,012 \pm 0,009$) e foram identificados como neurônios ocitocinérgicos. Os dois neurônios restantes foram identificados como intermediários, $\frac{X_{VP,O}}{X_{OT,O}} \sim 1$ (média = $0,8 \pm 0,6$; de 0,15 a 1,5). A comparação dos fenótipos descrita pelo protocolo eletrofisiológico e RT-qPCR para célula única, novamente, demonstrou baixa correlação, com somente quatro neurônios descritos com o mesmo fenótipo em ambos os métodos utilizados (Figura 14).

Figura 14. Caracterização molecular do fenótipo dos neurônios magnocelulares do PVN lateral.

A mostra a distribuição do fenótipo molecular dos neurônios previamente distinguidos pelo protocolo eletrofisiológico. Das três células identificadas como vasopressinérgica pela eletrofisiologia, 2 também foram caracterizadas como vasopressinérgica pelo RT-qPCR e uma como ocitocinérgica (**B**). Entretanto das 19 células ocitocinérgicas (presença das correntes retificadoras), somente 3 foram identificadas como ocitocinérgica pelo RT-qPCR. Seis células expressaram somente RNAm para vasopressina.

Isso significa que do total de neurônios identificados com o protocolo eletrofisiológico, 75% dos neurônios do SON e 77% dos neurônios do PVN lateral não corresponderam ao fenótipo molecular obtido pelo RT-qPCR.

4.6 Implicações Fisiológicas

A importância de caracterizar adequadamente o fenótipo dos neurônios magnocelulares pode ser facilmente apreciada em condições onde o equilíbrio hidroeletrólítico é desafiado. Sob estas condições, é razoável supor que os neurônios magnocelulares, expressando RNAm tanto para OT quanto para VP, priorizem a síntese e a liberação do peptídeo necessário para o reestabelecimento de equilíbrio hidroeletrólítico. Ou seja, a proporção dos fenótipos do SON poderia ser alterada. Portanto, para testar essa hipótese ratos foram submetidos à restrição hídrica ou à sobrecarga salina durante 7 dias (ver métodos 3.5.6). Em sequência, a proporção fenotípica em cada condição experimental foi avaliada e comparada com o grupo de animais controle. Como podemos observar na figura 15, nos animais submetidos a 7 dias de restrição hídrica houve um aumento de neurônios imunorreativos para vasopressina e uma redução no número de neurônios imunorreativos para ocitocina quando os comparamos com animais controle (VP= $60,4 \pm 3$ vs $84,1 \pm 5,3$ e OT = $61,7 \pm 3$ vs $48,5 \pm 1,6$; $p < 0,005$ e $p < 0,05$. Controle vs restrição hídrica, respectivamente). Entretanto, em relação ao grupo de animais submetidos a sobrecarga salina, nós não observamos diferenças significativas na proporção de neurônios VP ($60,4 \pm 3$ vs $64,5 \pm 4$; $p > 0,05$) e OT ($61,7 \pm 3$ vs $54 \pm 3,7$; $p > 0,05$) quando os comparamos com o grupo controle.

Figura 15. Desequilíbrio hidroeletrólítico altera a proporção de fenótipos dos MNCs. Fotomicrografias mostrando neurônios imunorreativos para VP (vermelho) e OT (verde) em situação controle (A), após restrição hídrica (B), e sobrecarga salina (C). A3- C3 representa a sobreposição das imagens. O Gráfico de barras em D resume o número de neurônios imunorreativos encontrados para cada condição experimental. A estatística foi realizada utilizando ANOVA One Way não paramétrica com pós-teste de Bonferroni ** $p < 0,005$. Barras horizontais = 15 μ m. OC = quiasma óptico.

Dos resultados até aqui apresentados, podemos concluir que o protocolo eletrofisiológico não é eficaz na discriminação dos fenótipos dos neurônios magnocelulares, quando se utiliza a configuração *whole cell*. Além disso, a caracterização do fenótipo neuronal utilizando o protocolo eletrofisiológico se torna crítico em situações onde a proporção fenotípica esta alterada. Já com relação aos resultados obtidos com as células gliais, o envolvimento destas na modulação nitrérgica dos MNCs parece interferir somente em neurônios ocitocinérgicos uma vez que nos vasopressinérgicos a L-arginina produziu o mesmo efeito observado previamente por nós (34, 147).

Como o RT-qPCR para célula única demonstrou 83 % de coerência entre a expressão de RNAm e a síntese de peptídeos para os neurônios do SON, este foi adotado por nós para caracterizar o fenótipo celular nos próximos experimentos.

4.7 Canais HCN e Modulação Nitrérgica

Uma vez que alterações na atividade intrínseca dos neurônios magnocelulares durante a modulação nitrérgica ocorrem por meio dos canais de cátions ativados por hiperpolarização e nucleotídeos cíclicos (HCN) (130), avaliamos ainda se esta modulação persiste quando a interação neurônio-glia está ausente. Para tal, utilizamos neurônios magnocelulares isolados e avaliamos o efeito do óxido nítrico sobre os canais HCN. A figura 16 apresenta os resultados obtidos. Aplicação de 500 μ M de L-arginina produziu uma redução significativa na corrente estacionária (-19 ± 5 pA vs $-6,8 \pm 1,8$ pA, em -135 mV). Adicionalmente, ajustando uma exponencial simples para calcular a constante de tempo do componente do processo de ativação da corrente I_h , nós observamos que na presença da L-arginina houve um aumento significativo quando comparado com o controle ($109,6 \pm 26$ ms vs $167,7 \pm 32$ ms, em -135 mV; $n=6$, $*p<0,05$). Assim, esses dados demonstraram que a modulação nitrérgica nos canais HCN ocorre independentemente das células gliais.

Figura 16. Efeito do óxido nítrico nos canais HCN em neurônios magnocelulares isolados. Em **A**, estão representados traçados típicos obtidos em um neurônio do grupo demonstrando o efeito da superfusão com L-arginina, seguida da aplicação do bloqueador específico ZD7288. Observa-se uma redução da corrente total sensível ao ZD7288. Em **D,E** a corrente subtraída (L-arginina – ZD7288) é evidenciada numa relação corrente voltagem e comparada com o grupo controle. Na relação *I-V* em **B**, nota-se que quando os neurônios são submetidos a L-arginina (500 μ M) há uma redução expressiva da amplitude da corrente total quando comparada com o controle (-19 ± 5 pA x $-6,8 \pm 1,8$ pA, em -135 mV, $n=9$ $p<0,05$). Em **C**, traçado representativo de um neurônio do grupo demonstrando a ativação da corrente, em -135 mV, ajustada com uma exponencial simples (em vermelho) para calcular a constante de tempo do processo. O grupo controle está representado em preto e o grupo submetido a L-arginina está representado em cinza. O gráfico de barras, em **D**, resume a média do grupo no controle e após o tratamento com L-arginina. Note o aumento na constante de tempo na presença da L-arginina.

4.8 Neurônios Magnocelulares do SON Produzem NO

Como observamos nos experimentos anteriores, a modulação nitrérgica da corrente I_h ocorre na ausência das células gliais. Entretanto, considerando que tais células são capazes de produzir NO (100), nossa próxima pergunta foi: Onde é produzido o NO?

Como a sintase do óxido nítrico foi encontrada nos neurônios magnocelulares e sua expressão no hipotálamo está correlacionada com aumentos da osmolalidade plasmática (21, 144) o próximo passo consistiu em avaliar se os neurônios do SON são capazes de produzir óxido nítrico e este por sua vez atuar autocrinamente ou paracrinamente. Inicialmente, foram feitos experimentos de fluorescência com neurônios isolados para verificar a produção de NO, em condições de aumento da osmolalidade, sem a interferência dos astrócitos. Para tanto, os neurônios foram dissociados e incubados com um marcador fluorescente da produção de NO (DAF-FM – 10 μ M) e com auxílio da microscopia confocal nós observamos as alterações na produção do NO durante uma situação controle (solução de Hank – Ver metodologia) seguido de um estímulo hiperosmótico de 330 mosm/(kg.H₂O) (Solução de Hank ajustada com manitol) e como controle positivo nós utilizamos um doador de NO (SIN 1, 100 μ M).

Como demonstrado na figura 17, durante o estímulo hipertônico houve um aumento na fluorescência indicando aumento na produção de NO. Para demonstrar que o aumento na produção de NO foi devido ao estímulo hipertônico, nós utilizamos um doador de NO (SIN1 100 μ M) como controle positivo e observamos um aumento ainda maior.

Figura 17. Hipertonicidade aumenta a produção de NO em neurônios do supraóptico. A - Evolução temporal da produção de NO de um único neurônio isolado do grupo na condição controle, durante o estímulo hipertônico e após tratamento com doador de NO (SIN1 100 μ M). As figuras no topo do gráfico representam “*surface plots*” do mesmo neurônio em cada condição experimental e a barra colorida, de cores frias a cores mais quentes, indica o aumento na produção de NO. **B** média $\Delta F/F_0$ (média $\pm$ epm) obtida de 13 células em 3 experimentos independentes. $\Delta F = (F - F_0)$ é a diferença entre a fluorescência obtida em um intervalo de tempo (F) e a fluorescência de fundo (“*background*”) (F_0). A média dos valores foi calculada com os maiores valores de $\Delta F/F_0$ para cada situação.

A identificação dos neurônios isolados como sendo magnocelulares foi realizada através de experimentos de imunofluorescência. Como mostrado na figura 18, nós encontramos neurônios com morfologias distintas, os circulares e os neurônios com axônios e dendritos. Quanto à marcação dos neurônios para determinação do fenótipo celular, observamos que apesar da especificidade dos anticorpos, varias colocalizações foram observadas.

Figura 18. Imunofluorescência para neurônios vasopressinérgicos e ocitocinérgicos do SON isolados. Os neurônios isolados foram incubados com anticorpos específicos para identificação fenotípica (vasopressina em vermelho e ocitocina em verde). A colocalização é mostrada em amarelo em **F** e **I**. Dois tipos de morfologia neuronal foram identificados após 24 horas em cultura: Neurônios circulares (**D**) e com axônios e dendritos (**G**). **A**, **B** e **C** são experimentos controles somente com anticorpo secundário e na presença de DAPI (marcador nuclear). Escala: 7,5 μm (**A-F**) e 25 μm (**G-I**).

Frente ao alto número de neurônios colocalizados e sabendo que os neurônios magnocelulares do núcleo supraóptico possuem neurônios com fenótipos intermediários, isto é, produzem os dois peptídeos, seria razoável pensar em um neurônio isolado com fenótipo intermediário. Todavia, a porcentagem de colocalização, indicando duplo fenótipo em condições basais é baixa e neste experimento observamos que maior parte células tiveram colocalização (~70%). Assim, na tentativa de sanar este problema nós repetimos o mesmo tipo de experimento, utilizando fatias do SON e avaliamos as colocalizações com o intuito de

investigar se o processo de dissociação dos neurônios estava alterando sua fisiologia. Como demonstrado na figura 19, utilizando os mesmos anticorpos, para imunofluorescência em fatias, nós não observamos a mesma intensidade de colocalizações.

Figura 19. Imunofluorescência em fatias do SON. **A**, neurônios vasopressinérgicos marcados com anticorpo anti-VP (1:500 Guinea Pig anti-(Arg⁸)-Vasopressina - Vermelho). **B**, neurônios marcado com anticorpo anti-OT (1:500 anti Rabbit - Verde). **C**, experimento controle somente com anticorpo secundário. Colocalização é mostrada em **D**. Note a ausência de colocalizações. **QO** = quiasma óptico.

Devido ao fato de não termos observado a mesma proporção de colocalizações na imunofluorescência com neurônios isolados e nas fatias, nós optamos por repetir a fluorescência na presença do DAF-FM e observar a produção de NO em fatias evitando assim alterações que possam ocorrer na fisiologia do neurônio durante o processo de dissociação. Os dados demonstram que durante o estímulo hipertônico ocorre um aumento na fluorescência indicando maior produção de NO que retorna aos níveis basais quando retiramos o estímulo.

Por outro lado, na presença do inibidor da enzima sintase do óxido nítrico não observamos aumentos na produção de NO (Figura 20) confirmando a especificidade do estímulo.

Figura 20. Hipertonicidade estimula a produção de NO nos neurônios magnocelulares do SON.

Em **A**, evolução temporal da produção do NO durante controle, o estímulo hipertônico e lavagem (círculos preenchidos) ou na presença do inibidor da sintase do óxido nítrico (círculos vazios). Durante o estímulo hipertônico houve um aumento da fluorescência e sua volta próximo aos níveis basais ocorreu com a lavagem, efeito não observado na presença do L-NAME. **B** resume os resultados obtidos em cada situação experimental. $\Delta F = (F - F_0)$ é a diferença entre a fluorescência obtida em um intervalo de tempo (F) e a fluorescência de fundo (“background”) (F_0). A média dos valores foi calculada com os maiores valores de $\Delta F/F_0$ para cada situação.

Com esses experimentos nós evidenciamos que os neurônios magnocelulares são capazes de produzir óxido nítrico em resposta a aumentos da osmolalidade, e os neurônios isolados nos permitem inferir que esta produção ocorre independente das células gliais. Entretanto, uma vez que os neurônios magnocelulares são osmosensíveis, o aumento na produção de NO pode ser atribuído, em princípio, ao aumento da frequência de disparo dos neurônios ocasionada pelo estímulo hipertônico. Desta forma, para dissociar os dois fenômenos nós repetimos o experimento anterior, mas ao mesmo tempo um neurônio que estava carregado com o indicador fluorescente DAF-FM foi registrado, e quando o estímulo hipertônico foi aplicado, injeções de corrente negativa foram aplicadas com o intuito de hiperpolarizar o neurônio evitando assim que o mesmo aumentasse sua frequência de disparo.

Desta forma, o neurônio permaneceu silente durante todo o estímulo. Como pode ser observado na figura 21 A e A1 o estímulo hipertônico novamente induziu um aumento na fluorescência, mesmo quando o neurônio estava silente. Estes resultados demonstram que o estímulo hipertônico por si só foi capaz de aumentar a produção de NO nos neurônios magnocelulares do núcleo supraóptico independentemente da frequência de disparo. Experimentos controles utilizando solução isotônica e despolarização do neurônio por injeção de corrente na ausência da hipertonicidade, também foram feitos e como demonstrado na figura 21 B, B1, C e C1 não houve alterações na fluorescência reforçando nossa hipótese de um efeito específico da hipertonicidade na produção de NO em neurônios magnocelulares do núcleo supraóptico.

Figura 21. Frequência de disparo não determina a produção de NO. Frequência de disparo dos neurônios foi alterada por injeções de corrente hiperpolarizante (diminuição) ou despolarizante (aumento) em células submetidas a condições de tonicidade indicados em cada situação experimental. (A) Decurso temporal da produção de NO em condições isotônicas e em repouso, após exposição das células a hipertonicidade e hiperpolarização e ao voltar para isotonicidade, como indicado pelas setas. (B) Fluorescência medida sob condições isotônicas e despolarização da célula, a fim de aumentar a

frequência de disparo. O painel (C) mostra uma situação em que as células foram expostas a hipotonicidade. A fluorescência das células registrada está representada pelos círculos abertos (**A1**, **B1** n=7 e **C1** n = 8) e as células vizinhas que serviram como controle positivo por círculos fechados (**A1** n=32; **B1** n=101 e **C1** n=86). Os gráficos de barras resumem a frequência de disparo dos neurônios registrados para cada caso. Os traçados nos gráficos são representativos de cada situação e as escalas de voltagem e tempo mostradas em C são válidas para todos os registros. Os dados foram expressos como média $\pm$ epm.

Resumidamente, os resultados até aqui apresentados nos permitem duas conclusões: 1) Durante aumentos da osmolalidade, há um aumento na produção de NO nos neurônios magnocelulares do SON, sugerindo uma ação parácrina e/ou autócrina e 2) A modulação nitrérgica dos canais HCN nos neurônios magnocelulares do núcleo não envolve células gliais. Frente a estas evidências as seguintes perguntas foram feitas: 1) Qual isoforma dos canais HCN é expressa nos SON? 2) Qual a importância funcional destes canais nos MNCs?, e 3) Qual a via de sinalização utilizada pelo NO na modulação desses canais?

4.9 Expressão dos Canais HCN por RT-qPCR Single Cell

As quatro isoformas dos canais HCN já foram descritas no sistema nervoso central (71). Entretanto, no SON há uma controvérsia quanto à isoforma predominante (96, 102). Nesse sentido, nós inicialmente realizamos experimentos com “single cell RT-qPCR” para avaliarmos a isoforma dominante. De trinta neurônios analisados nós observamos que 17 expressaram mais de uma isoforma e 13 somente uma delas. Os fenótipos das mesmas células foram molecularmente identificados, mas não encontramos diferenças de expressão entre eles. Como podemos observar na tabela 3, os MNCs vasopressinérgicos e ocitocinérgicos expressam RNAm para as quatro isoformas, sendo a do tipo 3 e 4 as predominantes.

# Célula	HCN1	HCN2	HCN3	HCN4	Fenótipo
1		●	●	●	OT
2				●	VP
3		●	●	●	VP
4			●		VP
5			●		OT
6		●			VP
7		●		●	VP
8			●		OT
9	●			●	OT
10			●		OT
11	●		●	●	VP
12				●	VP
13	●		●	●	VP
14		●		●	VP
15			●		VP
16		●		●	VP
17			●	●	VP
18				●	VP
19				●	OT
20			●	●	IT
21	●			●	OT
22	●		●	●	OT
23	●	●	●		OT
24		●	●		IT
25				●	VP
26		●			VP
27		●	●		VP
28		●	●		IT
29		●	●		VP
30		●			VP

Tabela 3. Expressão RNAm para isoformas dos canais HCN. Expressão dos canais HCN no supraóptico foi realizada por meio de *single cell* RT-qPCR. Neurônios vasopressinérgicos e ocitocinérgicos expressaram todas as isoformas, porém a do tipo 3 e 4 predominaram. Círculos coloridos indicam a expressão de uma determinada isoforma para cada célula analisada. Círculos Amarelos = HCN1, Vermelhos = HCN2, Verdes = HCN3 e Azuis = HCN4.

4.10 Efeitos Macroscópicos do ZD7288

Estudos electrofisiológicos demonstraram que os canais HCN são responsáveis por manter o nível do potencial de repouso em vários tipos de neurônios (43, 50, 125). Desta forma, para avaliarmos o papel funcional dos canais HCN na excitabilidade dos MNCs, registramos a atividade intrínseca dos neurônios magnocelulares em condição controle e na

presença do ZD7288, bloqueador desses canais. Como demonstrado na figura 22 A, B e C, houve uma redução significativa na frequência de disparo dos neurônios quando o ZD7288 foi adicionado à solução de perfusão ($4,3 \pm 0,5$ Hz vs $2,7 \pm 0,4$ Hz, $n=13$; $p<0,05$), demonstrando uma participação destes na excitabilidade dos MNCs. Adicionalmente, o número de potenciais de ação em resposta a valores crescentes de correntes positivas foi reduzido na presença do ZD7288 quando comparamos com o grupo controle ($73,7 \pm 2,4$ vs $64,1 \pm 2,9$ $n=12$; $p<0,05$), mais uma vez, demonstrando uma redução da excitabilidade dos neurônios (Figura 22 E e E1). Por outro lado, a resposta dos neurônios a injeções de corrente negativa (Figura 22 D) mostrou que a resistência de entrada dos mesmos, não foi estatisticamente diferente entre os grupos (Controle = $1,1 \pm 0,5$ G Ω vs ZD7288 = $1,0 \pm 0,4$ G Ω , $n=12$; $p>0,05$).

Figura 22. Canais HCN contribuem para excitabilidade dos neurônios do SON. Traçado representativo de um neurônio do grupo ilustrando os efeitos do ZD7288 na frequência de disparo dos MNCs e no número de potenciais durante injeções de corrente positiva (**A**, **E** e **E1**). Em **C**, o mesmo traçado exposto em **A**, ampliado para visualizar melhor os efeitos do ZD7288 na cinética do potencial de ação. A linha vermelha representa o ajuste de uma exponencial simples na fase de despolarização pós-potencial, e como demonstrado o ZD parece reduzir a constante de tempo deste processo. Os gráficos em **B** e **E2** resumem a média $\pm$ epm de 12 neurônios registrados. Como demonstrado em **D**, **D1** e **D2** a resistência de entrada dos neurônios, medida por injeções de corrente negativa (0 a -50 pA) não foi significativamente alterada na presença do ZD quando comparado ao controle.

4.11 Vias de Sinalização do NO

Como os canais HCN possuem sítios de ligação para cGMP, e este por sua vez, tem sido apontado na literatura como principal via de sinalização do NO, avaliamos se o mecanismo de modulação nitrérgica nesses canais, envolve a formação deste segundo mensageiro. Para tanto, utilizamos um inibidor seletivo da guanilato ciclase solúvel, H-(1,2,4) oxadiazolo[4,3-a] quinoxalin-1-one – ODQ (10 μ M, 30 μ M e 100 μ M), mas nenhuma alteração e evidência significativa de modulação endógena do NO utilizando essa via de sinalização foi observado (Figura 23). Também não observamos diferenças de modulação em relação ao fenótipo celular caracterizado pelo RT-qPCR em célula única.

Figura 23. Modulação nitrérgica nos canais HCN é independente de cGMP. A, B e C representam traçados de corrente evocados por pulsos de voltagem de -50 a -135 mV, com duração de 3 segundos, na presença de três concentrações distintas do inibidor da guanilato ciclase solúvel (ODQ 3 μ M, 30 μ M e 100 μ M), seguido do inibidor da corrente I_h , ZD7288 (50 μ M). Correntes I_h obtidas pela subtração digital (d,e). A1, B1 e C1 relação corrente x voltagem para o componente sensível a ZD7288. Não foi observado alterações significativas na corrente I_h em nenhuma das concentrações utilizadas. Controle vs ODQ: 3 μ M ($-33,3 \pm 7,6$ pA vs $33 \pm 6,8$ pA), 30 μ M ($30,6 \pm 4$ pA vs $-37,2 \pm 5,4$ pA) e 100 μ M ($-48,7 \pm 8,7$ pA vs $-45,3 \pm 13,3$ pA). $p > 0,05$ para todas as concentrações. Oito células foram registradas para cada concentração utilizada.

A partir desses resultados, utilizamos a concentração intermediária de ODQ (30 μ M) testada anteriormente, e acrescentamos à solução de perfusão o substrato do NO, L-arginina. Como demonstrado na figura 24, observamos que mesmo na presença do inibidor da guanilato ciclase, a L-arginina foi capaz de reduzir significativamente a corrente I_h , tanto em neurônios VP quanto OT, apontando, assim, uma via direta de modulação ou o envolvimento de outros segundos mensageiros, ainda desconhecidos.

Figura 24. Modulação nitrérgica dos canais HCN é independente de cGMP. A relação $I-V$ foi construída utilizando-se o mesmo protocolo descrito anteriormente e demonstra o efeito da L-arginina na presença do inibidor da guanilato ciclase. Mesmo na presença do bloqueador a L-arginina reduziu significativamente a corrente I_h sugerindo uma via independente de cGMP. (-48 ± 14 pA x $-24,7 \pm 10$ pA, $n=5$). * $p < 0,05$ - Teste t pareado.

4.12 Óxido Nítrico Modula os Canais HCN por Mecanismo de S-nitrosilação

A visão clássica de que o cGMP é o único mediador da modulação nitrérgica tem sido questionada nos últimos anos. Uma nova via de modulação, a S-nitrosilação, tem sido proposta como via alternativa (3). A S-nitrosilação é um mecanismo importante para estabilizar a reação do NO, prolongando sua atividade biológica, e tem sido apontada como uma modificação pós-traducional chave em canais iônicos (60) incluindo os HCN (155). Diante disso, além de investigarmos o envolvimento do cGMP na modulação nitrérgica, também investigamos se essa modulação ocorre por este mecanismos não convencional. Para tanto, utilizamos a técnica de *patch clamp* em modo *voltage clamp* para aplicação de pulsos de voltagem hiperpolarizantes (-50 a -135 mV) a partir de um pulso pré-condicionado de -50 mV. A corrente foi registrada numa condição controle, na presença de L-arginina e na presença de ZD7288 mais L-arginina, como nos registro anteriores expostos acima. Porém, neste caso, utilizamos um bloqueador, a N-etilmaleimida (NEM – 300 μ M), que oxida os resíduos de cisteína impedindo a S-nitrosilação. A figura 25 mostra os resultados obtidos em 11 neurônios magnocelulares na presença do inibidor da S-nitrosilação. Como podemos observar, quando os resíduos de cisteína são oxidados há um aumento na corrente I_h demonstrando que este processo pode ser uma via de modulação dos canais HCN ($-33,2 \pm 10$ pA vs $81,2 \pm 13$ pA em -135 mV, controle e nitrosilado respectivamente, $n = 11$; $p < 0,05$). De fato, quando a L-arginina foi adicionada ao banho não foi observado redução na corrente I_h ($-59,2 \pm 15$ pA vs -59 ± 11 pA em -135 mV; $n=10$; $p > 0,05$), como demonstrado previamente por nós (130), permitindo concluir que os canais HCN podem ser S-nitrosilados (Figura 25 B).

Figura 25. Modulação nitrérgica dos canais HCN é dependente de s-nitrosilação. Em A e B, traçados representativos de registros de corrente I_h na presença do bloqueador NEM, NEM + L-arginina seguido de ZD7288. Gráfico A1 mostra a relação $I-V$ da média de neurônios registrados na presença do bloqueador da S-nitrosilação (NEM – 300 μ M). Note que na presença do bloqueador houve um aumento na corrente basal indicando que o canal é modulado por oxidações dos resíduos de cisteína ($-33,2 \pm 10$ pA vs $81,2 \pm 13$ pA em -135 mV, controle e nitrosilado respectivamente, $n = 11$; $p < 0,05$). Em B1, o gráfico com a relação $I-V$ resume a média dos neurônios registrados na presença da L-arginina demonstrando que na presença do bloqueador da S-nitrosilação a L-arginina não foi capaz de alterar a corrente I_h apontando a via utilizada pelo NO para modular os canais HCN ($-59,2 \pm 15$ pA vs -59 ± 11 pA em -135 mV; $n = 10$; $p > 0,05$). Não foi observado diferenças entre os fenótipos celulares.

Apesar da N-ethylmaleimide ser um oxidante específico para resíduos de cisteína, testamos, ainda, o ácido ascórbico, que também atua como um agente denitrosilante ou redutor (138). Como demonstrado na figura 26, na presença do ácido ascórbico há um aumento na magnitude da corrente ($-9,8 \pm 14$ vs $-16,8 \pm 8,5$, $n = 6$; $p < 0,05$) sugerindo a presença de uma S-nitrosilação endógena.

Figura 26. Neurônios magnocelulares são s-nitrosilados endogenamente. O gráfico $I-V$ mostra o efeito da denitrosilação nos canais HCN. Como podemos observar, na presença do ascorbato houve um aumento na magnitude da corrente I_h indicando uma s-nitrosilação endógena ($-9,8 \pm 14$ vs $-16,8 \pm 8,5$, $n=6$; $p<0,05$). Não foi observado diferenças entre os fenótipos celulares.

4.13 Modulações Nitrérgica dos Canais HCN Durante Estimulo Hipertônico

Agudo

Partindo do princípio que a produção e liberação de VP e OT na circulação sistêmica são modificadas toda vez que ocorrem alterações na osmolalidade plasmática, e que o estímulo osmótico aumenta a produção do NO, nós também avaliamos o se estímulo hipertônico *per se* modulava os canais HCN. Como ilustrado na figura 27, a corrente I_h na presença da solução hipertônica (330 mosm/kg.H₂O) teve um valor médio de $-50,33 \pm 0,9$ pA, (em -135 mV) significativamente maior que na situação controle ($-20,1 \pm 7$ pA, em -135 mV). Observamos ainda, que na presença do bloqueador da NOS, um aumento adicional foi observado demonstrando que o NO produzido nessas condições experimentais tem também um efeito inibitório sobre os canais HCN (Figura 27 B e D).

Figura 27. Hipertonicidade modula os canais HCN em neurônios magnocelulares. Em A e B, traçados típicos obtidos de um neurônio do grupo na condição controle (a), na presença do estímulo hipertônico (b) ou hipertônico mais L-NAME (B-b), seguido da aplicação do bloqueador dos canais HCN ZD7288 (c). d,e representam o componente sensível ao ZD7288 em cada condição experimental. C e D, as relações I-V das médias das correntes sensíveis ao ZD7288 (corrente I_h) nas mesmas condições experimentais descritas acima. Note um aumento significativo da corrente I_h na presença do estímulo hipertônico quando comparado ao controle ($-50,33 \pm 0,9$ pA vs $20,1 \pm 7$ pA, $n=9$), e um aumento adicional na corrente quando o L-NAME foi adicionado ao banho ($-91,7 \pm 14$ vs $-50,33 \pm 0,9$). Diferenças estatísticas estão indicadas por * (* $p < 0,05$; ** $p < 0,005$). Não foi observada diferença entre os fenótipos celulares.

5. DISCUSSÃO

No presente trabalho, nós investigamos os possíveis mecanismos da modulação nitrérgica sobre os canais HCN bem como a origem deste neuromodulador. Até o momento demonstramos que o NO produzido nos neurônios magnocelulares do SON, modula a corrente I_h independentemente de cGMP, não envolve outros tipos de células nesta resposta e não há diferenças na modulação quanto ao fenótipo celular. Além disso, experimentos investigando mecanismos não convencionais de modulação como a S-nitrosilação mostram a presença de uma via de sinalização alternativa do NO. Evidenciamos ainda que aumentos da osmolalidade promoveram um aumento significativo da corrente I_h , mostrando que os canais HCN também são modulados pelo estresse osmótico, e que o NO produzido nessas condições, tem um efeito inibitório sobre os canais HCN. Destacamos ainda a importância de se diferenciar os fenótipos dos neurônios magnocelulares, bem como a sensibilidade de alguns métodos que são utilizados para este fim.

Como a excitabilidade dos MNCs está diretamente relacionada com a quantidade de neuropeptídeos liberados (40, 107), entender os mecanismos que a controlam implica em contribuir para o melhor entendimento da sua fisiologia. Dentre os mecanismos já descritos na literatura, o controle sináptico é um deles, e sua contribuição está relacionada ao balanço entre as entradas excitatórias e inibitórias, mediadas pelos neurotransmissores glutamatérgicos e GABAérgicos, respectivamente (37, 41). No entanto, outros fatores também podem alterar a excitabilidade destes neurônios independentemente da transmissão sináptica. Um deles é a alteração da osmolalidade plasmática, que aumenta ou reduz a excitabilidade neuronal com consequentes alterações na concentração de vasopressina e ocitocina circulante (24). Adicionalmente, tem sido demonstrado que tanto em condições isotônicas quanto durante alterações na osmolalidade plasmática, alguns neuromoduladores são capazes de, direta ou

indiretamente, controlar a excitabilidade dos MNCs (133, 136). Dentre estes o NO tem se mostrado um modulador da excitabilidade destes neurônios tanto via sinapse (141) quanto por ações diretas em canais iônicos (130).

5.1 Caracterização Fenotípica Baseada no Método Eletrofisiológico

Tão importante quanto desvendar os mecanismos de ação do NO em neurônios do SON, é realizar a caracterização adequada do fenótipo celular. Este parâmetro é importante no entendimento da fisiologia dos MNCs uma vez que nossos resultados e de outros tem mostrado que uma mesma molécula pode ter efeitos distintos dependendo do fenótipo celular (62, 120, 165). Eletrofisiologicamente, neurônios VP e OT podem ser distinguidos uns dos outros por vários procedimentos. No entanto, a maior parte dos métodos é baseada na resposta dos neurônios a um determinado peptídeo ou, por exemplo, pela sucção durante a amamentação (7, 54, 118), tornando o processo de diferenciação fenotípica complexo. Outra complicação na diferenciação dos MNCs, baseado apenas na eletrofisiologia surgiu com os estudos de Moos et al (97). Os autores demonstraram que durante a amamentação, alguns neurônios exibiam tanto características fásicas da vasopressina quando atividades em *burst* da ocitocina, características utilizadas para diferenciar os fenótipos na década de 70 a 80 (24, 119, 160). Esta constatação aumentou a possibilidade de que ambos os peptídeos poderiam ser sintetizados na mesma célula. No entanto, a descoberta das correntes retificadoras, supostamente expressa em OT, mas não em neurônios VP (8, 61, 139, 165) tem sido utilizado como critério para distinguir os fenótipos. Como já sugerido acima, este protocolo está sujeito a críticas, uma vez que os neurônios deste núcleo possuem três fenótipos: 1) Vasopressinérgico, caracterizado pela síntese de vasopressina; 2) ocitocinérgico, caracterizado pela síntese de ocitocina; e 3) um terceiro tipo, no qual VP e OT são sintetizados em concentrações equivalentes e são denominados de intermediários (52, 74, 94).

Neste estudo, observamos que de 63 neurônios caracterizados como ocitocinérgicos pela eletrofisiologia, 36 foram identificados como vasopressinérgicos pelo RT-qPCR (Figura 11), evidenciando que o protocolo eletrofisiológico não se coaduna com os resultados obtidos pela biologia molecular. Portanto, os nossos resultados indicam que a presença ou ausência de correntes retificadoras na relação $I-V$ não são parâmetros confiáveis para serem utilizados como identificadores de fenótipos dos MNCs. Nossos dados corroboram os de Zampronio et al. (165) que utilizaram ratos transgênicos expressando eGFP, juntamente com o gene da vasopressina, e observaram que nem todos os neurônios identificados como ocitocinérgicos, devido à ausência de eGFP, apresentavam correntes retificadoras. Esses achados reforçam a ideia de que este tipo de corrente não se restringe aos neurônios OT. Além disso, os neurônios que expressam ambos os peptídeos, conforme indicado por imunofluorescência, não foram incluídos no grupo de neurônios por eles analisados.

Desta forma, os resultados divergentes aqui relatados podem ser atribuídos a 2 fatores: 1) a presença de correntes retificadoras em células vasopressinérgicas e intermediárias, e 2) devido ao fato da primeira descrição do uso das correntes retificadoras e sua correlação com os neurônios ocitocinérgicos (mas não os seguintes, ver Hirasawa et al., 2003 e Zampronio et al., 2010) terem utilizado eletrodos finos, com duração das injeções de corrente 6 vezes maior do que em experimentos de *patch clamp* (8, 139, 140). De fato, estudos comparando experimentos realizados com registros intracelulares através de microeletrodos finos e *whole cell patch clamp*, mostraram que devido a grande corrente de vazamento na interface eletrodo/membrana, observada nos experimentos com eletrodos, a propriedade intrínseca dos neurônios pode mostrar-se alterada levando, por exemplo, a despolarização do potencial de repouso (82, 137). Além disso, estudo mais recente de Li e colaboradores (80) reporta que foram incapazes de replicar os resultados do protocolo eletrofisiológico porque o mesmo não era consistente com registros em *whole cell*.

5.1.2 Tripla Caracterização dos MNCs

Apear da expressão de RNAm ter sido correlacionada e utilizada como estimativa da síntese de proteína/peptídeo, a correlação entre eles ainda é um assunto controverso e os resultados descritos até agora têm sido relativamente inconsistentes. Coeficientes altos e baixos entre os dois parâmetros foram relatados para as proteínas, variando de 0,40 a 0,78 (55, 56, 88), e uma alta correlação para peptídeos (101). Frente a esse impasse, e com o intuito de evitar erros na caracterização fenotípica somente pela expressão de RNAm, foi realizada uma caracterização tripla em um único neurônio, comparando os resultados eletrofisiológicos não apenas com RT- qPCR, mas também com imunofluorescência (ver métodos 3.5). Como mostram a figura 12 e tabela 2, há uma forte correlação entre o fenótipo caracterizado pela imunofluorescência e RT - qPCR. Comparando os resultados obtidos pelos três métodos, observou-se que apenas 10 neurônios identificados pela eletrofisiologia corresponderam aos descritos pela imunofluorescência, e apenas 8 com imunofluorescência e RT- qPCR (Tabela 2). Este fato é particularmente crítico se consideramos situações em que a proporção relativa dos tipos neuronais está alterada.

Como demonstrado, a expressão de RNAm obtido pelo RT-qPCR teve uma correlação de 80% com a imunofluorescência. Contudo, os resultados do protocolo eletrofisiológico não corroboraram aqueles obtidos com imunofluorescência, reforçando a hipótese de que o critério da presença ou ausência de correntes retificadoras em neurônios magnocelulares ocitocinérgicos e vasopressinérgicos, respectivamente, não é determinante para distinguir o fenótipo dos mesmos.

5.1.3 Implicações Fisiológicas

O número de neurônios com fenótipo intermediário encontrado na caracterização pelo RT - qPCR (n=18) não nos surpreendeu, uma vez que 1 % - 3 % dos MNCs co-expressam

ambos os peptídeos sob condições fisiológicas. Além disso, quando o sistema é confrontado com situações de desafio, como lactação, esta percentagem pode aumentar para 24 %, e ambos os peptídeos podem ser encontrados na mesma vesícula neurosecretora. Sob estas condições, Mezey et al (94) sugeriram que os neurônios OT expressando pouca ou nenhuma VP se tornam capazes de sintetizar VP de modo a compensar a perda de água observada durante a lactação. Nossos resultados reforçam esta hipótese, uma vez que ratos submetidos à restrição hídrica mostraram um aumento nos neurônios imunorreativos a VP e uma diminuição no número relativo de neurônios OT (Figura 15). Esta é uma clara indicação de que os neurônios são capazes de alterar seu fenótipo em resposta a uma situação de desafio.

Este fenômeno pode ser explicado, pelo menos em parte, por dois fatores: 1) a presença de RNAm de OT e VP na mesma célula, permitindo que os neurônios sintetizem ambos ou apenas um dos peptídeos quando necessário (através de uma regulação pós-transcricional do RNAm), e/ou 2) por modulações dos genes envolvidos na expressão da OT e VP. Estas hipóteses estão apoiadas por estudos de hibridização *in situ* e RT-qPCR, confirmando a expressão de ambos os RNAs mensageiros na maioria dos MNCs, com um aumento evidente em um deles, quando o sistema é osmoticamente estressado (52, 73, 92, 159). A coexistência de ambos RNAm (s) parece ser uma propriedade de neurônios magnocelulares, uma vez que neurônios magnocelulares do núcleo paraventricular lateral também expressam RNAm para VP e OT (Figura 14), enquanto os neurônios vasopressinérgicos parvocelulares do núcleo supraquiasmático não expressam RNAm para OT (52). Em relação à modulação gênica, dados da literatura mostram que os genes que codificam VP e OT estão no mesmo cromossomo separados apenas por uma região intergênica, e são processados em sentidos de transcrição opostos (57, 95). O que se sabe ainda é que a identidade molecular fenotípica desses neurônios é regulada por genes específicos, e o consenso é que entre o domínio 288 pb e 116 pb exista um ativador específico para VP e um repressor que inibe a expressão de OT

nos MNCs, levando assim a expressão de um único peptídeo (117). No entanto, vários estudos têm demonstrado que a expressão de genes VP e OT pode ser influenciada por entradas sinápticas, bem como por estímulos osmóticos (10, 46, 92, 99).

Não foram observadas mudanças significativas na proporção de neurônios VP e OT em ratos com sobrecarga salina em comparação com o controle. Se considerarmos que neste modelo alterações na diurese e natriurese (Liberação de peptídeo atrial natriurético, inibição do transporte de Na^+ , supressão da liberação de renina, inibição da liberação de aldosterona, e aumento na liberação de ocitocina e vasopressina), são necessárias, é fácil entendermos porque não encontramos alterações na proporção relativa de fenótipos nesta situação (23, 63, 132, 148, 149).

Assim devido à alta especificidade oferecida pelo RT-PCR e sua alta correlação com a imunofluorescência, podemos dizer que o método eletrofisiológico utilizado para caracterizar o fenótipo dos neurônios magnocelulares do SON não é confiável quando usado isoladamente e em *Whole cell patch clamp*. Neste estudo a caracterização fenotípica foi realizada utilizando o RT-qPCR.

5.2 Células Gliais e Óxido Nítrico

Em condições experimentais que resultam em remodelamento da interação neurônio glia, um ponto importante a ser considerado é que a excitabilidade celular pode estar alterada, uma vez que a recaptação de glutamato da fenda sináptica esta comprometida. Neste sentido, o acúmulo deste neurotransmissor altera a atividade neuronal por reduzir a eficácia sináptica por meio da ativação de receptores metabotrópicos glutamatérgicos (112). No SON esta alteração na recaptação resulta em ativação de receptores metabotrópicos do tipo III gliais, os quais estão negativamente acoplados à liberação de neurotransmissores (14, 110). Apesar de não termos observado envolvimento das células gliais na modulação nitrérgica com o uso do

fluorocitrato, nossos resultados mostram diferenças significativas com o protocolo de sobrecarga salina, onde a L-arginina promoveu duas respostas distintas: um aumento, e uma redução na frequência de potenciais de ação. Com relação ao L-NAME, nós observamos que em todos os neurônios analisados o L-NAME produziu um aumento na frequência de potenciais de ação (Figuras 6 e 7). Desta forma, seria razoável pensar que os efeitos observados foram devidos às alterações no “clearance” de glutamato e não do NO. Todavia, como nossos experimentos foram realizados na presença de bloqueadores glutamatérgicos e GABAérgicos, nós descartamos esta possibilidade. Além disso, separando os neurônios de acordo com seu fenótipo, observou-se que aqueles que apresentaram uma redução na frequência de potenciais de ação foram molecularmente caracterizados como vasopressinérgicos, enquanto que os neurônios que apresentaram um aumento neste parâmetro foram caracterizados como ocitocinérgicos. Estes resultados reafirmam o fato de que uma determinada droga pode ocasionar diferentes efeitos dependendo do fenótipo do neurônio (62, 165) e da condição experimental (136). Adicionalmente, algumas moléculas podem, ainda, ocasionar efeitos diferentes em neurônios com mesmo fenótipo (120). Por outro lado, olhando para o modelo experimental utilizado, é importante lembrar que a sobrecarga salina apesar de ocasionar retração glial, ela também ocasiona alterações sistêmicas por acarretar um aumento na concentração de sódio circulante (neste estudo, a média foi de $168 \text{ mEq} \pm 10$), bem como a anorexia adaptativa induzida por alterações osmóticas (154). Neste sentido, o modelo de sobrecarga salina pode estar sujeito a críticas quando utilizado para esta análise.

Não constatamos participação das células gliais na modulação nitrérgica dos canais HCN uma vez que reduções significativas da corrente estacionária dos canais HCN foram observadas na presença da L-arginina, em neurônios isolados (Figura 16). Assim, descartamos

os efeitos do remodelamento glial nesta resposta e a participação dessas células na modulação nitrérgica nos canais HCN.

5.2 Excitabilidade Neuronal e Canais HCN

No sistema nervoso central estudos demonstraram que a maioria dos neurônios expressa uma ou mais isoformas dos canais HCN. Ao contrário da grande maioria dos canais iônicos, os HCN são ativados por hiperpolarização da membrana (152) e responsáveis por originar uma corrente conhecida como I_h . Uma corrente mista, para sódio e potássio, com uma razão de permeabilidade de aproximadamente 1:4, sendo bloqueada por concentrações milimolares de Cs^+ (152) e mais seletivamente por um agente bradicárdico, o ZD7288 (4-(N-ethyl-N-phenylamino)-1,2-dimethyl-6-(methylamino) pyrimidinium chloride) (158). Quatro isoformas dos canais HCN já foram identificadas (13). Entretanto no SON, os dados descritos até o momento são controversos (96, 102). Neste estudo, nós mostramos que a maioria dos neurônios expressaram RNAm de todas as isoformas dos canais, com predominância do tipo 3 e 4. Estes resultados corroboram dados de Monteggia et al. (96) mas não os de Notomi e colaboradores (102) que observaram uma predominância das isoformas 2 e 3 (Tabela 3). Somente 6 neurônios, de um total de 30 analisados, expressaram a isoforma do tipo 1, embora a literatura sugira que esta isoforma seja expressa seletivamente em alguns tipos celulares como células do hipocampo, Purkinje e cerebelo (124). O fato de encontrarmos RNAm para todas as isoformas também pode ser justificado pelo fato destes canais serem encontrados como heterodímeros (166).

Independente da isoforma expressa, estes canais são responsáveis por manter o potencial de repouso em vários tipos de neurônios (43, 50), e no SON Erickson e colaboradores (42) demonstraram em cobaias, que estes canais estão envolvidos no controle da ritmicidade dos MNCs. A ativação tônica destes canais ajuda a manter o potencial de

repouso em um nível despolarizado, enquanto a sua desativação leva o potencial de membrana para valores hiperpolarizados (124). Assim, avaliando a contribuição dos canais HCN na excitabilidade dos neurônios do SON evidenciamos que quando esses canais são bloqueados há uma redução na frequência de disparo, bem como no número de potenciais de ação frente a injeções de correntes despolarizantes (Figura 22). Todavia, não encontramos diferenças significativas na resistência de entrada dos neurônios na presença do ZD7288. Considerando a lei de Ohm, onde $V = R.I$, com um R_i elevado, pequenas alterações de corrente podem ocasionar alterações significativas no potencial de membrana. Em se tratando de células excitáveis, vale salientar que qualquer variação, mesmo que pequena, no potencial de membrana, pode ser suficiente para desencadear alterações na atividade celular. Assim, como exemplo, utilizando $\Delta I = 40$ pA, com R_i de $1,1 \text{ G}\Omega$ (como encontrado neste estudo), teremos uma variação no $V_m = -44$ mV. Entretanto pequenas variações do R_i de $1,1 \text{ G}\Omega$ para $1 \text{ G}\Omega$ para um mesmo $\Delta I = 40$ pA, resultará em uma variação no V_m de -44 mV para -40 mV, ou seja, 4 mV o que seria suficiente para causar grandes alterações na excitabilidade celular. O oposto será encontrado para R_i maiores que $1,1 \text{ G}\Omega$, ou seja, uma hiperpolarização do potencial de membrana. Portanto, considerando que o potencial de repouso criticamente determina o nível de excitabilidade dos neurônios e que, como descrito anteriormente, os canais HCN auxiliam na manutenção deste potencial podemos concluir que tais canais desempenham um importante papel na excitabilidade dos neurônios magnocelulares do SON. Nossos dados vão de encontro aos de Ghamari-Langroudi e Bourque (51), que demonstram um papel funcional da I_h nos neurônios magnocelulares do núcleo supraóptico e sua contribuição na excitabilidade bem como nos disparos fásicos e tônicos dos MNCs. Adicionalmente, reforçando nossos dados estudos tem demonstrado que os canais HCN contribuem na determinação da excitabilidade neuronal de outros tipos celulares (38, 87, 125).

5.3 Origem do NO e Modulação dos Canais HCN

Antes de nos atermos as vias de sinalização utilizada pelo NO nos neurônios do SON, nos preocupamos em investigar a origem deste mensageiro neuronal. Na literatura encontramos que a osmolalidade plasmática acima de 300 mosm/KgH₂O é um estímulo potente que aumenta a atividade elétrica dos MNCs em ratos (24, 84, 151). Adicionalmente, ainda tem sido demonstrado que alterações da osmolalidade plasmática entre 310 a 330 mosm/KgH₂O resultam em um aumento da expressão de RNAm para NOS no núcleo supraóptico indicando que o NO pode estar envolvido no controle da excitabilidade dos MNCs, especialmente em situações em que a osmolalidade é comprometida. De fato, nós demonstramos recentemente que nestas situações experimentais o NO atua como um mensageiro inibitório ocasionando reduções na frequência de potenciais de ação dos MNCs, bem como alterações nas propriedades intrínsecas dos neurônios (34). Entretanto, apesar do estímulo hipertônico modular a atividade elétrica dos neurônios magnocelulares, e aumentar a expressão de RNAm para enzima catalisadora da formação do NO, ainda era desconhecido se estes neurônios eram capazes de produzir este mensageiro neuronal. No presente estudo demonstramos que o estímulo hipertônico induz aumento da produção de NO, efeito este abolido pelo bloqueador da NOS, L-NAME (Figura 20). Este aumento ocorreu independentemente do aumento da excitabilidade neuronal (Figura 21), que é observado com o estímulo hipertônico uma vez que estes neurônios operam como osmorreceptores (107). Demonstramos ainda, que apesar das células gliais serem capazes de produzir NO (100), e que durante estresse osmótico estas são ativadas segundos antes dos próprios neurônios magnocelulares (104, 109, 112, 116, 131), o NO é produzido pelos MNCs uma vez que a mesma resposta foi observada nos neurônios isolados do SON (Figura 17). Nossos resultados coadunam-se com outros estudos que demonstraram um aumento de marcadores da produção do NO nos MNCs, tal como o NADPH – diaforase, RNAm e citrulina durante a estimulação

hipertônica aguda ou crônica (21, 69, 135, 146). Como a produção do NO necessita do complexo cálcio calmodulina para ativar a nNOS, nós acreditamos que os canais ativados por estiramento (20), e canais TRPVs (16), presentes nessas células e ativados durante o estímulo hipertônico, são as vias de entrada para cálcio e consequente ativação da NOS. No entanto, tais hipóteses são apenas especulativas e necessitam ser testadas.

5.4 Vias de Sinalização Nitrérgica nos Canais HCN

O estudo das vias de sinalização utilizadas pelo NO para modular os canais HCN têm evidenciado, nos últimos anos, ser a ativação da guanilato ciclase (cGC) pelo NO, com consequente aumento na síntese de cGMP, o principal mecanismo de atuação intracelular do modulador (6, 22, 162). As respostas mediadas pelo cGMP podem ocorrer por meio da ativação de proteínas quinases dependentes de cGMP, como também pela interação com canais iônicos ativados por nucleotídeos cíclicos (72) incluindo os HCN (31). Em diferentes populações neuronais como cerebelo, neurônios talâmicos e motoneurônios do hipoglosso e trigêmios, os canais HCN, tem sido apontados com alvo potencial de modulação do NO via cGMP, (1, 113, 155, 156). No núcleo supraóptico tem sido relatado que o nitroprussiato de sódio, um doador de NO, aumenta a concentração de cGMP nos neurônios magnocelulares (32) que por sua vez induz um aumento do acoplamento neuronal (161). Consequentemente, tem sido sugerido que o NO desempenha um papel autorregulatório dependente de cGMP nesses neurônios. Neste mesmo trabalho, Yang e colaboradores observaram que 8-Br cGMP, um análogo do cGMP, aumenta a excitabilidade dos MNCs, um achado contraditório em relação à ação inibitória do NO sobre os neurônios magnocelulares encontrado previamente por nós (34, 147) e por outros (83, 103, 141). Uma vez formado o cGMP pode desempenhar seu efeito por três vias distintas: 1) ativação de proteínas quinases com consequente alteração na concentração de cálcio intracelular; 2) ativação de fosfodiesterases que autorregulam sua

atividade; e 3) efeitos diretos sobre canais iônicos dependentes de nucleotídeo cíclico (45). Embora a maioria dos estudos aponte a guanilato ciclase como principal alvo do NO, alguns têm demonstrado que nos neurônios magnocelulares do SON, tanto em condições basais como na desidratação, a modulação nitrérgica da secreção de VP e OT (145), e sobre os canais para potássio ativados por cálcio do tipo BK (2) não envolve a formação de cGMP, em consonância com nossos resultados. Portanto, podemos observar que ainda há uma grande divergência a respeito do envolvimento do cGMP na sinalização do NO para diferentes situações.

Os achados do nosso estudo mostrando que cGMP não participa, como molécula sinalizadora, da modulação nitrérgica sobre as correntes I_h foi uma surpresa devido ao fato da cGC ser descrita como o principal alvo fisiológico do NO no sistema nervoso central (48, 134). Por um lado, ainda pode surgir a dúvida se a concentração de ODQ utilizada foi suficiente para inibir completamente a atividade da guanilato ciclase. Como o ODQ é solúvel em DMSO, e este por sua vez tem demonstrado excitar os neurônios MNCs, aumentar a concentração da droga seria problemático. Por outro lado, trabalhos que utilizaram concentrações menores do que a nossa, observaram o bloqueio da ação do NO tanto no supraóptico como no paraventricular (81, 162) nos apontando o envolvimento de outras vias de sinalização.

5.5 Modulação dos Canais HCN por S-nitrosilação

Nos últimos anos, estudos têm sugerido algumas vias não convencionais de sinalização nitrérgica. Neste contexto, a visão clássica do cGMP como mediador exclusivo da modulação nitrérgica foi questionada por resultados mostrando que o NO pode modificar proteínas através de reações químicas diretas (90). Uma dessas modificações pós-traducionais da proteína ocorre nos grupamentos tiol de cadeias laterais de resíduos de cisteína. Este processo

é denominado de S-nitrosilação, e ocorre através de um mecanismo químico complexo, sem o auxílio de enzimas (3). No sistema nervoso central este mecanismo foi descrito pela primeira vez em receptores glutamatérgicos do tipo NMDA, onde o resíduo de cisteína 399 da subunidade NR2A foi nitrosilado (30). Entretanto, outros estudos demonstram que o NO participa da modulação de proteínas de membrana incluindo canais iônicos, como os canais para potássio dependentes de cálcio (79), canais dependentes de nucleotídeos cíclicos (26), canais HCN (155), dentre outros (3). Como não há um bloqueador específico da S-nitrosilação, neste estudo nós utilizamos o N-etilmaleimida (NEM) que oxida os resíduos de cisteína obstruindo a S-nitrosilação para avaliar se a modulação nitrérgica observada nos MNCs ocorria por meio deste mecanismo não convencional. Assim, observamos que na presença do NEM houve um aumento na corrente I_h indicando que a oxidação dos resíduos de cisteína altera as propriedades intrínsecas dos canais HCN. Adicionalmente, quando a L-arginina foi adicionada ao banho, nós não observamos alterações na corrente I_h , sugerindo uma possível via de modulação neste canal (Figura 23). Estes resultados são consistentes com os dados de Wenker e colaboradores (155), onde se demonstrou que o NO modula os canais HCN de motoneurônios do hipoglosso via S-nitrosilação dos resíduos Cis531 da porção carboxiterminal no HCN1 e Cis341 do HCN2 localizados na alça citoplasmática entre os segmentos S4 e S5.

Considerando a variedade e ubiquidade de proteínas cuja função pode ser regulada por S-nitrosilação (78, 89), esta modificação pode ser um componente importante no repertório de regulação dos canais iônicos, como aqui evidenciado. Fisiologicamente, a vantagem deste mecanismo se deve a estabilidade que a reação confere ao NO. Desta forma o NO pode ser tanto armazenado como transportado pelo organismo na forma nitrosilada (RSNO). Um exemplo é seu transporte pela glutathione, e também sua ligação à albumina e hemoglobina (11, 70, 128). Embora a ligação do NO com os resíduos de cisteína ocorra de uma forma

covalente, mecanismos de denitrosilação, S-glutationationa, nitração da tirosina, e ascorbato, são responsáveis por reverter esta interação (11, 39). Neste estudo nós avaliamos a presença de S-nitrosilação endógena utilizando como agente denitrosilante o ascorbato e evidenciamos um aumento significativo na corrente I_h . Tais resultados confirmam a presença de nitrosilação endógena nos canais HCN dos MNCs corroborando resultados de Jaffrey et al (64) que identificaram os canais HCN como substratos neurais para S-nitrosilação endógena.

5.5 Modulação dos Canais HCN pelo NO Durante Estímulo Hipertônico

Ainda que estudos anteriores tenham mostrado uma modulação na excitabilidade dos MNCs durante estímulos hipertônicos (106, 107), no presente estudo, nós demonstramos que este fenômeno é acompanhado por um aumento na produção de NO nos MNCs (Figura 20), sugerindo que este mensageiro neural pode estar também envolvido na modulação da excitabilidade dessas células, nestas condições experimentais. Esses dados estão de acordo com estudos prévios do nosso laboratório, onde mostramos que durante o estímulo hipertônico, o NO inibi a atividade dos MNCs, resultando em uma hiperpolarização do potencial de repouso e alterações na constate de tempo da fase de despolarização pós-potencial (34), a qual está relacionada com o controle da excitabilidade desses neurônios (4, 19). Considerando que os canais HCN desempenham um papel importante na manutenção do potencial de repouso e que esses canais são, em parte, responsáveis pela fase de despolarização pós-potencial (51), nós avaliamos se o NO produzido durante o estresse osmótico também modula esses canais. Como demonstrado na figura 27, o estímulo hipertônico aumentou a corrente I_h e um aumento adicional foi observado durante a inibição da NOS, demonstrando que o NO produzido durante as alterações de osmolalidade possui um efeito inibitório sobre a corrente I_h . Desta forma, o efeito do NO durante o estresse osmótico também apontam os canais HCN como alvo desta modulação.

Nossos dados confirmam os achados de Kadowaki e colaboradores (69), onde os autores observaram que transcritos da NOS, tanto no PVN quanto no SON, aumentam após sobrecarga de salina reforçando a ideia de que o NO está envolvido nos mecanismos osmorregulatórios hipotalâmicos. Frente aos resultados inibitórios do NO sobre os canais HCN e consequentemente na excitabilidade dos MNCs durante o estímulo hipertônico, podemos especular que o NO é parte de um mecanismo de *feedback* negativo que evita a hiperatividade dos MNCs e, portanto, a liberação elevada de VP e OT pela neurohipófise. Nessas condições o sistema seria capaz de manter a síntese e liberação por tempo prolongado evitando a saturação deste sistema.

6. CONCLUSÕES

Em suma, nossos dados nos permitem concluir que: 1) Em relação à caracterização do fenótipo celular, utilizando pela primeira vez três técnicas diferentes para caracterizar um único neurônio, o presente estudo sugere que, em preparações *in vitro*, o protocolo eletrofisiológico sozinho não descreve fielmente os fenótipos dos neurônios magnocelulares. Isto é particularmente crítico em situações onde o sistema necessita rearranjar-se, a fim de manter o equilíbrio hidroeletrolítico. Sendo assim, a combinação da detecção de RNAm e imunohistoquímica parece ser a melhor escolha ao tentar caracterizar um único fenótipo. E por fim, a excitabilidade elétrica dos neurônios magnocelulares do núcleo supraóptico é essencialmente determinada por um efeito de NO sobre correntes I_h por um mecanismo dependente de S-nitrosilação. Deste modo, atuando em canais HCN, o NO representa outro modulador no complexo conjunto de mecanismos de controle da homeostase dos líquidos corporais.

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8. PUBLICAÇÕES

Effects of nitric oxide on magnocellular neurons of the supraoptic nucleus involve multiple mechanisms

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Abstract

Physiological evidence indicates that the supraoptic nucleus (SON) is an important region for integrating information related to homeostasis of body fluids. Located bilaterally to the optic chiasm, this nucleus is composed of magnocellular neurosecretory cells (MNCs) responsible for the synthesis and release of vasopressin and oxytocin to the neurohypophysis. At the cellular level, the control of vasopressin and oxytocin release is directly linked to the firing frequency of MNCs. In general, we can say that the excitability of these cells can be controlled via two distinct mechanisms: 1) the intrinsic membrane properties of the MNCs themselves and 2) synaptic input from circumventricular organs that contain osmosensitive neurons. It has also been demonstrated that MNCs are sensitive to osmotic stimuli in the physiological range. Therefore, the study of their intrinsic membrane properties became imperative to explain the osmosensitivity of MNCs. In addition to this, the discovery that several neurotransmitters and neuropeptides can modulate their electrical activity greatly increased our knowledge about the role played by the MNCs in fluid homeostasis. In particular, nitric oxide (NO) may be an important player in fluid balance homeostasis, because it has been demonstrated that the enzyme responsible for its production has an increased activity following a hypertonic stimulation of the system. At the cellular level, NO has been shown to change the electrical excitability of MNCs. Therefore, in this review, we focus on some important points concerning nitrergic modulation of the neuroendocrine system, particularly the effects of NO on the SON.

Key words: Magnocellular neurons; Supraoptic nucleus; Vasopressin; Oxytocin; Nitric oxide; Homeostasis

Nitric oxide as a messenger molecule

Studied in 1772 by Joseph Priestly (1), nitric oxide (NO) was taken, at first, to be a toxic gas. However, this view was changed when it was shown that NO was endogenously produced by living organisms. Initially characterized as an endothelium-derived relaxation factor (2), NO was later postulated to be a possible neurotransmitter in the central nervous system by Garthwaite et al. (3). In 1992, NO was named "Molecule of the Year", and its physiological and pharmacological importance became clear through the studies of Louis J. Ignarro, Robert F. Furchgott, and Ferid Murad, who were awarded the Nobel Prize in Physiology or Medicine in 1998 (4). These scientists showed that NO could act as a signaling molecule in the central nervous system, suggesting its role as a neurotransmitter/neuromodulator.

This view changed the classical concepts used to explain communication between neurons, because NO cannot be stored, released, or inactivated by conventional regulatory mechanisms. NO signaling in excitable tissues requires rapid and controlled release to specific cellular targets, since its average lifetime is on the order of a few seconds (5). In principle, NO could spread out from its site of production to influence different types of tissues (neuronal, glial, and vascular) that are not necessarily in anatomical juxtaposition, acting as an autocrine or paracrine signaling molecule. At the present time, there is vast literature focused on the study of the biological effects of NO. This messenger is involved in the function of a great diversity of tissues and is shown to be involved in many different physiological processes. For a review on other aspects of NO function, not addressed in this review, see Calabrese et al. (6).

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Synthesis of NO

In the organism, NO originates from L-arginine in a process catalyzed by three distinct NO synthase (NOS) enzymes: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS). eNOS and nNOS are constitutively expressed enzymes, which are stimulated by increases in intracellular calcium concentration (7). The immunological functions of NO are mediated by a calcium-independent iNOS that is activated by products released in inflammatory processes such as cytokines, interferon gamma, and interleukins 1 and 2 (8). However, all NOS enzymes use NADPH as an electron donor and require five cofactors as well as the presence of calmodulin to catalyze the oxidation of L-arginine to NO, with a stoichiometric formation of citrulline. The result of this reaction is one molecule of NO plus L-citrulline (9). L-citrulline is recycled back to regenerate L-arginine for a new NO synthesis, closing the cycle of NO production (Figure 1) (10). Since the amount of NADPH and citrulline correspond to the amount of NO produced, they were extensively used as markers of NO production in the brain including the supraoptic nucleus (SON) (11). Nowadays, other approaches can be used to detect NO production with the same precision: for example, fluorescent dyes like DAF and DAF-FM (12), or electrochemical sensors (13).

mRNA transcripts of all NOS isoforms are present in the hypothalamus, with the order of expression being nNOS>eNOS>iNOS (14). In the SON, the main isoform is nNOS (15), and its expression is likely to be controlled in an activity-dependent manner, i.e., increases in activity of the neuron induce an equivalent increase of nNOS in magnocellular neurons (16). However, the effects seen when nNOS is elevated are not completely understood. In the literature, there is a consensus that vasopressinergic

Figure 1. Nitric oxide synthesis. In the presence of NADPH, oxygen and co-factors, such as tetrahydrobiopterin (BH₄), flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), nitric oxide (NO) synthase (NOS) plus calcium (Ca²⁺), catalyze the breakdown of L-arginine to citrulline and NO. Citrulline will be recycled back to regenerated L-arginine and NO will produce autocrine and/or paracrine effects as well as negative feedback on NOS.

neurons constitute the major neuronal phenotypes expressing nNOS, suggesting a role for this new neurotransmitter in the mechanisms regulating fluid homeostasis (17).

Donors and inhibitors of NO

To help in the understanding of the biological functions of NO, exogenous sources and inhibitors of this neuro-modulator have been developed as research tools. To increase NO levels, both donors and substrates are used. Donors are compounds that release NO, and several compounds have already been described, for example, sodium nitroprusside (SNP), nitroso-*N*-acetylpenicillamine (SNAP), 3-morpholinosydnonimine (SIN-1), and diazeniumdiolates (NONOates).

Chemically, all donors have nitrate functionality within the molecule, and a nitroso functional group is present in all of these compounds (18). Some donors have functional nitrosothiol groups, *S*-nitrosothiols, whose decomposition is catalyzed by copper ions (Cu⁺) to form NO and disulfide. Interestingly, *S*-nitrosothiols were found endogenously, supposedly acting as NO stores for release when required (19). Another functionally important group is nitrosyl, commonly found in sodium nitroprusside (Na₂[Fe(CN)₅NO]-SNP), which is a mixed nitrosyl-cyano complex (20). Additionally, other metal complexes have been developed including a nitrosyl-ruthenium complex, which has the advantage of low cytotoxicity and readily releasing NO upon illumination (21). Although the classification of all NO donors is a complicated task, we can say that, when they show similar chemical structures, they usually have similar NO-releasing mechanisms.

SNP was used by several authors in their studies of the SON (22-24). SNP is a complex of ferrous ion with five cyanide anions (CN⁻) and a nitrosonium ion (NO⁺). Interaction of SNP with a reducing agent, such as thiols, leads to the formation of NO. Its use in biological systems has the inconvenience that formation of NO is accompanied by the formation of ferricyanide, a biologically active and toxic compound (25). To avoid the effects of ferricyanide, other types of donors have been used, for example, SNAP, SIN-1, and NONOates. Regarding SNAP, it is a derivative of the nitrosothiol group that seems to provide higher concentrations of NO, but micromolar concentrations of Cu²⁺ are required for its action (26). SIN-1 also produces NO and originates a breakdown product, SIN-1C, which is biologically inactive (27). A side effect of SIN-1 is the production of superoxide anion, which can react with NO and H⁺ to form peroxynitrite (ONOO⁻) and hydrogen peroxide (H₂O₂), both with deleterious effects on membranes (28). NONOates release NO spontaneously in solution at physiological pH and temperature. The NO derived from NONOates is not accompanied by the cytotoxic effects of

hydrogen, alkyl hydroperoxide, or hypoxanthine/xanthine. In addition, other NO donors have been developed, which promise advantages over the previous ones, such as spontaneous release of NO under controlled rates. In the literature, we can find descriptions of a variety of these, i.e., donors with higher levels of NO release without being photosensitive or releasing cyanide: for example, Rut-bpy (Cis-[Ru(bpy)₂(SO₃)(NO)]PF₆) (29) and/or donors with protection against hydrogen peroxide-mediated cytotoxicity diethylamine (DEA/NO) and propylamine propylamine (PAPA/NO) (30). The amount and duration of NO release depend on the pharmacological properties of each donor. Thus, some compounds could have a fast action in small quantities or a slow action when NO is released for long periods.

Besides donors, production of endogenous NO can be controlled by using inhibitors of NOS. These inhibitors can be divided into two groups: 1) inhibitors that target cofactor-binding sites and 2) inactive L-arginine analog molecules. The first interfere with flavin, calmodulin, or dioxygen binding sites. These NOS inhibitors have no selectivity for a particular isoform and interfere with the activity of other enzymes that need the same cofactors to become active (31).

In an opposite way, substrates analogous to L-arginine have a higher specificity toward the NOS isoforms. These inhibitors act as false substrates by binding to the L-arginine binding site (32). Although over one-hundred NOS inhibitors have been described as possible pharmacological tools (31) reducing or preventing the biological effects of NO (2,5), the majority of them are nonselective, and just a few compounds, such as 7-nitro-indazole, amidines, and some amino acid derivatives, are able to selectively inhibit nNOS (33,34). N^ω-nitro-L-arginine methyl ester (L-NAME), a nonselective NOS inhibitor, has been extensively used in studies of the SON (23,35,36).

Mechanisms of action

Guanylate cyclase

Although the number of newly discovered potential targets of NO increases continually, its major effect, under physiological conditions, appears to be mediated mainly through the activation of soluble guanylate cyclase (sGC), the intracellular NO receptor. sGC is a heterodimer composed of α - and β -subunits. Due to the electronic structure of NO, an uncharged molecule, it activates sGC by binding directly to the heme portion, leading to a conformational change that augments enzyme activity by forming a ferrous-nitrosyl-heme complex. At this point, there is conversion of GTP to cGMP followed by intracellular activation of several effectors (37). Some of the effectors were identified as 1) cGMP-dependent protein kinase, 2) cGMP-regulated phosphodiesterases, and 3) cGMP-gated ion channels (38). Although it seems

that the effects of NO are cGMP dependent, in the SON the evidence is controversial. Yang and Hatton (23) have shown that cGMP enhances dye coupling and excitability of supraoptic neurons. On the other hand, studies have shown that nitrergic modulation of magnocellular neurons of the SON involves an increase in the frequency of γ -aminobutyrate (GABA)ergic events (24) and that the signal transduction is independent of cGMP (39). The same type of result was observed in the neurosecretion of vasopressin (VP) and oxytocin (OT) in experiments with awake rats (39). In brain slices of the SON, using the outside-out configuration of the patch-clamp technique, results show that NO acts directly on the N-methyl-D-aspartate receptor (NMDA) channel complex, without mobilization of cGMP (40). However, to make the subject more complicated, our group has demonstrated that nitrergic modulation can be independent of synaptic events, suggesting a possible direct action on ion channels (35). As can be seen, the pathway used by NO to change the excitability of magnocellular neurons is still a matter of debate.

An alternative pathway

The classical view of cGMP being the exclusive mediator of the effects of NO has been questioned by findings suggesting that NO can modify proteins through direct chemical reactions (41). One of those alternative pathways involves S-nitrosylation, in which the NO molecule interacts with cysteine thiol groups in a covalent bond, resulting in an S-nitrosothiol complex (Figure 2). This mechanism has been described as an important NO reaction, which preserves its biological actions, and can be expressed as a key posttranslational modification of ion channels increasing or decreasing protein activity (42,43). Thus, protein functions can be controlled by either interaction or deletion of NO from the cysteine thiol group. For more details about S-nitrosylation, see Hess et al. (42).

In the central nervous system, this process was first described in NMDA-type glutamate receptors, where the cysteine (residue 399) in the NR2A subunit was nitrosylated (44). However, emerging results have demonstrated

Figure 2. S-nitrosylation at cysteine residues. After synthesis, nitric oxide (NO) reacts with cysteine residues (red) of proteins forming a nitrosylated compound.

that other proteins can also be nitrosylated, such as, calcium-activated potassium channels (45), cyclic nucleotide activated cation channels (46), and hyperpolarizing activated and cyclic nucleotide gated cation channels (47). In addition, Jaffrey et al. (48) verified that some proteins are endogenously nitrosylated, reinforcing the idea that this signaling mechanism for NO is of physiological significance.

NO effects on the mammalian SON

The observation that osmotic stimulation upregulated NOS as well as VP and OT mRNA expression in magnocellular neurosecretory cells (MNCs) of the SON was taken as evidence that NO could play a role as a neuromessenger involved in the control of neurohypophyseal hormone secretion (16). Additional immunohistochemical evidence colocalizing NOS with VP or OT and detection of NOS mRNA, NOS protein, and its product L-citrulline in MNCs lends further support to this idea. Moreover, *in vivo* and *in vitro* studies also produced a wealth of results that revealed a significant participation of NO in the control of VP and OT secretions (49,50).

Through a dynamic approach using confocal microscopy and NO-sensitive indicators, a basal production of NO at the cellular level was detected within the SON, but not the surrounding nuclei (51). Moreover, hyperosmotic stimulation induced NO production in MNCs in slices of the SON (52). This finding adds support to the hypothesis that osmotic stimulation induces an increase in NO production as a consequence of NOS overexpression (16,53).

Controversial effects of NO on VP and OT secretion

Results concerning the central effects of NO on VP

and OT plasma levels are not always coincident and are sometimes contradictory. Table 1 shows a collection of the main findings from experiments using intracerebroventricular (*icv*) injections of NO donors, L-arginine, or NOS blockers (L-NAME). Under physiological conditions, *icv* injection of L-NAME elicits an increase in both VP and OT plasma levels (54). This result indicates that NO tonically inhibits neurohormonal secretion. On the other hand, increased or unmodified effects on plasma VP levels were shown after *icv* injections of NO donors and L-arginine treatment (54-56).

Since increased plasma levels of VP and OT were observed after blockade of endogenous NO production, it would be expected that increased NO availability, after treatment with NO donors or L-arginine, would induce opposite effects. However, similar to the blocking of endogenous NO production, a larger NO availability also increased VP and OT plasma levels. On the contrary, *in vitro* studies reveal different effects of NO on neurohypophyseal hormone secretion. In rodent hypothalamic explants, NO suppressed VP secretion, an effect seen with NO donors SIN-1 and SNP (49,57). L-arginine also reduced VP release in this preparation, an effect reversed and reduced, respectively, by the NOS blocker L-NMMA and the addition of human hemoglobin, an NO scavenger (49). In microinjection experiments, interpretation of the results needs to take into consideration the microenvironments of the nuclei. Different brain nuclei have different sizes and can be damaged by microinjections with relatively large volumes. In situations like this, the effects observed are subjected to severe criticism because of the possibility of mechanical lesions and tissue edema. Furthermore, nuclei in the surroundings of the injection site can also be affected by the injected drug, and the final measured response may be misleading (58). A third and very important point is the concentration of drug used. As can be seen in Table 1, *icv* microinjections of donor and

Table 1. The hypothalamic-neurohypophyseal axis is modulated by the central nitrergic system.

<i>icv</i> treatment	Drug concentration (µg/µL)	Volume microinjected (µL)	Hormone		Reference
			VP	OT	
No donor (SNAP)	1.25 and 2.5	10	↑	NR	Ota et al. (56)
	1	5	NS	NS	Reis et al. (86)
L-arginine	50 and 100	10	↑	NR	Ota et al. (56)
	100	10	↑	NR	Cao et al. (87)
	40	0.5	NS	NS	Reis et al. (54)
NOS blocker (L-NAME)	54	5	↓	NR	Cao et al. (87)
	50	5	↑	↑	Kadekaro et al. (88)
	80	0.5	↑	↑	Reis et al. (54)

Plasma vasopressin (VP) and oxytocin (OT) levels from euhydrated rats treated with *icv* injections of NO donors, L-arginine or nitric oxide synthase (NOS) blocker. Upward and downward arrows indicate significant enhanced and reduced plasma levels when compared with the control group, respectively. SNAP: S-nitroso-N-acetylpenicillamine; L-NAME: N^ω-nitro-L-arginine methyl ester. NS: nonsignificant changes compared to the control group; NR: non-reported information.

substrate of NO resulted, at the higher doses, in an increase in the release of VP. Such an effect is opposite to that observed in *in vitro* studies, where the release of VP was inhibited. However, in experiments where the NOS enzyme was blocked, the results obtained with micro-injections are more similar to those obtained from *in vitro* experiments. Thus, although results from *in vivo* studies are controversial, findings from *in vitro* studies are more consistent, indicating a general inhibitory effect of NO on neurohypophysial hormone secretion. On the other hand, in dehydrated rats *icv* microinjections of L-NAME, an NOS blocker, induced an acute increase in OT, but not VP plasma levels, suggesting that the postulated tonic nitrgic inhibition of VP secretion is removed during dehydration (59). Such an effect was also reported after *icv* injection of angiotensin II (AngII), hypertonic solution treatment (60), and in hypovolemic rats (36). Besides this, NO seems to induce an increase in VP, but not in OT plasma levels induced by hypertonic blood volume expansion (61). Taken together, these findings indicate that, similar to what happens during hypovolemia, total and intracellular dehydration removes tonic inhibitory nitrgic modulation on VP neurons, but not on OT neurons. Therefore, it seems that nitrgic modulation on the hypothalamic-neurohypophysis axis can be strongly controlled by reflex responses activated by osmotic imbalance and depletion of body fluid compartments.

From the above discussions, the question that remains is: How could osmotic and volume challenges induce such diverse nitrgic effects on VP and OT secretions? It is known that dehydration and salt load induce overexpression of neuronal NOS mRNA in MNCs (53,62), a response controlled by the anteroventral third ventricular (AV3V) region (63). Thus, it is expected that 24-h dehydration would increase the levels of NO into the SON, with a consequent inhibition of VP and OT secretion. In order to address this problem, we should recall that hypovolemia, hypotension, and total dehydration, but not intracellular dehydration, significantly increase in AngII plasma levels. Circulating AngII may induce VP (64) and OT (65) secretion by acting on circumventricular organ neurons, where the blood-brain barrier is absent (66). Thus, circulating AngII may activate neurons at the subfornical organ (67), which sends axonal projections to the SON, increasing MNC activity via AngII release and activation of postsynaptic AngII receptors type-1 (AT1). This hypothesis is supported by experiments showing that administration of AT1 receptor antagonist suppresses the AngII response (68). Similarly, cellular dehydration induced by hypertonic solution activates subfornical organ neurons enhancing AngII transmission to MNCs (64). How can a blood-borne signal like AngII modulate the nitrgic system present in the SON? Experimental evidence shows that AngII could modulate nNOS mRNA expression in MNCs (69). Such

modulation is dependent on nNOS activity itself, since its blockade prevented AngII-induced nNOS mRNA overexpression in the SON. Therefore, we can assume that AngII receptor (AT) activation modulates the nitrgic system in MNCs. In neurons, increased NO production as a consequence of the AT1 receptor antagonist, losartan, is a response that may be blocked by an AT2 receptor antagonist, such as PC123319 (70). This indicates that, while AngII binding to AT1 receptors inhibits tonically produced NO, binding to AT2 receptors stimulates it. In this case, NO production was exclusively dependent on nNOS (70). How could the modulation of activity of AngII receptors explain selective nitrgic inhibition on OT secretion? According to Wang et al. (70), since AngII receptor activation stimulates or inhibits NO production, the balance between AT1 and AT2 activities may be decisive for VP and OT release. However, during dehydration, *icv* microinjection of losartan did not induce any changes in VP and OT plasma levels. On the other hand, *icv* losartan plus *icv* L-NAME elicited increased OT secretion. It is likely that, in MNCs, enhanced NO production is the consequence of both neuronal NOS stimulation via AT2 receptor activation and reduction of NOS inhibition via AT1 inactivation by losartan. Therefore, it seems that, when AT1 receptors are inactivated, AngII activates AT2 receptors in MNCs, eliciting overactivity of NOS, increased NO production, and inhibition of VP and OT secretion. Such a hypothesis would explain the increased OT plasma levels after *icv* L-NAME treatment, but would not explain nonsignificant changes on VP secretion. We could speculate that AT2 receptors are absent in VP neurons, but they have been detected in rodent VP neurons (71). Thus, to this point, we may assume that AngII may induce NO production through AT2 receptors, via nNOS activation, with consequent inhibition of both VP and OT secretion. However, another question remains: Why is OT, and not VP secretion, affected by L-NAME treatment? A possible explanation emerges from studies involving stimulation of AT2 receptors and consequent production of phospholipase A2 and arachidonic acid (72). Arachidonic acid is the substrate for cyclooxygenase enzymes (COX-1 and COX-2) to produce prostaglandins (PGs), also involved in the control of neurohypophysial hormone secretion. Indeed, central microinjections of different types of PGs elicit VP and OT secretion (73,74), an effect also observed in *in vitro* preparations (75). Interestingly, Yamaguchi et al. (73,74) observed that, in rats, brain PGs were synthesized during hyperosmolarity and volume depletion, but not under physiological conditions, suggesting that COX enzymes are activated by osmotic stress and hypovolemia. This is a plausible assumption, since COX-2 is also a constitutively expressed enzyme in the brain (76), lending support to the hypothesis that PGs participate in selective nitrgic inhibition on OT secretion during dehydration. Since NO can directly activate COXs (77,78), we

speculate that dehydration-induced NOS overexpression (79) would lead to an enhanced activation of the COX enzymes and synthesis of PGs, thereby inducing neurohypophyseal hormone secretion. Central microinjection of meclofenamate, a COX inhibitor, increased VP plasma levels during osmotic stimulation and hypovolemia, an effect not observed in euvoletic and euhydrated rats. Such results indicate that osmotic and volume imbalances stimulate central synthesis of PGs, which tonically induce the secretion of VP (73,74). Interestingly, it was reported that *icv* microinjections of PGE₂ or PGF₂ α elicit OT, but not VP secretion (77). The activation of SON neurons by PGE₂ occurs via binding to prostanoid receptors. In the SON, electrophysiological investigations showed that PGE₂ increases neuronal activity through postsynaptic PGE₂ and PGF₂ α receptors. Indirectly, PGE₂ induces MNC activation by reducing the inhibitory GABA inputs via presynaptic EP₃ receptors (80). Thus, we may speculate that, during dehydration, NO and PGs are two immediately synthesized factors with opposing effects to control MNC activity and neurohormonal secretion. As NO may induce additional synthesis of PGs through direct effects on COX, PG levels may increase during dehydration in parallel with NO production. The counterbalance between both factors maintains OT and VP secretions at optimal levels. Even when *icv* L-NAME treatment interrupts NO production, COX enzymes S-nitrosylated by NO may maintain the PG levels. In addition, the high sensitivity of OT neurons to PGE₂ and PGF₂ α may explain the exclusive increase in OT plasma levels after L-NAME treatment (77), since *icv* indomethacin microinjection suppressed L-NAME-induced OT secretion and did not change VP levels (81).

In summary, although *in vivo* experiments brought relevant contributions to understanding the control of neuroendocrine function by the brain nitrergic system, *in vitro* studies were also needed to unveil the nitrergic mechanisms controlling VP and OT secretion. In this regard it is worth noting that, although *in vivo* studies suggest that the activity of MNCs is modulated by synaptic neurotransmission, it is also well known that NO plays an important modulator effect by controlling their activity through both indirect (synaptic) and direct (intrinsic) mechanisms.

Indirect effects of NO on MNCs: synaptic mechanism

In vitro electrophysiological experiments were necessary to understand how NO induces changes in VP and OT plasma levels. Since release of VP and OT is correlated with the electrical activity of MNCs, several papers have investigated the effects of NO on the pattern and frequency of firing neurons of action potentials. To investigate how the effect of NO inhibits electrical activity of SON neurons, spontaneous excitatory (EPSCs) and

inhibitory (IPSCs) postsynaptic currents were recorded using the whole cell patch-clamp technique in unidentified SON neurons (82). NO reversibly increased only the frequency of IPSCs and did not change the amplitudes of either IPSCs or EPSCs. Since both IPSCs and EPSCs were recorded in the presence of tetrodotoxin, the spontaneous synaptic currents are, in fact, miniature IPSCs and EPSCs representing local release of GABA and glutamate, respectively (83). Contrary to the above results, it was reported that SNP, a donor of NO, and L-arginine, precursor of NO synthesis, increase both the frequency and amplitude of GABA_A miniature IPSCs in VP and OT neurons (24). This finding was taken to indicate that NO modulates GABA neurotransmission at both pre- and postsynaptic sites. Such evidence lends support to the idea that NO increases the presynaptic quantal release of GABA and the open probability (P_0) of GABA_A channels, since IPSC currents were abolished by the GABA_A channel blocker, picrotoxin (82). Although controversy still remains about a possible postsynaptic effect, the increase in amplitude elicited by L-arginine and the reversal of this effect by the nNOS inhibitor, 7-nitroindazole, suggest that postsynaptic GABA channel activity may be influenced by endogenous NO (24). Also, the NO donor, SNP, is known to negatively modulate glutamatergic neurotransmission in SON neurons (22). However, such evidence must be considered with caution, because it is well known that ferrocyanide ions, a byproduct of SNP photolysis, mimicked the effects of SNP on NMDA currents in neurons (25). Although Ozaki et al. (82) did not observe any effect of SNAP (NO donor) on the spontaneous EPSCs in SON neurons, there are reports that NMDA currents in striatal neurons are negatively modulated by other NO donors (27). In summary, endogenous NO acts indirectly on MNCs by enhancing fast inhibitory synaptic transmission through the induction of presynaptic GABA release, as well as by modulating the conductance and/or P_0 of postsynaptic GABA_A channels.

Direct effects of NO on MNCs: intrinsic mechanisms

NO may also inhibit the electrical activity of MNCs by acting independently of inhibitory synaptic input. Such a hypothesis has arisen from experiments where the effects of NO were observed in the presence of blockers of both GABAergic and glutamatergic neurotransmissions. Under these conditions, treatment of MNCs with L-arginine reduced their firing rate while treatment with L-NAME, an NOS blocker, produced the opposite effect. These observations are clear indications that endogenous NO directly inhibits the electrical activity of MNCs, independently of an effect via GABAergic neurotransmission. A more detailed analysis of the phenomenon showed that NO promotes a slower depolarization by increasing the

Figure 3. Direct and indirect mechanisms involved in the control of magnocellular neurosecretory cells (MNCs) firing frequency by nitric oxide (NO). NO controls the firing rate of the MNCs in order to prevent over-secretion of the neurohypophysial hormones through an indirect (increase in synaptic inputs) and/or a direct pathway by S-nitrosylation of hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels (see text). Traces shown on each side of the neurohypophysis are action potentials recorded directly from the MNCs under the conditions indicated. Increased firing rate (blue trace), induced by a hypertonic solution, is associated with an increased VP/OT secretion (blue arrow). NO decreases firing rates (red traces) in both basal and osmotic stress conditions. Voltage and time scales are the same for all records.

hyperpolarizing after potential amplitude (35), a component involved with spike frequency adaptation. Depolarization after potentials are determined by inward cation currents flowing through, but not only, hyperpolarization-activated and cyclic nucleotide-gated channels (HCN channels). Current flowing through the HCN

channels (I_h) is involved in the control of neuronal rhythmic activity, also regulating the spontaneous activity of MNCs. It has been observed in our lab that NO decreases the amplitude of I_h currents. This effect on the HCN channels seems to be independent of the soluble guanylyl cyclase-cGMP pathway (da Silva MP and Varanda WA, unpublished results), since the soluble guanylyl cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), did not prevent the effects of L-arginine. Alternative routes by which NO may modulate the kinetics of HCN channels may involve other intracellular messenger pathways (84) and/or the S-nitrosylation mechanism proposed to occur in other cell types.

Hyperosmolality and NO signaling in MNCs

Nitroergic signaling in MNCs seems also to be influenced by osmosensitive mechanisms. Blockade of NOS depolarizes the resting membrane potential both in hyperosmotic and isotonic conditions. Indeed, L-arginine significantly decreased the action potential firing rate elicited by hypertonicity, and blocking NOS induced a further increase in the frequency of action potential firing induced by the hypertonic solution. This suggests that, during the osmotic challenge, endogenous NO is synthesized, and modulates the electrical activity of MNCs (52). Since these results were obtained without major excitatory and inhibitory synaptic input, they suggest that MNCs exhibit intrinsic osmosensitivity, which may induce the synthesis of NO. How do intrinsic osmosensitive mechanisms affect nitroergic signaling in MNCs? A cationic conductance is augmented in MNCs during hyperosmotic stress, suggesting that intrinsic osmosensitivity may directly modulate the synthesis of NO. Recently, it was reported that an N-terminal variant of the transient receptor potential cation channel subfamily V member 1 (TRPV1) is essential for osmosensory transduction in supraoptic MNCs. A splice variant of the TRPV1 channel mediated hyperosmotic stimulus-induced depolarizing potential and action potential discharge (85). As the activation of TRPV channels is known to occur during

Table 2. Brain nitroergic system differentially modulates vasopressin (VP) and oxytocin (OT) secretion in rats under osmotic or volume challenge.

	Physiological conditions		Microinjection of AngII		Total dehydration		Intracellular dehydration		Hypovolemia	
	VP	OT	VP	OT	VP	OT	VP	OT	VP	OT
Effect of central NOS blockade	↑	↑	NS	↑	NS	↑	NS	↑	NS	↑

Brain nitroergic system was blocked by *icv* microinjection of L-NAME (50 $\mu\text{g}/\mu\text{L}$). Under physiological conditions, central nitric oxide synthase (NOS) blockade negatively modulates VP and OT secretion. On the other hand, *icv* microinjection of AngII (50 $\text{ng}/\mu\text{L}$), total (intracellular + extracellular) and intracellular dehydrations and hypovolemia remove the inhibitory effect on VP secretion and kept the inhibitory effects only on OT secretion. Upward arrows indicate significant increase compared to the control group. NS: nonsignificant changes compared to the control group. Data obtained from Refs. 33, 49, 55, and 56.

hypertonicity and may lead to NO synthesis, it is suggested that an osmotic stimulus may also indirectly elicit NO synthesis in MNCs through this pathway.

Concluding remarks

Although NO is a gas, its diffusion radius limits the extension of its actions. Thus, it is thought that endogenous NO produced within the SON represents an important neuromessenger to quickly control the activity of MNCs acting at synaptic elements and/or the MNCs themselves. NO may be seen as a fine tuner modulating MNC activity by increasing GABA neurotransmission and reducing I_h currents. In this way, NO is part of a feedback compensatory mechanism that is set to avoid overactivity

of MNCs and, hence, oversecretion of VP and OT. Osmotic stress increases NO levels in MNCs, indirectly through glutamatergic neurotransmission and, directly, possibly through TRPV activation. Increased NO levels would avoid excessive MNC activation, and hence VP and OT depletion. Figure 3 and Table 2 depict the relevant points involved in the control of VP and OT secretion as described earlier.

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***In vitro* differentiation between oxytocin- and vasopressin-secreting
magnocellular neurons requires more than one experimental
criterion**

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Highlights

- OT and VP neurons, in slices, cannot be truly distinguished by their I-V relationships
- “Intermediate” and VP neurons exhibit both outward and inward rectifying currents
- The proportional number of VP and OT neurons can change under water restriction
- Immuno labeling strongly correlates with VP and OT mRNA expression

Abstract

The phenotypic differentiation between oxytocin (OT)- and vasopressin (VP)-secreting magnocellular neurosecretory cells (MNCs) from the supraoptic nucleus is relevant to understanding how several physiological and pharmacological challenges affect their electrical activity. Although the firing patterns of OT and VP neurons, both *in vivo* and *in vitro*, may appear different from each other, much is assumed about their characteristics. These assumptions make it practically impossible to obtain a confident phenotypic differentiation based exclusively on the firing patterns. The presence of a sustained outward rectifying potassium current (SOR) and/or an inward rectifying hyperpolarization-activated current (IR), which are presumably present in OT neurons and absent in VP neurons, has been used to distinguish between the two types of MNCs in the past. In this study, we aimed to analyze the accuracy of the phenotypic discrimination of MNCs based on the presence of rectifying currents using comparisons with the molecular phenotype of the cells, as determined by single-cell RT-qPCR and immunohistochemistry. Our results demonstrated that the phenotypes classified according to the electrophysiological protocol in brain slices do not match their molecular counterparts because vasopressinergic and intermediate neurons also exhibit both outward and inward rectifying currents. In addition, we also show that MNCs can change the relative proportion of each cell phenotype when the system is challenged by chronic hypertonicity (70% water restriction for 7 days). We conclude that for *in vitro*

preparations, the combination of mRNA detection and immunohistochemistry seems to be preferable when trying to characterize a single MNC phenotype.

Keywords: magnocellular neurons; supraoptic nucleus; rectifying currents; vasopressin; oxytocin; molecular identification; electrophysiology

1. Introduction

Magnocellular neurosecretory cells (MNCs) located in the hypothalamic supraoptic nuclei are responsible for the synthesis and secretion of vasopressin (VP) and oxytocin (OT). Once released by the neurohypophysis to the circulation, these neuropeptides have different effects. Because they can be released independently (Antunes-Rodrigues et al., 2004), studies have been performed to identify parameters that would allow a quick and reliable phenotypic differentiation between MNCs.

From the 1960s until the end of the 1980s, the classical view of hormone production by the MNCs of the supraoptic nucleus relied on the premise that OT and VP are produced by mutually exclusive types of neurons (Sokol and Valtin, 1967, Mohr et al., 1988a). Later, it was shown that a third phenotype, which was able to produce both hormones, was also present in the supraoptic nucleus (Kiyama and Emson, 1990, Mezey and Kiss, 1991). Currently, evidence shows that any MNC can produce the mRNA for both VP and OT, albeit in different quantities. Thus, a given phenotype may be defined based on the predominantly expressed peptide, i.e., vasopressinergic neurons would produce circa two orders of magnitude more vasopressin than oxytocin and *vice versa*. The third, “intermediate” phenotype, from now on referred to as intermediate cells, would produce both peptides in equivalent amounts (Glasgow et al., 1999, Xi et al., 1999, Custer et al., 2007).

Distinguishing phenotypes among MNCs becomes relevant in experiments studying the modulatory effects of drugs on electrical activity because different results can be obtained depending on the phenotype of each cell (Hirasawa et al., 2003, Hoyda et al., 2007, Zampronio et al., 2010). In this regard, it has been suggested that OT and VP neurons can show different electrophysiological properties with a coupling between the firing rate pattern and the release of a given neuropeptide (Poulain and Wakerley, 1982). From these types of parameters, cells were identified by associating phenotypes to specific action potential firing characteristics: a firing pattern in which action potentials are intercalated with silent periods was considered typical of VP neurons, whereas a pattern showing bursts of high frequency firing would characterize OT neurons (Brimble and Dyball, 1977, Poulain et al., 1977, Yamashita et al., 1983). However, Moos and Ingram (1995) and Armstrong et al (1995, 2010) observed that *in vivo* and *in vitro*, both VP and OT neurons may fire action potentials at high or low frequency, phasically or in bursts, or they may even be silent. These findings suggest that the action potential firing pattern should not be used as the single criterion to distinguish between OT and VP phenotypes.

Another procedure to electrophysiologically distinguish between these two neuronal types was suggested by Stern and Armstrong (1995, 1997) based on their observation that for negative current injections, the current-voltage relationship of VP neurons is linear, while that of OT neurons is not. The authors report that this difference occurs because OT neurons exhibit the so-called sustained outward rectifying potassium current (SOR), which is activated upon the application of negative current steps (Stern and Armstrong, 1995, Armstrong and Stern, 1998). In addition, experiments using immunolabeled neurons or neurons expressing eGFP have suggested that OT neurons have not only SOR but also a hyperpolarization-activated inward rectifying current (IR);

this property could also be used to distinguish them from VP neurons (Hirasawa et al., 2003, Zampronio et al., 2010). Although these protocols have been used, they clearly disregard the existence of intermediate neurons. Studies using single-cell RT-qPCR demonstrated that all MNCs express both OT and VP mRNA, albeit in different quantities (Glasgow et al., 1999, Xi et al., 1999).

In this paper, we combined real-time single-cell RT-qPCR, immunofluorescence and patch-clamp recordings of MNCs in slices of the supraoptic nucleus of male rats to determine the reliability of the different methods for detecting a particular type of MNC. In addition, we also used water restriction and salt-loading rat models to investigate changes in the proportion of phenotypically different cells. Our results show that the *whole cell* voltage clamp electrophysiology protocol is not sensitive enough to discriminate between MNC phenotypes. This inadequacy mainly occurs because vasopressinergic and intermediate neurons can both exhibit non-linear current-voltage relationships, and oxytocin neurons exhibit a linear relationship. Furthermore, we also demonstrated that in rats subjected to a water restriction protocol, the proportion of OT and VP neurons changes, suggesting that MNCs are, in principle, able to synthesize and modulate the quantities of both peptides.

2. Material and methods

2.1. Ethics

The experimental protocols used in the present study were approved by the Ethical Committee for Animal Experimentation of the Ribeirão Preto Medical School, University of São Paulo (023/2012) in accordance with the Guide for Care and Use of Laboratory Animals of the National Institute of Health (USA).

2.2. Hypothalamic Slices Preparation

Hypothalamic brain slices from male *Wistar* rats (25-30 days old) were prepared as described previously (da Silva et al., 2013). In short, following decapitation, the brain was rapidly removed from the cranial vault and submerged in cooled ($0^{\circ}\text{C} - 4^{\circ}\text{C}$), oxygenated (95% O_2 – 5% CO_2) artificial cerebro-spinal fluid solution (ACSF) containing (in mM) 121 NaCl, 2.5 KCl, 26 NaHCO_3 , 25 glucose, 2 sodium lactate, 0.4 sodium ascorbate, 2 CaCl_2 and 1 MgCl_2 that was saturated with carbogen ($\text{pH} = 7.4$ and osmolality = 310 mosm/kg H_2O (Fiske Mark 3 Osmometer, USA)). Next, 230 μm thick coronal slices containing the supraoptic nucleus were obtained using a Leica vibratome (model VT 1200 S, Heidelberg, Germany). They were incubated for at least 1 hour at 36°C in a modified ACSF containing (in mM) 121 NaCl, 4 KCl, 2 CaCl_2 , 1 MgCl_2 , 26 NaHCO_3 , 10 glucose and 1.25 NaH_2PO_4 (constantly gassed with carbogen, $\text{pH} = 7.4$ and osmolality = 295 mosm/kg H_2O) for metabolic reestablishment.

2.3. Electrophysiology

Magnocellular neurons of the supraoptic nucleus were visualized using infrared differential contrast optics with images captured by a CCD digital camera (SensiCam qe, The Cooke Corporation, Romulus, MI, USA). Anatomical and morphological characteristics were used for their identification (Paxinos and Watson, 2007). Electrophysiological recordings were made using the *voltage clamp* mode in the *whole cell* configuration of the patch clamp technique. Micropipettes were made from borosilicate glass capillaries and had resistance ranging from 4 to 6 $\text{M}\Omega$ when filled with the following pipette solution (in mM): 140 potassium gluconate, 10 KCl, 0.75 CaCl_2 , 1 MgCl_2 , 10 HEPES, 1 EGTA, 2 Na-ATP and 0.25 Na- GTP ($\text{pH} = 7.3$, and osmolality ranging from 285 to 295 mosm/Kg H_2O). The “Milli Q” water used to prepare pipette solutions was treated overnight with Diethylpyrocarbonate (DEPC) (Sigma Chemical Corporation, St. Louis, MO, USA). Recordings were performed using

an Axon 200B patch clamp amplifier (Molecular Devices, Foster City, CA, USA), and signals were low-pass filtered at 2 KHz and digitized at 10 KHz using a Digidata 1440A (Molecular Devices) controlled by Clampex (Molecular Devices, Foster City, CA, USA). Recordings started 5 minutes after achieving the *whole cell* configuration, and the membrane capacitance and series resistance were compensated (40-60%) using the amplifier settings. The liquid junction potential between the pipette and bath solutions was calculated (-15 mV) using Clampex, and the values presented here were corrected accordingly. All data were analyzed using Clampfit (Molecular Devices) and OriginPro 8 (OriginLab Corporation, Northampton, MA, USA).

2.3.1. Electrophysiological Phenotypic Differentiation Protocol

The electrophysiological protocol used to differentiate OT from VP cells was based on that described by others (Armstrong and Stern, 1998, Hirasawa et al., 2003). Briefly, the discrimination is based on the presence or absence of sustained outward and/or hyperpolarized-activated rectifying potassium currents. Under *voltage clamp*, hyperpolarizing voltage pulses from -40 mV to -130 mV from a holding potential of -40 mV and lasting 250 ms were applied, and steady-state current-voltage (I-V) relationships were constructed. In this context, linear relationships defined VP neurons, and non-linear relationships defined OT neurons.

2.4. Molecular Identification of Phenotypes

Subsequent to the electrophysiological recordings, the cytoplasm of each cell was collected into the recording pipette and transferred to a reaction tube containing reverse transcription master mix in DEPC-treated water (High Capacity cDNA Reverse Transcription Kit, Life Technologies Corporation, Carlsbad, CA, USA). The reverse transcription was performed immediately after the recording in a thermocycler (Master

Cycler, Eppendorf, Hamburg, Germany) using the protocol recommended by the reverse transcription kit manufacturer.

2.4.1. cDNA Pre-Amplification Reaction

Because the amount of cDNA obtained from a single-cell is very small, a pre-amplification step was performed using the TaqMan PreAmp Master Mix Kit (Life Technologies Corporation, Carlsbad, CA, USA) with the following hydrolysis probes: Rn00564446_g1 (oxytocin) and Rn00566449_m1 (vasopressin). All reagents were from Life Technologies Corporation and were used in accordance with the pre-amplification master mix kit instructions. Afterward, the cDNA samples were quantified (1766 ± 19 ng/ μ l, $n = 74$) in a spectrophotometer (Nanodrop 1000, Thermo Scientific, Wilmington, DE, USA) and stored at -20°C until used in the real-time PCR reaction.

2.4.2. Real-Time PCR

qPCR was performed in a StepOnePlus thermocycler (Life Technologies Corporation, USA). Reactions were conducted in singleplex and in triplicate for each gene with the same hydrolytic probes described before and using the kit *TaqMan Universal PCR Master Mix* (Life Technologies Corporation). The quantification cycle (Cq, as defined by Bustin et al (2009)) was automatically calculated by the equipment's software. For each plate, the amplification efficiency (E_A) of the reaction for each gene was calculated from calibration curves based on serial dilutions of a pool of cDNA from all samples. E_A , in percentage, was calculated using the following equation:

$$E_A = \left(10^{\left(\frac{-1}{m}\right)} - 1\right) \times 100 \quad \text{Equation 1}$$

where m is the slope of the line resulting from the calibration curve.

In this paper, the average amplification efficiencies for vasopressin and oxytocin were $70 \pm 1.2\%$ and $72 \pm 0.6\%$, respectively ($n = 13$ plates). Because the efficiencies of the probes were lower than 95%, each plate had its own standard curve.

2.4.2.1. Analysis of the RT-qPCR Results

In this study, the relative expression of the two genes, oxytocin and vasopressin, was determined based on two assumptions: (1) The quantity of material in a given cycle is a function of the initial quantity according to equation 2 (Heid et al., 1996):

$$X_{T,n} = X_{T,0} \times (1 + E_A)^n \quad \text{Equation 2}$$

where $X_{T,n}$ is the number of target molecules “T” in the each cycle; $X_{T,0}$ is the initial number of target molecules; E_A is the target amplification efficiency, calculated by equation 1; and n is the number of cycles.

(2) The amounts of the target genes are equal at the cycle where C_q was calculated, i.e.,

$$X_{VP,C_q} = X_{OT,C_q} \quad \text{Equation 3}$$

where X_{VP,C_q} and X_{OT,C_q} are the quantities of vasopressin and oxytocin cDNA, respectively, in the quantification cycle (C_q).

From equations 2 and 3,

$$\frac{X_{VP,0}}{X_{OT,0}} = \frac{(1+E_{OT})^{C_{qOT}}}{(1+E_{AVP})^{C_{qVP}}} \quad \text{Equation 4}$$

where $\frac{X_{VP,0}}{X_{OT,0}}$ expresses the ratio between the initial amount of vasopressin cDNA and the initial amount of oxytocin cDNA. Therefore, neurons in which this ratio was greater than 1 were considered vasopressinergic neurons, and those in which the ratio was smaller than 1 were considered oxytocinergic neurons. A ratio equal to 1 was

considered a characteristic of an intermediate phenotype, in which the VP and OT cDNAs are expressed in similar quantities.

2.5. Electrophysiology, RT-qPCR and Immunofluorescence in a Single Neuron

To avoid mistakes related to the expression of cDNA and its correlation with peptide production, we performed a triple characterization in a single neuron using not only electrophysiology and RT-qPCR but also immunohistochemistry. For this purpose, we added an intracellular tracer, biocytin (Life Technologies Corporation), into the patch pipette solution (final concentration = 0.2%) and performed electrophysiology measurements in a new group of neurons (n=22). Subsequent to the electrophysiological measurements, the cytoplasm of each cell was collected for single-cell RT-qPCR following the same steps described previously in section 2.4. After these procedures, the slice was fixed overnight with 4% paraformaldehyde in phosphate-buffered saline (PBS – 0.01 M) and then dehydrated in 30% sucrose. Both steps were conducted at 4 °C. After dehydration, the slices were blocked and permeabilized in PBS containing 10% normal horse serum and 0.1% Triton X-100 for 1 hour at room temperature. Then, the slices were incubated overnight at 4 °C with primary antibodies: guinea pig anti-OT (1:2000, cat. n° T-5021 Peninsula Laboratories, San Carlos, CA, USA) and rabbit anti-VP (1:2000, (Arg⁸)-Vasopressin, cat. n° 4563, Peninsula Laboratories, San Carlos, CA, USA). Subsequently, the slices were washed using 0.01 M PBS and incubated with secondary antibodies: donkey anti-Rabbit conjugated to fluorescein isothiocyanate (FITC) for VP and donkey anti-guinea pig conjugated to Cy5 for OT (Jackson Immuno Research Lab, West Grove, PA, USA - both diluted 1:250). At the same time, the biocytin-labeled recorded neurons were revealed by reaction with streptavidin (Alexa fluor 405 conjugate, Life Technologies Corporation, Carlsbad, CA, USA), which was diluted 1:500. The slices were then washed three times in PBS and mounted onto glass

slides with Fluoromount (Sigma Chemical Corporation, St. Louis, MO, USA). Fluorescence images were acquired sequentially (interval of 0.3 μm) in a confocal microscope (TCS SP5, Leica, Heidelberg, Germany), and the images were analyzed using the software ImageJ (W.S. Rasband/National Institute of Health, USA - <http://rsb.info.nih.gov/ij>). For each slice, the biocytin-positive neuron was identified, and *x* and *z* projections were collected sequentially to analyze the colocalization.

2.6. Physiological Challenges and Phenotypes

To estimate the relative proportions of OT and VP MNCs, male rats (25-30 days old) were divided in three distinct groups: euhydrated, water-restricted, and salt-loaded rats ($n=4$ per group). In the euhydrated group, the rats had free access to water for 7 days. In the water-restriction model, the rats only had access to 30% of the volume of water ingested by the euhydrate group; the water was offered daily at the beginning of the dark phase of the cycle. In the salt-loaded model, the rats only had access to a 1.8% NaCl solution *ad libitum* for 7 days instead of water. On the seventh day, the rats were anesthetized (tribromoethanol: 250 $\text{mg}\cdot\text{kg}^{-1}$, i.p.; Sigma Chemical Corporation, St. Louis, MO, USA) and perfused transcardially with 0.1 M PBS followed by 4% paraformaldehyde (PFA). Sections (30 μm thick) of the hypothalamic supraoptic nucleus were made using a HM 505 N cryostat (Microm, Waldorf, Germany) and were stored in a cryoprotectant solution (0.1 M PBS: 0.3% ethylene glycol, 0.2% glycerol, v/v) at -20°C until processed. All immunofluorescence experiments were performed at the same time using exactly the same protocol described above, and the images were acquired sequentially (one frame every 0.3 μm) from six medial sections of the supraoptic nucleus *per* rat under a confocal microscope (TCS SP5, Leica, Heidelberg, Germany). After the image acquisition, VP and OT immunoreactive neurons were blindly counted bilaterally using image stacks produced with the Cell Counter Plugin of

Image J. The counts from all slices were averaged, and the results were plotted as the mean $\pm$ SEM. The proportion of each phenotype encountered in each experimental condition (euhydrated, water-restricted, and salt-loaded) was compared with that in the euhydrate rats using one-way ANOVA with the Bonferroni post hoc test (Prism 5 – GraphPad Software, Inc., La Jolla, CA, USA). P values less than 0.05 were considered significant.

3. Results

3.1. Electrophysiological Characterization of MNCs

We first set out to investigate whether rectifying currents are indeed a good criterion to determine the phenotype of MNCs. We performed electrophysiological measurements followed by single-cell RT-qPCR in 74 neurons from the supraoptic nucleus and correlated the presence/absence of rectifying currents to the ratio of VP mRNA to OT mRNA.

The electrophysiological identity of the neurons was assessed by recording currents in response to -10 mV voltage steps (250 ms), which were applied from -40 mV to -130 mV from a holding potential of -40 mV every 30 seconds. This protocol was applied 5, 10 and 15 minutes after breaking into the cell, and the steady-state currents were measured at the end of the pulse. As suggested in the literature, non-linear *I-V* relationships (indicating the presence of SOR and/or IR) were taken as indicative of OT neurons, whereas linear relationships suggested VP cells.

As shown in the figure 1A to 1D, four distinct groups of *I-V* relationships were found: three showed rectifying currents, and one showed a linear relationship. Among the 74 recorded neurons, 9 exhibited SOR, 39 presented IR, 15 exhibited both SOR and IR currents, and 11 showed a linear *I-V* relationship (Fig. 1E).

The presence of SOR was observed at voltages more positive than -60 mV, and the presence of IR was found at voltages more hyperpolarized than -70 mV; both currents have been used as criteria to identify OT neurons. Therefore, the 63 neurons that presented inward and/or outward rectification were classified as OT, and the 11 that exhibited a linear response were classified as VP neurons (Fig. 1F).

For comparison, the electrophysiological protocol was also used to characterize 22 magnocellular neurons from the lateral paraventricular nucleus (PVN). The same four I - V relationships reported for SON were also found in these neurons. Thus, out of 22 neurons, 5 exhibited SOR, 11 showed IR, 3 showed both SOR and IR, and only 3 had a linear response. According to this criteria, 19 neurons were classified as oxytocinergic, and 3 were considered vasopressinergic neurons (supplemental material Fig. 1S).

3.2. RT-qPCR Characterization

After the electrophysiological characterization, while still in *whole cell* mode, the cytoplasm of each recorded cell was aspirated into the patch pipette, and the mRNA was immediately transcribed into cDNA.

3.2.1. Positive Control for cDNA Pre-Amplification

Because the amount of mRNA collected from single cells was very small and needed to be pre-amplified prior to the PCR step, we first designed a control experiment for the pre-amplification step to determine whether this process could somehow distort the proportions of VP and OT cDNA in the samples and therefore affect the phenotypic characterization. For this purpose, we recorded currents from 10 neurons, collected the

cytoplasm, and after the transcription phase; for these same neurons, the cDNA was pre-amplified once and then subjected to the PCR procedures. The product of this first PCR step was then subjected to a second pre-amplification and a subsequent PCR quantification. As shown in the Table 1, the pre-amplification step did not change the $\frac{X_{AVP,O}}{X_{OT,O}}$ ratio of the individual neurons, demonstrating that this procedure did not affect the phenotypic characterization.

Table 1 here

3.2.2. PCR Phenotype Results

From the 74 neurons characterized, 13 had the ratio $\frac{X_{VP,O}}{X_{OT,O}} < 1$, indicating a larger proportion of oxytocin cDNA. The mean $\pm$ SEM of the $\frac{X_{VP,O}}{X_{OT,O}}$ ratio for these cells was 0.07 ± 0.02 , and the ratio ranged from 0.002 to 0.3. Forty-three neurons showed $\frac{X_{VP,O}}{X_{OT,O}} > 1$ and were identified as vasopressinergic (ratio ranged from 80.7 to 3097.5, with a mean of 554.3 ± 205). In addition, eighteen neurons showed a $\frac{X_{VP,O}}{X_{OT,O}} \sim 1$ (mean = 1.2 ± 0.1 , ranging from 0.7 to 1.7) and were classified as intermediate cells (Fig. 2).

Comparing the results obtained using the two methods (Fig. 3), we observe that out of the eleven neurons that were considered vasopressinergic according to the electrophysiology recordings, seven were also identified as vasopressinergic by RT-qPCR, one was characterized as oxytocinergic, and three were considered as intermediate. The surprising aspect of these results is that 85% of the neurons were identified as OT by electrophysiology, in clear contrast with the results of RT-qPCR, as shown in Fig. 3.

For comparison with the results for the MNCs from the PVN, single-cell PCR was also performed in the 22 previously described neurons. From those 22 neurons, 16 were identified as vasopressinergic with ratio $\frac{X_{VP,O}}{X_{OT,O}} > 1$ (ranging from 109.7 to 2296.3, average = $4,433.4 \pm 2,849.8$), and 6 expressed only VP cDNA. Four neurons had a ratio < 1 (ranging from 0.0007 to 0.04, with mean of 0.018 ± 0.009) and were classified as oxytocinergic. The other 2 neurons were identified as intermediate, $\frac{X_{VP,O}}{X_{OT,O}} \sim 1$ (mean = 0.8 ± 0.6 ; from 0.15 to 1.5). In Fig. 1S (supplemental material), these results are compared with the results obtained from the electrophysiological protocol. We can see that the correlation with RT-qPCR was also low because out of the 19 neurons identified as oxytocinergic by electrophysiology, only 3 were in agreement with RT-qPCR (supplemental material). Thus, approximately 75% of the neurons from the SON and 77% from the lateral PVN that were identified using the electrophysiological protocol did not match the molecular phenotype obtained by RT-qPCR.

3.3. Electrophysiology, mRNA and Peptide Relationships

Because protein levels are highly regulated by cells, the correlation between cDNA levels and protein and/or peptide expression is complex. VP and OT are peptides; to overcome this problem, we performed experiments combining electrophysiology, RT-qPCR and immunofluorescence for VP and OT, all on a single neuron (for detail, see methods section 2.5).

Out of 22 immunocytochemically identified neurons from 5 rats, 11 were immunoreactive for vasopressin, and 11 were immunoreactive for oxytocin. The single-cell RT-qPCR of the 11 vasopressinergic neurons indicated that 9 were also vasopressinergic and that the other two were intermediates. A similarly strong correlation between RT-qPCR and immunofluorescence was obtained for oxytocinergic

neurons, i.e., out of 11 oxytocinergic neurons, 11 were also identified as OT by PCR (Fig.4).

Regarding the electrophysiological results for these neurons, we observe that the majority ($n = 19$) presented inward and/or outward rectification (supposedly OT), and only 3 neurons had a linear $I-V$ relationship (supposedly VP). However, comparing the electrophysiological data with the two previous methods, we show that these results were positively correlated with the results from immunofluorescence for only ten neurons (9 OT and 1 VP). Of these, 8 showed positive correlation with both immunofluorescence and RT-qPCR, and the other two neurons were characterized as intermediate by RT-qPCR (Table 2, Fig. 4 C). These results support our hypothesis that the presence or absence of rectifying currents is not a good parameter to discriminate MNC phenotypes.

Table 2 here!!

3.4. Physiological Implications

The importance of properly characterizing the phenotype of MNCs can be readily appreciated in conditions where the hydroelectrolytic balance is challenged. Under these conditions, MNCs that express both OT and VP mRNA could be induced to synthesize one or the other peptide if necessary. To test this hypothesis, we designed experiments using two models in which rats were subjected either to water restriction or to salt overload for 7 days (see methods 2.6). As shown in figure 5, in the water restriction model, we observed a significant increase in the number of immunoreactive vasopressin neurons, as well as a decrease in oxytocinergic immunoreactive neurons compared with that in the rats in the control group (VP = 60.4 ± 3 vs 84.1 ± 5.3 and OT = 61.7 ± 3 vs 48.5 ± 1.6 ; $p < 0.005$ and $p < 0.05$, respectively). However, in the salt-loaded

rats, we did not observe a significant difference between the numbers of VP (60.4 ± 3 vs 64.5 ± 4 ; $p > 0.05$) and OT (61.7 ± 3 vs 54 ± 3.7 ; $p > 0.05$) neurons.

4. Discussion

In the present study, we analyzed the adequacy of using rectifying currents to accurately distinguish between MNC phenotypes. We compared electrophysiological results with single-cell RT-qPCR and performed a triple characterization with immunohistochemistry for OT and VP. We also analyzed the relative numbers of OT to VP cells using immunohistochemistry in rats subjected to water restriction or salt loading; in these situations, the populations of OT and VP neurons can change relative to those in the control.

4.1. MNC differentiation based on rectifying currents

Under certain circumstances, it is essential to know the MNC phenotypes to fully understand the action of specific substances on their excitability because different responses can be obtained for each phenotype (Hoyda et al., 2007, Price et al., 2009, Zampronio et al., 2010). From an electrophysiological point of view, VP and OT neurons can be distinguished from each other using several procedures. However, most of these procedures are based on the response of neurons to particular peptides or, for example, during the sucking reflex (Poulain and Wakerley, 1982, Armstrong, 1995, Gouzenes et al., 1998), making the process of phenotypic differentiation somewhat difficult. Another complication in differentiating MNCs based solely on electrophysiology arises from the work of Moos et al. (1995). They demonstrated that during breastfeeding, some neurons displayed both vasopressinergic-type phasic activity and oxytocinergic-type burst activity. This finding raises the possibility that both peptides could be produced by the same cell. However, the discovery of rectifying

currents, which are supposedly expressed in OT but not VP neurons (Stern and Armstrong, 1995, Armstrong and Stern, 1998, Zampronio et al., 2010), has been used as a criterion to discriminate between OT and VP neurons.

The idea that oxytocinergic but not vasopressinergic neurons show an outward rectifying current was first proposed by Stern and Armstrong (1995). In their experiments, the authors found differences in the intrinsic membrane properties of VP and OT neurons of both virgin and lactating female rats. Later, Hirasawa and collaborators described a second type of current that was present only in OT neurons, the inward rectifying current that is activated at membrane potentials more negative than -90 mV (Hirasawa et al., 2003). Since then, both types of rectifying currents have been used as a criterion to characterize oxytocinergic MNCs. However, our electrophysiological results showed that out of 63 neurons that were characterized as oxytocinergic due to the presence of rectifying currents, 36 were identified as vasopressinergic by RT-qPCR, and surprisingly, 18 neurons showed mRNA expression for both OT and VP in equivalent amounts, characterizing them as intermediate (Fig. 3). Immunofluorescence experiments confirmed this discrepancy because out of 11 VP-positive neurons, 8 also showed rectifying currents (Fig. 4). Therefore, our results indicate that the presence or absence of rectification in the $I-V$ relationship cannot be used as a safe criterion for defining MNC phenotypes. Additionally, even when using transgenic rats expressing eGFP, Zampronio et al (2010) observed that not all neurons that were identified as oxytocinergic due to the absence of eGFP had rectifying currents. This finding reinforces the idea that this type of current is not restricted to OT neurons. Furthermore, neurons that expressed both peptides, as indicated by immunofluorescence, were also not included in the group of neurons analyzed. The divergent results found by us can be attributed to the following: 1) the presence of

outward and inward currents in vasopressinergic and intermediate cells and 2) the use of sharp electrodes when obtaining the first description of the outward rectifier current (but not the following ones, see Hirasawa et al., 2003 and Zampronio et al., 2010) and its correlation with oxytocinergic neurons. For those recordings using sharp electrodes, the current injections lasted six times longer than patch-clamp experiments (Stern and Armstrong, 1995, 1997, Armstrong and Stern, 1998). In fact, studies comparing results from sharp electrodes and whole cell patch clamp experiments show that due to the larger leakage current observed in the case of sharp electrodes, the intrinsic membrane properties of the neuron can be changed, leading, for example, to a depolarization in the resting membrane potential (Staley et al., 1992, Li et al., 2004). In contrast, our results are consistent with a recent study of Li et al (2007) in which they were unable to reproduce the electrophysiological phenotype findings reported by others. In our experiments, the input resistance ($VP = 997 \pm 150 \text{ M}\Omega$ and $OT = 1012.3 \pm 230 \text{ M}\Omega$) and capacitance ($OT = 20 \pm 4.3 \text{ pF}$; $VP = 23 \pm 6.8 \text{ pF}$) were not different between VP and OT neurons, in accordance with the findings of Armstrong et al. (1994).

4.2. Electrophysiology, RT-qPCR and Immunofluorescence

The measurement of mRNA expression is commonly used in the literature as an estimation of protein/peptide synthesis. However, this correlation is still a controversial matter, and the results described so far have been relatively inconsistent. High and low correlation coefficients between the two parameters have been reported for proteins and have ranged from 0.40 to 0.78 (Gygi et al., 1999, Guo et al., 2008, Maier et al., 2009); a high correlation has been reported for peptides (Murray et al., 1999). To avoid mistakes due to the phenotype characterization by mRNA expression alone, we performed a triple characterization in a single neuron by comparing the electrophysiological results not only with RT-qPCR but also with

immunofluorescence (see methods 2.5). As seen in figure 4, our results show a very strong phenotype correlation between immunofluorescence and RT-qPCR. Out of 22 neurons analyzed, 11 were characterized as oxytocinergic by immunofluorescence and also by RT-qPCR. The other 11 were identified as vasopressinergic by immunofluorescence: 9 were also classified as vasopressinergic by RT-qPCR, and 2 were intermediate. In contrast, the electrophysiological results for the same 22 cells did not match the phenotype determined by both immunofluorescence and/or RT-qPCR. According to the electrophysiological protocol, 19 neurons showed rectifying currents related to oxytocinergic cells, and only 3 had a linear $I-V$ relationship that identified them as vasopressinergic neurons. When we compare the results obtained by these three methods, we observe that the electrophysiology classifications matched the immunofluorescence categorization for only 10 neurons, and the electrophysiology classified matched both the immunofluorescence and RT-qPCR classifications for only 8 neurons (Table 2). This fact is particularly critical when considering situations where the relative proportion of neuronal types changes.

4.3. Physiological Implications

The number of neurons with an intermediate phenotype found in the RT-qPCR experiments ($n=18$) is not surprising because 1% - 3% of MNCs coexpress both peptides under physiological conditions. Furthermore, when the system is confronted with challenging situations, such as lactation, this percentage may increase to 24%, and both peptides can be found in the same neurosecretory vesicle. Under these conditions, Mezey et al (1991) suggested that OT neurons expressing little or no VP gain the ability to synthesize VP to compensate for the loss of water observed during lactation. Our results are consistent with this hypothesis because the rats subjected to water restriction

showed an increase in immunoreactive VP neurons and a decrease in the relative number of OT neurons. This result clearly indicates that neurons have the ability to change their phenotype in response to a particular physiological situation (Fig. 5). This phenomenon can be explained, at least in part, by two factors: 1) the presence of both OT and VP mRNA in the same cell, which allows neurons to synthesize both or just one of the peptides when necessary (via a post-transcriptional regulation of mRNAs) and/or 2) changes in the modulatory control of the genes involved in the expression of OT and VP. These hypotheses are supported by *in situ* hybridization and RT-qPCR studies, which confirm the expression of both mRNA in most MNCs with an evident increase in one of them when the system is osmotically stressed (Kawata et al., 1988, Meeker et al., 1991, Glasgow et al., 1999, Xi et al., 1999). The coexistence of both types of mRNA seems to be a property of magnocellular neurons because magnocellular neurons from the lateral paraventricular nucleus also express both VP and OT mRNA (Supplemental data), whereas parvocellular vasopressinergic neurons from the suprachiasmatic nucleus do not express OT mRNA (Glasgow et al., 1999). The VP and OT genes are found in the same chromosome, are separated only by an intergenic region and are processed in opposite transcriptional directions (Mohr et al., 1988b, Hara et al., 1990). The molecular phenotypic identity of these neurons is likely regulated by specific genes, and the consensus is that the domain between 288 bp to 116 bp contains a VP-specific activator and a repressor that inhibits its expression in OT-MNCs, thereby leading to the cell-type specific expression of only one neuropeptide (Ponzio et al., 2012).

Besides this, several studies have demonstrated that the expression of VP and OT may be influenced by synaptic inputs and osmotic stimuli (Baldino et al., 1988, Meeker et al., 1991, Murphy and Wells, 2003, Gainer, 2012). In fact VP and OT neurons receive both excitatory (glutamate) and inhibitory (GABA) synaptic inputs

(Decavel and Van den Pol, 1990, Meeker et al., 1991). Interestingly GABA may act as an excitatory neurotransmitter in vasopressinergic cells, which lack the K^+/Cl^- cotransporter 2 (KCC2), and as inhibitory neurotransmitter in oxytocinergic neurons (Haam et al., 2012). MNCs are also subjected to an autocrine regulation via the release of OT and VP from dendrites (Gouzenes et al., 1998, Chevaleyre et al., 2000). The local released VP is known to increase the firing rate during osmotic stimulation and hemorrhage (Ludwig et al., 1994, Neumann et al., 1994, Ota et al., 1994), and OT during lactation (Wang et al., 2006). How these control mechanisms can alter the MNCs phenotype is still a matter of debate.

We did not observe significant changes in the numbers of VP and OT neurons in chronic salt-loaded rats compared with that in the controls. Given that in this model, the natriuretic system is also required to operate, it is easily understood why we did not find changes in the relative proportion of phenotypes in this situation (Verbalis et al., 1991, Huang et al., 1995, Sjoquist et al., 1995). In summary, this study is the first to use three different techniques to characterize a single neuron, and the results suggest that in *in vitro* preparations, the electrophysiological protocol alone does not faithfully describe the phenotypes of MNCs. This finding is particularly critical in situations where the system needs to rearrange itself to maintain homeostasis. The combination of mRNA detection and immunohistochemistry seems to be preferable when trying to characterize a single MNC phenotype.

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Legend to Figures

Figure 1. Electrophysiological Differentiation of Magnocellular Neurons. A, B and C: Representative traces of putative oxytocinergic neurons, as determined using the

electrophysiological protocol. The presence of a sustained outward rectifying potassium current (**A**), inward rectifying current (**B**) or both (**C**) identified OT neurons. **D**: representative traces showing the absence of the rectifier currents that characterize a putative VP neuron. **A1, B1, C1** and **D1**: Averaged $I-V$ curves for each case. Points are the mean $\pm$ SEM. The dotted lines at **A1** and **B1** represent the voltage at which SOR and IR, respectively, are identified. Bar graphs summarize the different types of $I-V$ relationships from 74 recorded neurons (**E**) and the classification of phenotypes according to the presence (OT) or absence (VP) of rectifier currents (**F**). Note that according to this protocol, the majority of the cells that expressed one type of rectifier current were classified as OT.

Figure 2. Molecular Phenotype of MNCs. The bar graph summarizes the classification of the 74 neurons by RT-qPCR; the neurons were previously recorded using the electrophysiological protocol. Using this method, 17.6% (n=13) had ratios smaller than 1 and were classified as oxytocinergic neurons; 58.1% (n=43) showed ratios higher than 1 and were characterized as vasopressinergic neurons. In addition, 24.3% (n=18) showed ratios of approximately 1; in these neurons, VP and OT cDNA were found in equivalent amounts, and they were considered intermediate neurons.

Figure 3. Correlation between Electrophysiological and Molecular Phenotypes. The bar graph compares the phenotypes identified by electrophysiology (black bars) and RT-qPCR (gray bars). Of the eleven neurons that were considered VP according to electrophysiology, 9% (n=1) were recognized as OT, 64% (n=7) were considered VP, and 27% (n=3) were classified as intermediate by RT-qPCR. In contrast, out of the 63 neurons identified as oxytocinergic by electrophysiology, 57% of the neurons (n=36) were identified as vasopressinergic, 24% (n=15) were considered intermediate, and only 19% (n=12) were classified as oxytocinergic, indicating that the presence of rectifying

currents must not be considered a reliable parameter for the phenotypic characterization of MNCs.

Figure 4. Characterization of MNC Phenotype Using Three Distinct Methods.

Photomicrography of two representative neurons immunohistochemically identified as OT and VP. Patched neurons loaded with biocytin and visualized with streptavidin are shown in red (**A1** and **B1**). Neurons that were immunoreactive for vasopressin are shown in green (**A2** and **B2**), and those that were immunoreactive for oxytocin are shown in blue (**A3** and **B3**). The phenotypes were identified by colocalization with biocytin-labeled recorded neurons (**A4** and **B4**). The bar graph displays the number of cells with a particular phenotype observed with a particular method. The scale bars correspond to 15 μ m. Note the strong agreement between the results of immunofluorescence and RT-qPCR and the low correlation of these methods with electrophysiology.

Figure 5. Challenging Homeostasis Changes the Proportion of MNC Phenotypes.

A1-C2, Photomicrographs showing immunoreactive VP (red) and OT (green) neurons in the supraoptic nucleus of euhydrated, water-restricted or salt-loaded rats. **A3-C3** are merged images, showing the colocalization of OT and VP in the same neuron (yellow). Scale bar = 25 μ m. OC = optic chiasm. **D**) The bar graph summarizes the number of immunoreactive neurons found for each experimental condition. A nonparametric one-way ANOVA with the Bonferroni post hoc test was used to test for differences between groups (** $p < 0.005$).

Table 1: Positive control of pre amplification

Sample	Phenotype: 1° Pre-Amp	Phenotype: 2° Pre-Amp
1	VP	VP

2	VP	VP
3	OT	OT
4	VP	VP
5	VP	VP
6	VP	VP
7	VP	VP
8	VP	VP
9	OT	OT
10	INTERMEDIATE	INTERMEDIATE

Table 2 – Comparison of the distinction between OT and VP neurons by the three methods: electrophysiology, RT-qPCR and immunofluorescence in single cells.

Cell #	Electrophysiology	RT-qPCR Ratio(Phenotype)	Immunofluorescence
1	IR (OT)	3.10 (VP)	VP
2	IR (OT)	* (OT)	OT
3	IR (OT)	143.6 (VP)	VP
4	SOR (OT)	* (VP)	VP
5	IR (OT)	0.02 (OT)	OT
6	SOR/IR (OT)	0.01 (OT)	OT
7	LINEAR (VP)	0.01 (OT)	OT
8	IR (OT)	0.01 (OT)	OT
9	SOR (OT)	25.61 (VP)	VP
10	LINEAR (VP)	4.91 (VP)	VP
11	IR (OT)	1.2 (INTERMEDIATE)	OT
12	IR (OT)	* (VP)	VP
13	LINEAR (VP)	0.01 (OT)	OT
14	SOR (OT)	* (VP)	VP
15	SOR (OT)	0.03 (OT)	OT
16	IR (OT)	0.01 (OT)	OT
17	IR (OT)	1.4 (INTERMEDIATE)	OT
18	IR (OT)	* (VP)	VP
19	IR (OT)	* (VP)	VP
20	IR (OT)	0.05 (OT)	OT
21	IR (OT)	75.69 (VP)	VP
22	IR (OT)	25.31 (VP)	VP

*cells that had only one type of mRNA expressed.

Gaseous Modulators in the Control of the Hypothalamic Neurohypophyseal System

Nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H₂S) are gaseous molecules produced by the brain. Within the hypothalamus, gaseous molecules have been highlighted as autocrine and paracrine factors regulating endocrine function. Therefore, in the present review, we briefly discuss the main findings linking NO, CO, and H₂S to the control of body fluid homeostasis at the hypothalamic level, with particular emphasis on the regulation of neurohypophyseal system output.

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The synthesis of organic molecules and more complex compounds was essential for the emergence of life on Earth (107). Those events were most likely favored by the ancient ocean environment, which was sodium (Na⁺)-rich in the form of sodium bicarbonate (NaHCO₃) (85). Therefore, unicellular organisms were surrounded by an aqueous Na⁺-enriched environment to which biochemical reactions and other essential physiological processes were adapted. The posterior development of multicellular organisms introduced the concept of intracorporeal extracellular fluid (ECF), which is also rich in Na⁺ and whose composition and volume should be precisely regulated by appropriate physiological systems (5, 76, 103).

Despite the wide variety of habitats colonized by vertebrates, most species have the ability to maintain the volume and ionic composition of their ECF within a narrow range of values compatible with life (103). Considering that life in terrestrial environments is characterized by frequent challenges to body fluid homeostasis and that terrestrial vertebrates are susceptible to desiccation, the maintenance of ECF volume and composition is a constant endeavor. Therefore, the emergence of specialized and highly effective physiological systems implicated in the acquisition and retention of water and electrolytes (especially Na⁺) was essential for the evolutionary success of these animals (97, 103).

Considering age- and gender-related characteristics, as well as variations among species and habitats, water constitutes between 60 and 80% of vertebrate body weight (74, 103). In the adult human, ~65% of body weight consists of water, of which 45% is located intracellularly, with the remaining 20% in extracellular compartments (5, 103). The ions Na⁺, bicarbonate (HCO₃⁻), and chloride (Cl⁻) are the primary solutes in the ECF; they play an important role in the control of volume and osmolality of this compartment (25, 76). Although the ionic concentrations between the intra- and extracellular compartments differ

consistently, the osmolality is the same. Because water permeates biological membranes with abandon, the main challenge faced by organisms involves minimizing the alterations in cellular volume mediated by osmotic pressure-driven water movement. In this regard, Na⁺ is particularly important because of its great hygroscopic property, and, as a result, several renal, hormonal, and cerebral mechanisms target Na⁺ management (input/output rate) in terrestrial vertebrates, ensuring its persistence in the body (25).

Under resting conditions, the combined efforts of membrane transport systems assure relatively stable ionic concentrations in the intracellular compartment, which prevents changes in cellular volume caused by osmotic gradient-derived water movement. Within this context, the addition of Na⁺ to the ECF promotes a transitory increase in the osmolality of this compartment. However, the presence of highly efficient behavioral and neuroendocrine effector systems guarantees a rapid increase in water ingestion and reabsorption, increasing ECF volume in proportion to the amount of Na⁺ added. Therefore, ECF volume and osmolality homeostasis are achieved primarily by the balance between water and Na⁺ input (ingestion, production by metabolism) and their output (excretion) (FIGURE 1). In mammals, small amounts of sodium and water are eliminated by the gastrointestinal tract, sweat glands, skin, and respiratory system. However, the decisive primary route for the excretion of these elements is the kidneys, which can eliminate concentrated or diluted urine according to requirements (6, 76). Despite being the main targets for the neuroendocrine control of hydromineral balance, the kidneys cannot recover water and Na⁺ lost through other systems. This task is accomplished by the development of thirst and Na⁺ appetite, which are essential physiological responses that lead to motivated behaviors for the search and acquisition of water and salt from the environment (33, 40). In the following sections,

the present review will discuss the general mechanisms regulating hydromineral balance, focusing on how gaseous modulators act, particularly at the hypothalamic level, to control water and Na⁺ management.

Sensory Pathways Regulating Hydromineral Balance

It was initially hypothesized that neurons were the primary regulators of osmolality/Na⁺-sensing in the central nervous system (CNS). In fact, primary osmosensory neurons in the organum vasculosum of the lamina terminalis (OVLT) transduce hypertonicity through activation of transient receptor potential vanilloid (TRPV) channels, resulting in membrane depolarization, an effect absent in TRPV1 knockout animals (22, 23). Furthermore, studies conducted by Noda (83) revealed that glial cells

isolated from the subfornical organ (SFO) are sensitive to increased ECF Na⁺ concentrations and that specific Na(x) channels are located in processes originating from SFO ependymal cells and astrocytes. The importance of glial cells in salt homeostasis was further highlighted by the finding that the blockade of astrocyte metabolism significantly reduced the hyperosmolality-induced activation of hypothalamic magnocellular neurons (MNs) (129). More recently, it has been demonstrated that MNs also display intrinsic osmosensitive properties, which mediate osmotic-induced hormone release (96). MNs were also shown to express Na(x) (82), suggesting that these cells are particularly sensitive to extracellular Na⁺ concentrations. Taken together, these results indicate that 1) osmosensitive neurons are located primarily in the lamina terminalis (which comprises the SFO and OVLT) and hypothalamus (MNs), and 2) glial cells may be required to promote subsequent neuronal activation following an osmotic challenge.

In contrast to osmotic stimuli, in which the primary sensory system is located within the CNS, changes in circulating volume/pressure are detected primarily by peripheral mechanoreceptors, which respond to the altered stretch of arterial vascular walls (arterial baroreceptors), or by increasing volumes in cardiac chambers, large systemic veins, and the pulmonary circulation (low-pressure/volume receptors). Action potentials triggered in these sensory nerve endings ascend to the nucleus of the solitary tract (NST) in the brain stem, where the first synapse is established. From the NST, multisynaptic pathways reach the hypothalamus, allowing volume-/pressure-mediated control of hormone release by MNs (5) (FIGURE 2).

Integrated Neuroendocrine Responses to Hydroelectrolytic Imbalances

In the CNS, the hypothalamic neurohypophyseal system plays an essential role in the control of hydromineral balance (6). The paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus receive numerous inputs from central/peripheral osmoreceptors and mechanoreceptors. Therefore, the hypothalamus represents a key site for the integration of hydromineral homeostasis through generation of neuroendocrine and autonomic effector responses (89, 102). The PVN contains not only MNs (which constitute the totality of neuroendocrine cells in the SON) but also parvocellular preautonomic and neuroendocrine groups. MNs from both the PVN and SON, in turn, are implicated in the synthesis of arginine vasopressin (AVP) and oxytocin (OT), which are transported axonally to the posterior pituitary, where they are

FIGURE 1. Main routes for water and sodium management in the adult organism

The main input is provided by water and sodium ingestion with fluids and food, although some water also can be produced by endogenous metabolism. The main final route for water and sodium excretion is represented by the kidneys. However, water can be lost during humidification of the inspired air and also through sweating. Sweat is the product of sweat glands (schematically represented at left) and also contains sodium, although at smaller concentrations than those found in plasma.

released into the circulation. Previous studies reported that, in response to imbalances in ECF volume and/or osmolality, as during hemorrhage, water deprivation, and salt loading, the increased excitability of MNs is associated with increased plasma concentrations of AVP and OT (19). Therefore, the secretion of AVP, OT, and atrial natriuretic peptide (ANP), along with the activation of the renin-angiotensin and autonomic systems, constitute the main effector responses that act coordinately to maintain body fluid homeostasis (5).

The Hypothalamic Neurohypophyseal System as a Target for Gaseous Modulators

The concept of neurotransmission was recently challenged by emerging evidence suggesting that gaseous neuromodulators, such as nitric oxide

(NO), carbon monoxide (CO), and hydrogen sulfide (H₂S), strongly affect neuronal excitability and homeostatic processes (42, 43, 91, 92, 112, 113). NO, CO, and H₂S are diffusible, highly membrane-permeable molecules with a short half-life that are produced on demand, presumably by neurons. These features determine the autocrine and paracrine actions attributed to these mediators (35, 123, 131). In addition to producing vasodilator effects on smooth muscle cells (37, 49, 57, 114), these gaseous molecules were shown to actively participate in neurotransmission (28, 57, 71). At the hypothalamic level in particular, it has been demonstrated that these compounds modulate osmotic-induced neurohormone release (53, 92, 113). The current understanding of how these gaseous neuromodulators participate in hydromineral balance is discussed in the following sections.

FIGURE 2. Sensory pathways implicated in the control of neurohypophyseal hormone secretion

Inputs to the hypothalamic neurohypophyseal system arise from the lamina terminalis in the central nervous system (CNS), where primary osmosensory/Na⁺ receptors are located, and from mechanoreceptors, located in the periphery, particularly in the cardiovascular system. The nucleus of the solitary tract (NST) in the brain stem integrates afferent visceral information and sends projections to the hypothalamus, controlling neurohormone release, as well as to the lamina terminalis, modulating water and salt appetite. The main effects of neurohypophyseal peptides are concentrated in the control of renal management of water (vasopressin, AVP) and sodium (OT). OT also stimulates the secretion of atrial natriuretic peptide (ANP) by the heart. The brain ANPergic system also contributes to some extent to the integrity of peripheral ANP secretion and to the modulation of behavior. AP, area postrema; ME, median eminence; PVN, paraventricular nucleus of the hypothalamus; SON, supraoptic nucleus of the hypothalamus.

NO: From an Endothelium-Derived Relaxing Factor to a Neuromodulator

Initially identified as an endothelium-derived relaxing factor (39) and later as a modulator in the CNS (79), NO has been widely studied in the last 27 years as a gaseous messenger. Its importance became apparent when Louis J. Ignarro and collaborators described NO as an endogenously produced signaling molecule (30). At that time, this discovery was responsible for the recognition of NO as the “molecule of the year.” Needless to say, the 1998 Nobel Prize in Physiology or Medicine was awarded jointly to Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad for their discoveries concerning NO actions in the cardiovascular system.

NO is a diatomic free radical biosynthesized from L-arginine through a catalytic process carried out by the enzyme NO synthase (NOS), which exists as two distinct isoforms: 1) the constitutive isoform, consisting of neuronal- and endothelial-derived NOS, whose activity is dependent on intracellular calcium; and 2) the inducible isoform, which is calcium-independent but is stimulated by others molecules, such as cytokines (36, 60). The constitutive isoform is found in neurons and also in glial, muscle, and endothelial cells (79, 116), whereas the inducible isoform is primarily expressed in macrophages (13). In addition to the enzyme itself, NO synthesis also requires cofactors, such as nicotinamide adenine dinucleotide phosphate (NADPH), and the presence of oxygen. In the case of constitutive NOS, the formation and binding of a calcium-calmodulin complex is also required. In turn, inducible NOS, whose action is calcium-independent, exhibits a permanently activated calmodulin subunit. Independent of the enzyme isoform, this reaction produces equimolar amounts of NO and L-citrulline (44).

Studies have demonstrated the involvement of NO in several functions within the CNS, such as plasticity, circadian rhythms, neurotransmission, and hormone secretion (8, 38, 92). As a gaseous molecule, NO is not stored. However, a calcium-dependent and NOS-independent mechanism for NO release from S-nitrosothiol stores has been reported in isolated pancreatic acinar cells (21). In addition, NO does not require a receptor system; instead, NO, like other gaseous molecules, diffuses through the plasma membrane and acts in a paracrine and/or autocrine manner (123).

The physiological functions of NO are primarily exerted through the activation of guanylyl cyclase (GC). This mechanism was first characterized in different tissue homogenates by several groups in 1977 (7, 55, 77). These authors demonstrated that

NO donors added to tissue homogenates increased GC activity, as well as cyclic GMP (cGMP) levels. Since then, GC has been described as the major intracellular target for NO. This enzyme exists as a heterodimer containing α - and β -subunits, which are structurally composed of a COOH-terminal catalytic domain (cyclase), a central dimerization sequence, and a NH₂-terminal segment, the latter of which is where the heme prosthetic group is located. Due to the molecular structure of NO, this gas complexes with iron contained in the GC heme group, consequently increasing GC activity and cGMP synthesis (100). The increase in the intracellular concentration of cGMP, in turn, is followed by several events, such as 1) the activation or inhibition of specific phosphodiesterase isoforms, 2) the regulation of protein kinase G, followed by phosphorylation of specific proteins, and 3) the modulation of nucleotide-gated ion channels (94).

In addition, NO can directly react with cysteine thiol groups (S-nitrosylation), resulting in post-translational modification of proteins. This process was initially characterized in *N*-methyl-D-aspartate (NMDA) glutamate receptors (20) and later was found to occur in different types of proteins (51), including ion channels (121, 128), further validating its physiological significance. Although the binding of NO to cysteine residues occurs in a covalent manner, the mechanisms of denitrosylation, S-glutathionylation, and tyrosine-nitration are responsible for reversing this interaction (11).

Concerning the involvement of NO in hydroelectrolytic balance, initial evidence was provided by experiments showing that the presence of NOS protein and messenger RNA (mRNA), L-citrulline, and NADPH in the nuclei was primarily involved in homeostatic control (14, 78, 126). These findings were further confirmed by the increased NOS expression in response to challenges such as hypovolemia, dehydration, and salt load (54, 110, 111). Although GC mediates most NO actions, its participation in the signaling pathways controlling hydromineral balance has not been fully elucidated. Accordingly, some reports support a role for GC in hydroelectrolytic homeostasis (17, 127), whereas others demonstrate a GC-independent mechanism of operation (88, 106).

The main biological effects of this gaseous neuromodulator on endocrine function are predominantly described on the PVN and SON. In this regard, NO was shown to control the electrical excitability of MNs and, consequently, neuroendocrine output. Although the effects of NO are not unanimous in the literature, most reports suggest that MN activity and neuropeptide secretion are under tonic inhibition by NO. Conversely, few works have demonstrated that NO is apt to stimulate hormone release by the neurohypophysis. It

has been reported that intracerebroventricular (icv) microinjection of L-NAME in anesthetized rats resulted in a decrease in both basal and reflex-induced AVP release, whereas L-arginine induced the opposite response (18). Although anesthetics are known to inhibit neuronal excitability (4), the same pattern of responses was observed by Ota and colleagues in conscious rats (87). In their study, the administration of SNAP, an NO donor, or L-arginine increased basal concentrations of plasma AVP.

However, most in vitro experiments and electrophysiological recordings on SON explants or brain slices reported a decreased firing rate as the major effect of NO on MNs; this response was reversed by L-NAME administration, a NOS inhibitor, as well as by hemoglobin, a NO scavenger (66). The inhibitory tonus provided by NO on the intrinsic excitability properties of MNs is also preserved in response to osmotic stress. Accordingly, it has been demonstrated that hypertonicity produces an increase in NOS expression and NO production, which is followed by a decrease in the firing frequency of MNs (26, 110). Assuming that MNs function as osmoreceptors themselves and that hypertonicity is a potentially threatening condition that requires increased hormone secretion, it has been hypothesized that the hypertonicity-evoked NO inhibitory effect would exist as a protective mechanism through which reduced excitability would prevent overstimulation of MNs, thus avoiding saturation of the neurohypophyseal system (66). Based on the aforementioned findings, two main mechanisms underlie the inhibitory actions of NO: 1) stimulation of GABAergic inputs to MNs, consequently decreasing neuronal activity (98), and 2) changes in intrinsic neuronal properties, suggesting a direct action on ion channels (26, 112). Regarding this latter effect, unpublished observations from our group suggest that NO may affect the activity of channels responsible for pacemaker generation in MNs, the so-called hyperpolarized activated cyclic nucleotide cation channels.

The aforementioned mechanisms of NO action at the cellular level can be applied to many aspects of whole-animal physiology. Accordingly, the icv microinjection of L-NAME accentuated the ANG II-induced release of AVP, whereas the icv administration of L-arginine, the substrate for NO production, inhibited the secretion of both AVP and OT induced by diverse paradigms (53, 92, 93). Corroborating these results, Ahern and coworkers described an NO-mediated effect on large conductance calcium-activated potassium channels in posterior pituitary axon terminals, leading to a local inhibition of impulse-induced hormone secretion (2). Therefore, taken together with previous findings, these results suggest that

NO, acting simultaneously at MN cell bodies and terminals, produces a tonic inhibition of neuronal excitability and, consequently, neuropeptide secretion. The main mechanisms underlying the inhibitory actions of NO on MN activity are summarized in **FIGURE 3**.

NO has recently revealed itself as an important signaling molecule involved in the control of hydroelectrolytic balance. Although other brain targets may be equally important to the development of appropriate homeostatic responses, most recent reports suggest that the main actions of NO within the neurohypophyseal system are concentrated on the inhibitory control of MN excitability. These findings highlight NO as an extremely versatile and highly sensitive molecule, potentially implicated in the precise management of a great variety of hydroelectrolytic-derived imbalances.

CO and H₂S: From Environmental Pollutants to Biologically Active Molecules

CO is a colorless, odorless and tasteless gaseous molecule that has been an object of interest in the scientific community for centuries. Claude Bernard (12) initially described the effect of this gas in the organism, reporting blood color changes in different animal species submitted to CO inhalation. Later on, Haldane (48) showed that CO binds with a 200- to 300-fold greater affinity to hemoglobin than oxygen, resulting in hypoxia. Since then, thousands of studies have focused their attention on CO poisoning. In 1993, Snyder's group observed the enzyme heme oxygenase (HO) in the CNS. Subsequently, it was demonstrated that HO is colocalized with the mRNA encoding the soluble isoform of the enzyme GC (sGC) (114), which can be activated by CO (15). The main effect observed following sGC activation is an increase in cyclic guanosine monophosphate (cGMP) intracellular concentrations, which were later demonstrated to be the main signaling pathway implicated in the CO-mediated actions as a neuromodulator. In addition to the modulation of neurotransmission, several other functions have been attributed to CO, such as vasodilation, anti-inflammation, anti-apoptosis, and anti-oxidation (47).

CO can be produced endogenously by the activity of HO isoforms or by lipid peroxidation (125). The enzymatic process consists of heme group cleavage by HO, producing equal amounts of CO, Fe²⁺, and biliverdin. There are three distinct isoforms of HO presently described: 1) the inducible isoform, HO-1, which can be activated by numerous stimuli (130); 2) the constitutive isoform, HO-2, which is mainly responsive to glucocorticoids; and

3) HO-3, whose function is not well established (71). The first evidence suggesting that CO is produced by the CNS was provided by studies demonstrating the presence of HO mRNA in the olfactory bulb, hippocampus, cerebellum, habenula, and piriform cortex, among other brain regions (114). One year later, the expression of both the constitutive and inducible isoforms of HO in the thalamus, hypothalamus, and cerebellum was reported (115). The nonenzymatic process for CO generation, in turn, consists of the lipid peroxidation of unsaturated fatty acids residing in cellular membranes (117, 124).

Regarding the control of endocrine function, CO modulates both the hypothalamic-pituitary-adrenal and gonadal axes (32, 72), as well as the neurohypophyseal system. An inhibitory role for CO

on neurohypophyseal hormone release has been reported under some experimental paradigms. Kostoglou-Athanassiou's group showed that hemin, a CO precursor, decreased KCl-induced OT and AVP release by hypothalamic explants, an effect prevented by addition of HO inhibitors or hemoglobin, a CO scavenger (62, 73). Studies from the same group also demonstrated that the incubation of rat hypothalami with HO inhibitors or hemoglobin reversed the endotoxin-induced inhibitory effect on AVP release (61). Similar results were found *in vivo* by Giusti-Paiva and colleagues (41), who demonstrated that the icv administration of hemin reversed LPS-induced increases in AVP and OT secretion, whereas the injection of zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG), a nonspecific HO inhibitor, produced opposing effects.

FIGURE 3. Overview of nitric oxide (NO) effects on magnocellular neurons (MNs)

NO controls the excitability of MNs by two distinct mechanisms: 1) activation of GABAergic synaptic inputs and 2) modulation of neuronal intrinsic properties (see text for more details). The left scheme shows that both mechanisms result in decreased MN firing frequency. Considering that MN firing rate and the pattern of action potentials discharge in these cells determine the release of neuropeptides, the inhibitory effects of NO on neurohypophyseal terminal probably decrease hormone secretion. On the other hand, when NO synthesis is blocked by L-NAME, as represented at right, the endogenous tonic inhibition is abolished, resulting in increased firing frequency and hormone release.

Conversely, Gomes and coworkers (42, 43) demonstrated that ZnDPBG prevents hyperosmolality-induced increases in ANP and OT release by the medial basal hypothalamus *in vitro*. Furthermore, Reis and colleagues (91) recently reported an increase in the number of HO-1 immunoreactive neurons in the PVN and SON of 48-h-water deprived rats. These authors also demonstrated that the application of CrMP, an inhibitor of HO activity, on brain slices from water-deprived rats significantly decreased the firing rate of MNs. Therefore, the majority of reports agree that CO may positively regulate neurohypophyseal hormone secretion induced by challenges to hydromineral homeostasis.

More recently, H₂S appeared as the third gaseous molecule with potential targets in endocrine systems. It is commonly produced from organic material decay or by industrial activities (45), and exists as a colorless and malodorous gas that partly dissociates into hydrosulfide (HS⁻) in aqueous solutions at pH 7.4 (86). Although the main clinical signs of H₂S poisoning have been known since the first half of the 20th century, the toxicological mechanisms underlying H₂S actions were only elucidated recently. In this regard, HS⁻ has proven to be a more potent inhibitor of cytochrome oxidase than cyanide, impairing mitochondrial electron transport, and, in addition, demonstrated the potential to denature protein structure through the reduction of disulfide bridges (10, 46).

Endogenous H₂S production was first noted by Stipanuk and Beck in 1982 (99). They found that both rat liver and kidneys could generate H₂S through cysteine desulfhydration. This process is mediated by three enzymatic pathways: 1) cystathionine γ -lyase (CSE), 2) cystathionine β -synthase (CBS), and 3) cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (3MST). Conversely, endogenous H₂S production in the brain was demonstrated 7 years later (120). Such findings drove forthcoming investigations on H₂S actions on central, cardiovascular (105), and immune systems (65). As a result, Abe and Kimura (1) provided evidence that CBS mRNA is expressed in the CNS of rats (hippocampus, cerebellum, cortex, and brain stem) and that exogenous H₂S facilitates the induction of long-term potentiation in the hippocampus by modulation of NMDA receptors. Furthermore, it has been recently demonstrated that the enzyme CBS is expressed in the PVN (101). Accordingly, H₂S has been shown to participate in the hypothalamic control of heart rate and blood pressure (27, 67), body temperature during hypoxia (64), and corticotropin-releasing hormone (CRH) release (29).

Regarding hydromineral balance, Khademullah and Ferguson (56) recently showed through

electrophysiological approaches that exogenous H₂S increases the excitability of PVN MNs, which most likely produce direct effects on AVP and OT secretion. In addition, a recent report of our group demonstrated that water deprivation increases the hypothalamic activity of H₂S-generating enzymes and that the icv administration of an H₂S donor potentiates neurohypophyseal hormone secretion, as well as decreases hypothalamic nitrite/nitrate contents in water-deprived rats, indicating a reduced NO production (24). Although there are very few reports in the literature devoted to the investigation of H₂S action in hypothalamic neurotransmission, a possible site-specific and complex interaction between NO, CO, and H₂S and their specific enzymatic/intracellular machineries will likely appear in the near future as a promising field of investigation.

Integration of Gaseous Modulators in the Control of Endocrine Function

Gaseous neuromodulators have challenged conventional concepts of cell signaling. It has been hypothesized that synthesizing enzymes represents the major regulatory mechanism determining local gaseous production, although evidence showing that NO can be produced by a NOS-independent mechanism has been provided. The finding that NOS, HO, and CBS enzymes may colocalize in discrete brain regions, as discussed later, also suggests that a mutual relationship may exist. However, due to the small number of cross-talk studies on the subject, many putative mechanisms are speculated, and these theories are transposed to hydromineral homeostasis based on previous findings in other similar areas.

NO actions are mainly mediated by binding to the sGC heme domain, which increases intracellular cGMP concentrations. Much like NO, CO also displays affinity for the heme group. However, CO increases sGC activity by only 4-fold compared with the 200-fold increase produced by NO (70). Thus CO binding leads to a net reduction in sGC activity compared with NO binding. In addition, NOS contains a heme group in the oxygenase domain (3), which also constitutes a CO-binding site (75) and is implicated in the inhibition of NOS enzymatic activity at high CO concentrations (108). The molecular structure of HO is also characterized by the presence of a heme group (95), which was shown to complex with NO, resulting in HO inhibition (119). Conversely, NO was shown to induce HO-1 gene transcription through an interaction with DNA response elements and, consequently, increases HO-1 protein expression and enzymatic activity (31, 34, 68). Therefore, CO and NO not only activate the same intracellular pathway (sGC) but

also show a positive interaction at both the enzymatic and molecular levels.

HO inhibition decreases *in vitro* OT release by the medial basal hypothalamus, an effect that

occurs in parallel with increased NOS activity (42). Furthermore, hypertonicity-induced ANP release is associated with the inhibition of NOS activity, a response prevented by the addition the HO inhibitor ZnDPBG to the incubation medium (43). These findings are also supported by results obtained from cerebellar cell cultures, which showed that endogenous CO inhibits the NO-induced increase in cGMP production through conformational changes in sGC (50).

Distinct from NO and CO, H₂S is produced in the CNS through two different pathways: CBS and 3-MST. CBS, a heme-containing piridoxal phosphate-dependent enzyme (9), generates H₂S by condensation of cysteine and homocysteine (59), whereas 3MST, an enzyme very similar to rhodanese, which does not contain a heme prosthetic group (81), produces H₂S from 3-mercaptopyruvate (59). Due to the presence of a heme group in CBS, NO and CO can both bind to this enzyme, although CO binds with a 200-fold greater affinity than NO. This event culminates with a decrease in CBS activity (72, 104). Similarly, the production of H₂S by CBS is suppressed by NO and CO in the rat brain (1, 80). Furthermore, H₂S alters the function of tetrahydrobiopterin (BH₄), a NOS co-factor, consequently altering the tightening of NOS dimerization and reducing enzymatic activity (63). In addition, H₂S and NO can directly react with each other, generating S-nitrosothiol, a molecule that is unable to stimulate sGC (122), thus leading to inhibition of NO-mediated actions. Nevertheless, in smooth muscle cell preparations, H₂S was shown to potentiate the relaxing effects of NO (49). Moreover, H₂S increases HO-1 expression in a dose-dependent manner in macrophages by activation of extracellular signal-regulated kinase (ERK) (84), suggesting a possible genomic-mediated mechanism regulating HO function.

As discussed in the previous sections, NO and CO were already shown to modulate the secretion of AVP, OT (91, 109), and CRH (90, 109). The modulatory actions of H₂S, in turn, have also been demonstrated on CRH (29), ACTH, and corticosterone release (69), as well as on PVN neurons (56), directly affecting AVP and OT secretion (24). Therefore, the hypothalamic neurohypophyseal and pituitary-adrenal systems appear as potential targets in which these gaseous modulators could locally interact to modulate endocrine function.

The complex interaction of the three gaseous modulators and their respective enzymes is represented in **FIGURE 4**. Taken together, evidence from the literature indicates that there is a subtle control of the entire gaseous system, which operates so that under physiological conditions the production of each molecule is within adequate levels to exert the required biological actions. Conversely, when

FIGURE 4. Potential mechanism by which osmotic stimulation regulates NOS/NO, HO/CO, and CBS/H₂S systems to control neurohypophyseal hormone secretion

Increased activity of MNs caused by osmotic stimulation induces NOS and HO activation, which increases, respectively, NO and CO levels. CO stimulates MNs to secrete AVP and OT. NO, in turn, inhibits AVP and OT release both directly (via a cGMP-dependent pathway) and indirectly (increasing GABA release). There is also evidence that osmotic stimulation increases H₂S production via CBS activation, which increases cyclic AMP (cAMP) intracellular levels. However, the mechanism by which H₂S affects MNs activity remains unknown. Note the complex negative feedback mechanisms provided by gaseous molecules at enzymatic level (NOS, HO, CBS, sGC). The simultaneous activation of CO- and NO-mediated pathways by an osmotic stimulus results in a local and self-limited mechanism to control hormone secretion, allowing a sustained but not exaggerated secretory response (see text for more details). Arrows, stimulatory pathways; dashed lines with flat head, inhibitory pathways. AC, adenylyl cyclase; AVP, arginine vasopressin; CBS, cystathionine-β-synthase; cAMP, cyclic AMP; cGMP, cyclic GMP; CO, carbon monoxide; GABA, gamma-aminobutyric acid; HO, heme oxygenase; H₂S, hydrogen sulfite; L-arg, L-arginine; L-cys, L-cysteine; MNs, magnocellular neurons; NO, nitric oxide; NOS, nitric oxide synthase; OT, oxytocin; sGC, soluble guanylyl cyclase.

homeostasis is disturbed and an effector response is required, the systems react in a coordinated way so that the altered production of one gaseous modulator directly impacts the others. Within this context, the main strategy used to accomplish this very dynamic process is the modulation of enzymatic activity, either through a direct interaction (rapid response) or through alterations in gene expression (more delayed effect).

Conclusions and Perspectives

In addition to the well documented and interdependent actions of NO and CO in the control of hydromineral balance, H₂S appears to be the third element deserving inclusion within this complex homeostatic network. In this three-way system, the intriguing question of which gaseous modulator is altered first in response to an osmotic stimulus remains unanswered so far. The rapid and site-restricted mechanism of action of gaseous modulators suggests that alterations in enzymatic activity may take place very shortly after the challenge is delivered. However, studies have demonstrated that gene expression may also be affected to some extent. Accordingly, preliminary evidence indicates that a time-dependent mechanism, related to the persistence of the osmotic stimulus, may also induce more prolonged responses on gaseous modulatory systems. Therefore, it appears that the temporal frame by which an interaction is observed is particularly important to characterize a gaseous modulator as either inhibitory or stimulatory.

Interestingly, although gaseous molecules can in theory act as paracrine or autocrine messengers, the synthesizing enzymes are not equally distributed among cell types. CBS is primarily localized in astrocytes, 3MST is found predominantly in neurons (58), and NOS and HO have been identified in both cell types (16, 118). As such, following an osmotic challenge, astrocytes are apparently activated earlier than neurons as a requirement for intact effector responses. This differential enzymatic expression should give insight into the period at which each gaseous modulator may be predominantly recruited. ■

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Electrophysiological Properties of Rostral Ventrolateral Medulla Presympathetic Neurons Modulated by the Respiratory Network in Rats

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The respiratory pattern generator modulates the sympathetic outflow, the strength of which is enhanced by challenges produced by hypoxia. This coupling is due to the respiratory-modulated presympathetic neurons in the rostral ventrolateral medulla (RVLM), but the underlining electrophysiological mechanisms remain unclear. For a better understanding of the neural substrates responsible for generation of this respiratory-sympathetic coupling, we combined immunofluorescence, single cell qRT-pCR, and electrophysiological recordings of the RVLM presympathetic neurons in *in situ* preparations from normal rats and rats submitted to a metabolic challenge produced by chronic intermittent hypoxia (CIH). Our results show that the spinally projected catecholaminergic C1 and non-C1 respiratory-modulated RVLM presympathetic neurons constitute a heterogeneous neuronal population regarding the intrinsic electrophysiological properties, respiratory synaptic inputs, and expression of ionic currents, albeit all neurons presented persistent sodium current-dependent intrinsic pacemaker properties after synaptic blockade. A specific subpopulation of non-C1 respiratory-modulated RVLM presympathetic neurons presented enhanced excitatory synaptic inputs from the respiratory network after CIH. This phenomenon may contribute to the increased sympathetic activity observed in CIH rats. We conclude that the different respiratory-modulated RVLM presympathetic neurons contribute to the central generation of respiratory-sympathetic coupling as part of a complex neuronal network, which in response to the challenges produced by CIH contribute to respiratory-related increase in the sympathetic activity.

Key words: bulbospinal RVLM presympathetic neurons; chronic intermittent hypoxia; intrinsic electrophysiological properties; respiratory-sympathetic coupling

Introduction

Oscillations in the sympathetic outflow are modulated by the respiratory rhythm in rats (Numao et al., 1987; Haselton and Guyenet, 1989) and humans (Badra et al., 2001; Dempsey et al., 2002), and this modulation contributes to the neurogenic vasomotor tone (Bachoo and Polosa, 1985). This phenomenon is in part a consequence of activation of pulmonary stretch receptors (Zhou et al., 2002). However, the respiratory modulation of sympathetic activity persists even after vagotomy (Adrian et al., 1932; Barman and Gebber, 1980; Haselton and Guyenet, 1989), suggesting the existence of a central neural coupling between the respiratory pattern generator and the neurons involved with the generation of the sympathetic activity. The strength of this respiratory-sympathetic coupling is enhanced by challenges,

such as acute (Dick et al., 2004), chronic hypoxia (Zoccal et al., 2008; Moraes et al., 2012a) and increased respiratory drive (Malpas, 1998; Taylor et al., 1999) to provide a fine tuning in the cardiovascular and respiratory responses under these circumstances.

It has been suggested that the neuronal coupling between the respiratory and sympathetic activities occurs, at least in part, at the level of the rostral ventrolateral medulla (RVLM), where the bulbospinal presympathetic neurons are located (Reis et al., 1989; Guyenet et al., 1990; Dampney, 1994). This brainstem region contains two major groups of presympathetic bulbospinal glutamatergic neurons: one expresses all enzymes required for production of adrenaline (C1 group) and the other correspond to noncatecholaminergic neurons (Ruggiero et al., 1994; Stornetta et al., 2002). Most of the bulbospinal RVLM presympathetic neurons have a respiratory-related modulation of their activity (Terui et al., 1986; McAllen, 1987; Haselton and Guyenet, 1989; Miyawaki et al., 1995).

Despite the existence of a large body of electrophysiological evidence, from both extracellular (Guyenet and Brown, 1986; Morrison et al., 1988; Haselton and Guyenet, 1989; Kanjhan et al., 1995) and intracellular recordings (Dembowsky and McAllen, 1990; Lipski et al., 1995a,b, 1996; Zagon and Spyer, 1996) in anesthetized animals, several fundamental aspects such as (1)

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Figure 1. Recordings of arterial pressure, respiratory muscles, and nerves *in vivo* and *in situ* preparations of control and CIH rats. Raw and integrated (J)EMG records of inspiratory (diaphragm-DiA), expiratory muscles (abdominal-Abd) simultaneously with pulsatile arterial pressure (PAP) and mean arterial pressure (MAP) in conscious, freely moving rats from control (**Ai**) and CIH (**Aii**) rats. Note the presence of late-expiration (late-E) activity in the Abd (↑) and the increase in the MAP after CIH. Raw and integrated (J) records of thoracic sympathetic nerve (tSN), abdominal nerve (AbN), and phrenic nerve (PN) activities of *in situ* preparations from the same control and CIH rats shown in **Ai** (**Bi**) and in **Aii** (**Bii**), respectively. Note the presence of late-E event (↑) in the tSN and AbN after CIH.

their respiratory-related synaptic inputs; (2) their relation to C1 group of neurons; and (3) their intrinsic electrophysiological properties, remain unclear. Therefore, in the present study we used whole-cell patch recordings, in unanesthetized preparations with preserved networks, to get a better understanding of the modulation of bulbospinal RVLM presympathetic neurons induced by respiratory network. Experiments were performed using the *in situ* working heart-brainstem preparation (Paton, 1996) to evaluate the respiratory-related synaptic inputs, the pacemaker activity, the different ionic currents, and membrane properties of the respiratory-modulated RVLM presympathetic neurons in normal rats and in rats submitted to a metabolic challenge produced by chronic intermittent hypoxia (CIH).

Our results show that the respiratory-modulated RVLM presympathetic neurons constitute a heterogeneous neuronal population, regarding the intrinsic electrophysiological properties, respiratory synaptic inputs, relation to C1 group of neurons, as well in the control of sympathetic outflow in response to a metabolic challenge produced by CIH.

Materials and Methods

Animals and ethical approval. Experiments were performed on male Wistar rats (85–140 g) obtained from the Animal Care Facility of the University of São Paulo, Ribeirão Preto, Brazil. The animals were maintained in standard environmental conditions (23 ± 2°C; 12/12 h dark/light cycle) with water and chow *ad libitum*. All experimental protocols were approved by the Ethical Committee on Animal Experimentation of

Figure 2. Electrophysiological identification of the nonmodulated and respiratory-modulated RVLM presympathetic neurons *in situ* preparations. **Ai**, Hyperpolarizing potentials and inhibition of spike discharge frequency (3 superimposed sweeps, no polarizing current, membrane potential, −57 mV) evoked by stimulation of the ipsilateral ADN with bursts of 3 pulses (***). **Aii**, Antidromic action potentials evoked by stimulation (*) of the T8–T12 segment of the spinal cord. Three superimposed sweeps pretriggered by sEPSPs are shown. The arrow indicates spontaneous excitatory postsynaptic potential preceding spontaneous spikes. Note that spinal stimulation was followed by antidromic responses that occurred ~6 mV more negative than the threshold at which spontaneous action potentials were induced by the ongoing synaptic activity. **Bi**, The ADN-evoked potentials (10 averaged sweeps) recorded intracellularly when the firing of the cell was stopped by a continuous negative current (−25 pA). **Bii**, Reversal of evoked inhibitory postsynaptic potential (holding current of −200 pA) evoked by stimulation of the ADN. **Ci–Dii**, sEPSPs, measured by hyperpolarizing the neurons, in the non-modulated (**Ci**), inspiratory-modulated (**Cii**), postinspiratory modulated (**Di**), and inspiratory-inhibited (**Dii**) RVLM presympathetic neurons.

the School of Medicine of Ribeirão Preto, University of São Paulo (protocols #093/2009 and #064/2010).

Chronic intermittent hypoxia. The CIH and control groups of rats were housed in collective cages and maintained in Plexiglas chambers (volume, 210 L) equipped with gas injectors of O₂ and CO₂ and sensors of O₂, CO₂, humidity, and temperature. The CIH group was exposed to 5 min of normoxia (fraction of inspired O₂, FiO₂, of 20.8%) followed by 4 min of hypoxia (fraction of inspired O₂, FiO₂, of 6%), remaining at this level between 30 and 40 s. After hypoxia, O₂ was injected to return FiO₂ back to 20.8%. This cycle with duration of 9 min was repeated over the time of 8 h a day (from 9:30 A.M. to 5:30 P.M.) for 10 d. During the remaining 16 h, the animals were exposed to 20.8% FiO₂. Control rats were exposed to 20.8% FiO₂ 24 h a day for 10 d as described in a previous study from our laboratory (Zoccal et al., 2008).

Respiratory and cardiovascular measurements in conscious animals. Electromyogram (EMG) of respiratory muscles were used to evaluate inspiration and expiration index in freely behaving conditions after submitting the animal to the hypoxic ($n = 16$) or normoxic ($n = 13$) protocols. Two days before starting these protocols, rats were anesthetized with tribromoethanol (250 mg/kg, i.p.; Aldrich) and bipolar Teflon-coated stainless steel EMG electrodes were implanted in the diaphragm (DIA) and abdominal muscles (Abd). Wires were tunneled under the skin and attached to an electrical socket positioned on the back of the

Figure 3. Phenotype of the nonmodulated and respiratory-modulated RVLM presympathetic neurons. **Ai–Aii**, Representative voltage traces of one nonmodulated RVLM presympathetic neuron (**Ai**) and the corresponding photomicrograph (**Aii**) showing biocytin (green) and TH (red) immunohistochemical staining. Note that this RVLM presympathetic neuron is a positive TH-ir cell (yellow). **Aiii**, Amplification plots of TH, VGLUT₂, and β actin from qRT-PCR from another nonmodulated RVLM presympathetic neuron. The fluorescent emission (ΔRn) is plotted against the number of cycles. Note that this neuron expresses TH and also VGLUT₂. **Bi–Bii**, Representative voltage traces of one inspiratory-modulated RVLM presympathetic neuron (**Bi**) and the corresponding photomicrograph (**Bii**) showing biocytin (green) and TH (red) immunohistochemical staining. Note that this RVLM presympathetic neuron is also a positive TH-ir cell (yellow). **Biii**, Amplification plots of TH, VGLUT₂, and β actin from qRT-PCR from another inspiratory-modulated RVLM presympathetic neuron. Note that this neuron expresses TH and also VGLUT₂. **Ci–Cii**, Representative voltage traces of one postinspiratory-modulated RVLM presympathetic neuron (**Ci**) and the corresponding photomicrograph (**Cii**) showing biocytin (green) and TH (red) immunohistochemical staining. Note that this RVLM presympathetic neuron is a negative TH-ir cell. **Ciii**, Amplification plots of TH, VGLUT₂, and β actin from qRT-PCR from another postinspiratory-modulated RVLM presympathetic neuron. Note that this neuron does not express TH but expresses VGLUT₂. **Di–Dii**, Representative voltage traces of one inspiratory-inhibited RVLM presympathetic neuron (**Di**) and the corresponding photomicrograph (**Dii**) showing biocytin (green) and TH (red) immunohistochemical staining. Note that this RVLM presympathetic neuron is also a negative TH-ir cell. **Diii**, Amplification plots of TH, VGLUT₂, and β actin from qRT-PCR from another inspiratory-inhibited RVLM presympathetic neuron. Note that this neuron does not express TH but expresses VGLUT₂. Scale bar, 20 μ m.

rat's neck. At the end of the experimental protocol (CIH or normoxia), rats were anesthetized with tribromoethanol and a catheter was inserted into the abdominal aorta through the femoral artery for arterial pressure measurement ($n = 39$; control: 13 rats with and 6 rats without EMG measurements; CIH: 16 rats with and 4 rats without EMG measure-

ments). The catheter was tunneled subcutaneously and exteriorized through the skin in the back of the rat's neck. One day after, the arterial catheter was connected to a pressure transducer, and in turn, to an amplifier (Bridge Amp, ML221, ADInstruments). The electrical socket of the EMG was also connected to an amplifier (Bioamplifier, Insight). The arterial pressure and EMG (0.5–5 kHz of bandpass) signals were acquired by a data acquisition system (CED 1401, Cambridge Electronic Design) controlled by a computer running the software Spike 2 (Cambridge Electronic Design, CED). The respiratory and cardiovascular parameters were recorded in conscious, freely moving rats under normoxic conditions during 60 min.

Ventral approach in the working heart–brainstem preparation. Immediately after recordings in freely behaving conditions ($n = 39$) or after submitting the animal to the control ($n = 67$) or CIH protocols ($n = 26$), the rats were prepared for an *in situ* working heart–brainstem preparation, as previously described (Paton, 1996). Briefly, the animals were deeply anesthetized with halothane (AstraZeneca), transected caudal to the diaphragm, exsanguinated, and submerged in ice-cold artificial CSF (ACSF) containing the following (in mM): 125 NaCl, 24 NaHCO₃, 3 KCl, 2.5 CaCl₂, 1.25 MgSO₄, 1.25 KH₂PO₄, 10 dextrose. They were then decerebrated at the precollicular level, therefore rendered insentient and skinned. To expose the ventral medullary surface for neuronal recordings, the preparations were placed supine and all muscles and connective tissues covering the basilar surface of occipital bone were removed. Then, the basilar portion of the occipital bone was removed to expose the ventral surface of the medulla in the anteroposterior extension from the vertebral arteries to the pontine nuclei as previously described (Moraes et al., 2012b). Preparations were then transferred to a recording chamber and the descending aorta was cannulated and retrogradely perfused with ACSF containing an oncotic agent (1.25% Polyethylene glycol, Sigma), a neuromuscular blocker (vecuronium bromide, 3–4 μ g/ml, Cristália Produtos Químicos Farmacêuticos), and continuously gassed with 5% CO₂ and 95% O₂ using a peristaltic pump (Watson-Marlow 502s). For voltage-clamp experiments of isolated T-type calcium currents, the ACSF consisted (in mM): 118 NaCl, 3 KCl, 1 MgSO₄, 24 NaHCO₃, 1 NaH₂PO₄, 1.5 CaCl₂, 10 dextrose. The perfusate was warmed to 31–32°C and filtered using a nylon mesh (pore size: 25 μ m, Millipore).

Electrophysiological measurements. Sympathetic and phrenic nerves were isolated and their activities recorded using bipolar glass suction electrodes. Phrenic nerve (PN) activity was recorded from its central end. The activity of thoracic sympathetic nerve (tSN) was recorded from the sympathetic chain at the level of T8–T12. All signals were amplified, band-pass filtered (Bioamplifier, Insight; 0.5–5 kHz), and acquired with an A/D converter (CED 1401, Cambridge Electronic Design, CED) controlled by a computer running Spike 2 software (Cambridge Electronic Design, CED).

Blind whole-cell patch-clamp recordings were obtained from medullary RVLM presympathetic neurons (one neuron per rat) with electrodes

Table 1. Average values of electrophysiological properties of nonmodulated and all respiratory-modulated RVLM presympathetic neurons recorded from control and CIH rats

	Control				CIH			
	Group I (n = 9)	Group II (n = 12)	Group III (n = 12)	Group IV (n = 12)	Group I (n = 10)	Group II (n = 12)	Group III (n = 14)	Group IV (n = 10)
Capacitance (pF)	39 ± 1.9	36 ± 1.7	40 ± 1.8	42 ± 1.8	40 ± 3.6	41.5 ± 2.8	38 ± 2.7	40 ± 1.2
Frequency (Hz)	6 ± 1.1	22 ± 3*	13 ± 2	8 ± 1.9	6.4 ± 2.2	20 ± 2.4	15 ± 3.1	9 ± 2
RMP (mV)	−53 ± 2.2	−51 ± 2.3	−52 ± 3.2	−54 ± 3.4	−52 ± 4	−50 ± 2	−53 ± 2.1	−53 ± 2
R _i (MΩ)	335 ± 1.1**	162 ± 6.5 [#]	245 ± 5 ^{##}	515 ± 9	348 ± 8	159 ± 7	259 ± 5	509 ± 16.6
Excitability (spikes)	46 ± 6**	90 ± 8.7 ⁺	61 ± 7	64 ± 6	49 ± 6	95 ± 7.7	59 ± 6.7	74 ± 5
AHP (mV)	7 ± 1.1 ^{###}	3 ± 0.6	3.3 ± 0.7	6 ± 1.3	7.2 ± 2.2	2.8 ± 0.9	3 ± 0.9	6.2 ± 2.3

Excitability: values in response to injection of 100 pA of positive current. * $p < 0.05$ different in relation to groups I, III, and IV from control rats; ** $p < 0.001$ different in relation to the groups II, III, and IV from control rats; $^{\#}p < 0.001$ different in relation to the groups II and III from control rats; $^{++}p < 0.05$ different in relation to the group III from control rats. CIH produced no changes in the electrophysiological properties of the 4 groups of RVLM presympathetic neurons characterized, and the differences among the groups in CIH were also similar to those observed in neurons from control rats.

filled with a solution containing the following (in mM): 130 K-gluconate, 4.5 MgCl₂; 14 tris-phosphocreatine, 10 HEPES; 5 EGTA; 4 Na-ATP; 0.3 Na-GTP; 0.2% biocytin; pH 7.3, and ~300 mOsmol and had resistances of 3–8 MΩ when tested in bath solution. Only for voltage-clamp experiments of T-type calcium currents, the electrodes were filled with a solution containing the following (in mM): 110 CsCl₂, 30 TEA, 1 CaCl₂, 5 EGTA, 2 MgCl₂, 4 Na-ATP, 0.5 Na-GTP, 10 HEPES, pH 7.3, and ~300 mOsmol. The electrodes were mounted on a micromanipulator (MHW-3; Narishige) and positioned onto the ventral surface of the medulla under visual control (binocular microscope; Zeiss) using surface landmarks (trapezoid body, rootlets of the hypoglossal nerve, and basilar artery) and coordinates of RVLM presympathetic neurons for orientation as previously described (Moraes et al., 2012b). The RVLM presympathetic neurons were also characterized by its inhibitory response to baroreflex activation (by electrically stimulating either contralateral or ipsilateral aortic depressor nerves—ADN; 0.2 ms, 3 pulses at 400 Hz) and the presence of antidromic responses to stimulation of spinal segment T8–T12 (0.2 ms). Antidromic responses were distinguished from synaptic excitation by using near threshold stimulation of descending axons, and at this intensity of stimulation synaptic events were absent (absence of spontaneous excitatory postsynaptic potential preceding evoked spikes). Voltage- and current-clamp experiments were performed using an Axopatch-200B integrating amplifier (Molecular Devices) and pClamp acquisition software (version 10.0, Molecular Devices). Gigaseals (>1 GΩ) were formed, and whole-cell configuration was obtained by suction. Recordings with access resistance larger than 20 MΩ were not used.

Ionic currents were measured under voltage-clamp with the cell's membrane potential initially held at −70 mV and expressed as pA/pF to normalize for cells of different sizes. Activation properties of outward transient potassium current (I_A) were evaluated by applying 10 mV voltage steps (2 s) from −80 to 60 mV, from conditioning step (500 ms) of either −110 or −40 mV. After off-line subtraction of the currents evoked with holding voltage of −110 and −40 mV, the I_A current was isolated. To maximize I_A and reduce sodium currents, TTX (0.5 μM; Tocris Bioscience) was added to the perfusion solution. The voltage-dependent inactivation of I_A was estimated using conditioning steps

Figure 4. Repetitive firing properties of nonmodulated and respiratory-modulated RVLM presympathetic neurons from control rats. Plots of representative traces of instantaneous spike discharge frequency versus time and interspike interval (ISI) versus time obtained from representative control nonmodulated (**Ai, Aii**), inspiratory-modulated (**Bi, Bii**), postinspiratory-modulated (**Ci, Cii**), and inspiratory-inhibited (**Di, Dii**) RVLM presympathetic neurons in response to injection of 100 pA of positive current.

of 1 s ranging from −130 to −30 mV in 10 mV increments followed by a test pulse of 500 ms to −30 mV. To measure subthreshold sodium-persistent conductance (I_{NaP}), 30 mV/s ramp from −85 to 15 mV were applied. The riluzole-sensitive (10 μM; Tocris Bioscience) component of the ramp-

Figure 5. Spike discharge frequency and intrinsic electrophysiological properties of nonmodulated RVLM presympathetic neurons from control and CIH rats. Raw and integrated (*J*) records of PN, tSN, and nonmodulated RVLM presympathetic neurons activities from control (**Ai**) and CIH (**Aii**) rats. Note the presence of late-expiration (late-E; $\uparrow$) in tSN and no changes in the spike discharge frequency of nonmodulated RVLM presympathetic neuron after CIH. In the presence of synaptic blockade, intrinsic spike discharge frequency (**Bi, Bii**), excitability properties (**Ci, Cii, Ciii**), and input resistance [R_i (**Di, Dii, Diii**)] of nonmodulated RVLM presympathetic neurons were not affected by CIH. Note that upon larger hyperpolarizing pulses in **Di** and **Dii**, the membrane potential presented a shift toward more positive values as a time-dependent conductance was slowly turned on (*).

evoked currents was taken as I_{NaP} . Hyperpolarization-activated inward currents (I_h) were activated, in the presence of TTX, using a series of hyperpolarizing voltage pulses (from -60 mV to -130 mV, in 10 mV increments), from a -70 mV conditioning pulse. The ZD7288- (a selective blocker of I_h current) sensitive ($40 \mu\text{M}$; Sigma-Aldrich) component of the hyperpolarizing-evoked currents represents the I_h . T-type calcium currents were activated, in the presence of TTX, using a series of depolarizing command pulses (from -90 to 0 mV, in 10 mV increments), from a hyperpolarizing conditioning pulse (-90 mV). The recording of all ionic current were also performed in the presence of blockers of fast synaptic transmission in the perfusion solution [2.5 – 6.0 mM kynurenic acid, $20 \mu\text{M}$ bicuculline (free-base), and $1 \mu\text{M}$ strychnine; Sigma-Aldrich] and all signals were low-pass filtered at 1 kHz and sampled at 5 kHz.

In current clamp, all data were low-pass filtered at 5 kHz and digitized at the rate of 20 kHz. The intrinsic electrophysiological properties of respiratory-modulated RVLM presympathetic neurons were also evaluated in the presence of blockers of fast synaptic transmission in the perfusion solution.

Data analyses. All analyses of nerve recordings were performed off-line in rectified and smoothed (50 ms) signals using Spike 2 software with

custom-written scripts. The respiratory modulation of sympathetic activity was analyzed using phrenic-triggered averages of tSN (generated from 1 – 2 min epochs of the recordings), which were divided into three parts: inspiration (coincident with inspiratory PN discharge), postinspiration (post-I; first half of expiratory phase), and late-expiration (late-E; second half of expiratory phase). The percentage of tSN activity in each phase was assessed by the measurement of the area under the curve and normalized by the total area, i.e., sum of areas obtained in inspiratory, post-I and late-E.

All analyses of neuronal recordings were performed using Clampfit 10 (Molecular Devices), GraphPad 5, and a custom script written in Spike 2 software. The peak conductance for each voltage (G_v) of the IA was calculated as $G_v = I_{\text{peak}} / (V_m - E_K)$, where I_{peak} is peak current, V_m membrane voltage, and E_K is the potassium equilibrium potential calculated from the Nernst equation (Hille, 2001). The relationship between normalized conductance and membrane potential (V_m) was fitted by a Boltzmann function of the form: $G = 1 / [1 + \exp \{ (V_h - V_m) / V_c \}]$, where G is the relative membrane conductance, V_h is the voltage at half-maximal conductance, and V_c is the slope factor. Voltage-dependent inactivation of the IA was fitted with an equation of the form: $I / I_{\text{max}} = 1 / [1 + \exp \{ (V_m - V_h) / V_c \}]$, where I_{max} is the maximal current recorded. Current–voltage relationship of I_h was determined from steady-state currents. The voltage dependence of I_h activation was obtained from tail currents measured at -80 mV following a series of hyperpolarizing steps; those data were normalized and fitted with also an equation of the form: $I / I_{\text{max}} = 1 / [1 + \exp \{ (V_m - V_h) / V_c \}]$. Current–voltage relationship of T-type calcium currents was determined from the peak currents after the capacitive transient.

Current-clamp recordings were analyzed off-line to measure: (1) resting membrane potential (RMP), (2) input resistance, (3) repetitive discharge characteristic of the neuron (frequency and interspike intervals of action potentials; the last was measured as the time difference between the peaks of the action potentials in response to 100 pA of positive current injection), (4) duration of delayed excitation, and (5) amplitude of the after hyperpolarization potential (AHP). The input resistance was determined via linear regression applied to the linear portion of the voltage–current (V – I) relationship. Considering that the RVLM presympathetic neurons always presented spontaneous spiking, it was difficult to clearly determine a stable membrane potential that could unequivocally be defined as the resting potential. To address this issue, we constructed an all-points histogram of 1 min of membrane potential value recorded in each experimental condition, and the resting membrane potential value was considered to be the value at which the cells spent most of the time, i.e., the peak of the distribution (da Silva et al., 2013). A liquid junction potential of 15 or 2 mV (T-type calcium current) were corrected off-line.

Immunofluorescence. All preparations used for immunofluorescence were perfused transcardially first with PBS (0.1 M) and then with 4% paraformaldehyde (PFA) and brains removed and postfixed in 4% PFA for 2 – 5 d. Transverse sections ($50 \mu\text{m}$ thick) were cut through the RVLM with a vibrating microtome (Leica) and collected into a cryoprotectant solution and stored at -20°C before further processing. The immuno-

fluorescence was performed with free-floating sections. Sections were blocked and permeabilized in PBS containing 10% normal horse serum and 0.5% Triton X-100 for 1 h at room temperature. After three PBS washes, the slices were incubated in primary antibody mouse anti-tyrosine hydroxylase (TH; Millipore, 1:1000) for 24 h at 4°C. After three PBS washes, the sections were incubated in secondary antibodies Alexa 488-conjugated streptavidin (1:1000; Invitrogen) and Alexa 647-conjugated donkey anti-mouse (1:500; Jackson Laboratories) for 1 h at room temperature. Slices were washed three times in PBS and mounted in Fluoromount (Sigma-Aldrich). Images were collected on a Leica TCS SP5 confocal microscope equipped with 488 and 633 nm laser lines and tunable emission wavelength detection. For each preparation, biocytin-positive neurons were identified and confocal z stacks were collected sequentially for the other channel to detect the TH-positive neurons and verify the colocalization.

Single-cell qRT-PCR. The cytoplasm of the neuron was pulled into a patch pipette with a slight negative pressure. We only applied negative pressure to pull the cytoplasm into a patch pipette in neurons presenting gigaseals and access resistance up to 20 M Ω at the end of whole-cell patch-clamp records to give us a high success rate of identifying neuronal phenotypes using single-cell qRT-PCR. The cytoplasm was then placed into a microtube containing High Capacity cDNA Reverse Transcription Kit reagents (Life Technologies) and nuclease-free water for subsequent transcription in a thermocycler (Mastercycler Gradient—Eppendorf). A preamplification of the cDNA was performed using the TaqMan Pre-Amp Master Mix Kit (Life Technologies) with the following probes: Rn00562500_m1 (TH), Rn00584780_m1 (VGLUT₂) and NM_031144.2 (β -Actin; reference gene). The preamplification protocol consisted of a hold temperature at 95°C during 10 min and 14 cycles of 95°C and 60°C during 15 s and 14 min, respectively. The reactions for the single-cell qRT-PCR were performed in singleplex and triplicate (StepOnePlus System, Applied Biosystems) using the same probes described above and the TaqMan Universal PCR Master Mix kit (Life Technologies) according to the manufacturer's recommendations.

Statistical analyses. Results are expressed as mean $\pm$ SEM and compared using Student's unpaired *t* test or one-way ANOVA, in accordance with the requirements of each experimental protocol. Differences were considered significant when *p* < 0.05.

Results

Cardiovascular and respiratory recordings in awake rats and in *in situ* preparations

In a specific group of control and CIH rats we recorded arterial pressure, Abd, and DIA electromyogram in conscious, freely moving condition before identifying and characterizing the respiratory-modulated RVLM presympathetic neurons in *in situ* preparations. We observed that CIH produced a significant increase in the arterial pressure (105 ± 2 vs 86 ± 1 mmHg; *p* < 0.0001; *n* = 39) and in the Abd activity during the late-E phase (*n* = 29; Fig. 1*Ai,Aii*). These animals, in *in situ* preparations, also presented late-E activity in the AbN and an increase in the tSN

Figure 6. Spike discharge frequency and intrinsic electrophysiological properties of inspiratory-modulated RVLM presympathetic neurons from control and CIH rats. Raw and integrated (*f*) records of PN, tSN, and inspiratory-modulated RVLM presympathetic neurons activities from control (*Ai*) and CIH (*Aii*) rats. Note the presence of late-expiratory (late-E; $\uparrow$) in tSN and no changes in the spike discharge frequency of inspiratory-modulated RVLM presympathetic neuron after CIH. In the presence of synaptic blockade, intrinsic spike discharge frequency (*Bi, Bii*), excitability properties (*Ci, Cii, Ciii*), and input resistance [*Ri* (*Di, Dii, Diii*)] of inspiratory-modulated RVLM presympathetic neurons were not affected by CIH.

activity during the same phase of respiratory cycle (55 ± 2 vs $33 \pm 5\%$; *n* = 39; *p* < 0.0001; Fig. 1*Bi,Bii*). After the characterization of the effects of CIH in the respiratory-sympathetic coupling of awake rats and *in situ* preparations, we used these rats (*n* = 39) and others (*n* = 93) to identify and characterize the different respiratory-modulated presympathetic neurons in the RVLM and their contribution to the respiratory-related increase in the sympathetic activity observed after CIH.

Identification of nonmodulated and respiratory-modulated RVLM presympathetic neurons in *in situ* preparations

We were able to perform intracellular recordings of 132 presympathetic neurons in the RVLM region, which responded with a hyperpolarization and a transient inhibition of the spike discharge frequency in response to stimulation of the ADN (Fig. 2*Ai*). These neurons send projections to the spinal cord [antidromic latencies ranged from 2.2 to 10 ms (average 5.2 ± 0.7

Figure 7. Spike discharge frequency and intrinsic electrophysiological properties of postinspiratory-modulated RVLM presympathetic neurons from control and CIH rats. Raw and integrated (*f*) records of PN, tSN, and postinspiratory-modulated RVLM presympathetic neurons activities from control (*Ai*) and CIH (*Aii*) rats. Note the presence of late-expiration (late-E; $\uparrow$) in tSN and an increase in the spike discharge frequency of presympathetic neuron in the same phase of respiratory cycle after CIH. In the presence of synaptic blockade, intrinsic spike discharge frequency (*Bi*, *Bii*), excitability properties (*Ci*, *Cii*, *Ciii*), and input resistance [*Di*, (*Di*, *Dii*, *Diii*)] of postinspiratory-modulated RVLM presympathetic neurons were not affected by CIH.

ms)] and had axonal conduction velocity that ranged from 1 to $4.5 \text{ m} \cdot \text{s}^{-1}$ [(average $2.5 \pm 0.3 \text{ m} \cdot \text{s}^{-1}$); $n = 132$; Figure 2*Aii*]. The baroreceptor inhibition (latency: $27 \pm 1.6 \text{ ms}$; amplitude: $5 \pm 0.9 \text{ mV}$; $n = 132$) suggests a chloride conductance, as demonstrated by reversals in the polarity of these potentials in response to a continuous application of negative current (Fig. 2*Bi*, *Bii*). A prominent feature of respiratory-modulated RVLM presympathetic neurons recorded in *in situ* preparations, an experimental condition in which the sympathetic and respiratory networks are preserved, was a high level of synaptic “noise.” During baseline conditions, individual action potentials were usually interspersed by transient increase or decrease in their frequency discharge in the different phases of the respiratory cycle. When the firing was eliminated by a small continuous hyperpolarizing current, it was possible to observe the presence of spontaneous excitatory postsynaptic potentials (sEPSPs) and synaptic inhibition in the different phases of the respiratory cycle (Fig. 2*Ci*, *Cii*, *Di*, *Dii*). Considering these different synaptic inputs, we were able to

clearly classify four groups of respiratory-modulated RVLM presympathetic neurons, in accordance with the firing pattern of each neuron in the different phases of the respiratory cycle: (1) neurons with a very regular pattern of action potential discharge; (2) neurons discharging action potentials in an irregular pattern with an inspiratory modulation; (3) neurons discharging action potentials in an irregular pattern with a pos-I modulation; and (4) neurons discharging action potentials in a regular pattern with an inspiratory inhibition.

To further investigate the respiratory- and nonrespiratory-related rhythmic activity of RVLM presympathetic neurons, we analyzed whether synaptic inputs, intrinsic electrophysiological properties, ionic currents, or the relation to C1 group of neurons were different in these four groups of RVLM presympathetic neurons. Considering that the exposure to CIH enhanced the central coupling between respiratory and sympathetic activities (Fig. 1*Ai*, *Aii*, *Bi*, *Bii*), the effects of a metabolic challenge produced by CIH on respiratory synaptic inputs and intrinsic electrophysiological properties of these neurons were also evaluated.

Group I (nonmodulated)

Catecholaminergic neurons belonging to the spinally projecting C1 group were identified using the single-cell qRT-PCR technique and immunofluorescence, while glutamatergic neurons were identified using single-cell qRT-PCR. All tested nonmodulated RVLM presympathetic neurons were positive TH-ir cells using immunofluorescence (control: 5 neurons; CIH: 5 neurons; Fig. 3*Ai*, *Aii*) and all tested neurons (control: 4 neurons) using single cell qRT-PCR for the presence of VGLUT₂ also were positive (Fig. 3*Aiii*). During control condition, reduced spike discharge frequency with a

small continuous hyperpolarizing current revealed few sEPSPs without relation to any specific phase of the respiratory cycle (Fig. 2*Ci*). After synaptic blockade, this group of neurons showed a resting membrane potential of $-53 \pm 2.2 \text{ mV}$, intrinsic spike discharge frequency of $6 \pm 1.1 \text{ Hz}$, and an input resistance of $335 \pm 1.1 \text{ M}\Omega$ ($n = 9$; Table 1). The average number of actions potentials in response to 100 pA of positive current was 46 ± 6 spikes (Table 1). A small decrease in the instantaneous spike discharge frequency and increase in the interspike interval in response to positive current injection were also observed (Fig. 4*Ai*, *Aii*). During larger negative current steps, an initial hyperpolarization followed by a shift of the membrane potential toward more positive voltages (sag) was also seen, suggesting the presence of a hyperpolarization-activated inward conductance (Fig. 5*Di*).

Nonmodulated RVLM presympathetic neurons from CIH hypertensive rats, with increased sympathetic activity during the late-E phase, presented no significant changes in the spike discharge

frequency (3.2 ± 0.9 vs 3 ± 1 Hz; $n = 19$; Fig. 5*Ai,Aii*) or any change in the intrinsic electrophysiological properties, such as resting membrane potential, intrinsic spike discharge frequency (Fig. 5*Bi,Bii*), number of spikes in response to positive current injection (Fig. 5*Ci,Cii,Ciii*), or input resistance (Fig. 5*Di,Dii,Diii*) when compared with nonmodulated RVLM presympathetic neurons from control rats ($n = 19$; Table 1). These data suggest that this group of TH-positive RVLM presympathetic neurons is not involved in the increased sympathetic activity observed after CIH.

Group II (inspiratory-modulated)

All tested RVLM presympathetic neurons with inspiratory modulation were positive TH-ir cells using immunofluorescence (control: 8 neurons; CIH: 8 neurons; Fig. 3*Bi,Bii*) and all tested neurons (control: 4 neurons) also expressed VGLUT₂ (Fig. 3*Biii*), as shown by single-cell qRT-PCR experiments. Reducing spike discharge frequency with a small continuous hyperpolarizing current revealed sEPSPs clearly correlated with the inspiratory phase (Fig. 2*Cii*), which may be responsible for the observed increase in their spike discharge frequency during inspiration. After synaptic blockade, this group of RVLM presympathetic neurons had a resting membrane potential of -51 ± 2.3 mV, intrinsic spike discharge frequency of 22 ± 3 Hz, and an input resistance of 162 ± 6.5 M Ω ($n = 12$; Table 1). The average number of action potentials in response to 100 pA of positive current was 90 ± 8.7 spikes. A sustained profile in the instantaneous spike discharge frequency and interspike interval responses to positive current injection (Fig. 4*Bi,Bii*) were also observed.

Similar to nonmodulated RVLM presympathetic neurons, positive TH-ir inspiratory-modulated neurons seem not to be involved in the increased sympathetic activity observed after CIH because the spike discharge frequency (5 ± 1.3 vs 4 ± 1.9 Hz; $n = 24$; Fig. 6*Ai,Aii*), resting membrane potential, intrinsic spike discharge frequency (Fig. 6*Bi,Bii*), number of spikes in response to positive current injection (Fig. 6*Ci,Cii,Ciii*), and input resistance (Fig. 6*Di,Dii,Diii*) were not significantly different from control rats ($n = 24$; Table 1).

Group III (postinspiratory modulated)

All tested RVLM presympathetic neurons with post-I modulation were negative TH-ir cells using immunofluorescence (control: 9 neurons; CIH: 7 neurons; Fig. 3*Cii*) and all tested neurons (control: 3 neurons) also expressed VGLUT₂ (Fig. 3*Ciii*), as shown by single cell qRT-PCR. Reducing their spike discharge frequency with a small continuous hyperpolarizing current revealed sEPSPs during the post-I phase (Fig. 2*Di*), which may be responsible for the increase in

Figure 8. Effects of CIH on the synaptic inputs to the postinspiratory-modulated RVLM presympathetic neurons. sEPSPs, measured by hyperpolarizing the neurons, during different phases of respiration of postinspiratory-modulated RVLM presympathetic neurons from control (*Ai*) and CIH (*Aii*) rats. Note that CIH produced an increase in the frequency and amplitude of sEPSPs only during late-E (*Aiii*, *Aiv*). sEPSCs, measured at -70 mV, during different phases of respiration of the same postinspiratory-modulated RVLM presympathetic neurons from control (*Bi*) and CIH (*Bii*) rats shown in *A*. Note that CIH also produced an increase in the frequency and amplitude of sEPSCs only during late-E (*Biii*, *Biv*). The superimposed sEPSPs (*Aiii*, *Aiv*) and sEPSCs (*Biii*, *Biv*) were event triggered.

their spike discharge frequency observed during post-I phase. Under conditions of synaptic blockade, their resting membrane potential was -52 ± 3.2 mV, intrinsic spike discharge frequency was 13 ± 2 Hz, and input resistance was 245 ± 5 M Ω ($n = 12$; Table 1). The average number of action potentials in response to 100 pA of positive current was 61 ± 7 spikes (Table 1). A temporal increase in the instantaneous spike discharge frequency and decrease in the interspike interval responses to positive current injection were observed (Fig. 4*Ci,Cii*).

RVLM presympathetic neurons from hypertensive CIH rats, with post-I modulation, showed a significant increase in the spike

Figure 9. Spike discharge frequency and intrinsic electrophysiological properties of inspiratory-inhibited RVLM presympathetic neurons from control and CIH rats. Raw and integrated (*f*) records of PN, tSN, and inspiratory-inhibited RVLM presympathetic neurons activities from control (**Ai**) and CIH (**Aii**) rats. Note the presence of late-expiration (late-E; $\uparrow$) in tSN and no changes in the spike discharge frequency of inspiratory-inhibited RVLM presympathetic neuron after CIH. In the presence of synaptic blockade, intrinsic spike discharge frequency (**Bi**, **Bii**), excitability properties (**Ci**, **Cii**, **Ciii**), and input resistance [R_i (**Di**, **Dii**, **Diii**)] of inspiratory-inhibited RVLM presympathetic neurons were not affected by CIH. Note that after large hyperpolarizing current injection in **Di** and **Dii**, the inspiratory-inhibited RVLM presympathetic neurons expressed a delay in the rate of depolarizing and in the generation of action potentials ($\uparrow$).

discharge frequency at the late-E phase (35 ± 3 vs 4 ± 1 Hz; $p < 0.0001$; $n = 26$; Fig. 7*Ai,Aii*), which is well correlated with late-E tSN activity. Regarding the intrinsic electrophysiological properties, the data show that the spike discharge frequency (Fig. 7*Bi–Bii*), number of spikes in response to positive current injection (Fig. 7*Ci,Cii,Ciii*), and input resistance (Fig. 7*Di,Dii,Diii*) of RVLM presympathetic neurons, showing post-I modulation, were not affected by CIH ($n = 26$; Table 1). This set of data indicates that increased sympathetic activity in *in situ* preparations of CIH rats is not due to changes in the intrinsic electrophysiological properties of the postinspiratory-modulated RVLM presympathetic neurons, but rather dependent on inputs from other groups of neurons, which are also part of the intact network. Therefore, on the next step, we evaluated the excitatory synaptic inputs to these neurons, by recording both spontaneous excitatory postsynaptic currents (sEPSCs) and sEPSPs, during

different phases of the respiratory cycle. Using this approach, we analyzed the possible source of the increased spike discharge frequency of this population of RVLM presympathetic neurons during the late-E phase. The results show that CIH increased the frequency of sEPSPs (12 ± 2.4 vs 2.2 ± 0.9 Hz; $p = 0.001$; $n = 26$; Fig. 8*Ai–Aiv*) and sEPSCs (14 ± 2 vs 2 ± 0.5 Hz; $p < 0.0001$; $n = 26$; Fig. 8*Bi–Biv*) as well as their amplitudes [(sEPSPs: 19 ± 3 vs 9 ± 2 mV; $p = 0.01$; $n = 26$; Fig. 8*Ai–Aiv*) (sEPSCs: 155 ± 3 vs 25 ± 4 pA; $p < 0.0001$; $n = 26$; Fig. 8*Bi–Biv*)] only in the late-E phase. These findings indicate that the excitatory inputs, probably from expiratory neurons, drive the increased RVLM presympathetic neurons activities and, as a consequence, induce the increased sympathetic activity observed in CIH rats.

Group IV (inspiratory-inhibited)

All tested RVLM presympathetic neurons with inspiratory inhibition were negative TH-ir cells using immunofluorescence (control: 7 neurons; CIH: 5 neurons; Fig. 3*Di,Dii*) and all tested neurons (control: 3 neurons) also expressed VGLUT₂ (Fig. 3*Diii*), as shown by single cell qRT-PCR. Reducing their spike discharge frequency with a small continuous hyperpolarizing current revealed a strong inhibition during the inspiratory phase (Fig. 2*Dii*). Under conditions of synaptic blockade, their resting membrane potential was -54 ± 3.4 mV, intrinsic spike discharge frequency was 8 ± 1.9 Hz, and input resistance was 515 ± 9 M Ω ($n = 10$; Table 1). The average number of actions potentials in response to 100 pA of positive current was 64 ± 6 spikes (Table 1). In contrast to postinspiratory-modulated RVLM presympathetic neurons, it was possible to observe a spike frequency adaptation with an increase in the interspike interval in response to positive current injection in the inspiratory-inhibited RVLM presympathetic neurons (Fig. 4*Di,Dii*). After large hyperpolarizing current injection, the inspiratory-inhibited RVLM presympathetic neurons also expressed a delay in the rate of depolarization and in the generation of action potentials (Fig. 9*Di*). The current that underlies this delay excitation seems to be similar to a transient potassium current, activated upon depolarization from hyperpolarized potentials.

Despite the fact that inhibitory-modulated RVLM presympathetic neurons were negative TH-ir, similar to the postinspiratory-modulated neurons, they are not involved in the increased sympathetic activity after CIH, because the spike discharge frequency (6 ± 1.3 vs 6.4 ± 2.2 Hz; $n = 20$; Fig. 9*Ai,Aii*), resting membrane potential, intrinsic spike discharge frequency (Fig. 9*Bi–Bii*), number of spikes in response to positive current injection (Fig. 9*Ci,Cii,Ciii*), or input resistance (Fig. 9*Di,Dii,Diii*) were similar to

the neurons from control rats ($n = 20$; Table 1). The basic electrophysiological properties of the different populations of respiratory-modulated RVLM presympathetic neurons from control and CIH rats are summarized in the Table 1 and the differences between these properties in the four groups, when present, are indicated.

Hyperpolarization-activated inward conductance does not modulate the pacemaker activity of nonmodulated RVLM presympathetic neurons

Only the group I of RVLM presympathetic neurons exhibits a hyperpolarization-activated inward conductance (Fig. 5*Di–Dii*). Here, we examined the contribution of this inward conductance to pacemaker activity of group I of RVLM presympathetic neurons. Small injections of negative current (-25 pA) cause a hyperpolarizing membrane response that is maintained throughout the duration of the current pulses (Fig. 5*Di,Dii*). In contrast, larger hyperpolarizing pulses produced a “sag” (Fig. 5*Di,Dii*). This sag is consequence of the activation of a time-dependent inward conductance. Under voltage-clamp, hyperpolarizing voltage steps (-70 to -130 mV), from a holding potential of -70 mV, revealed a slowly activating inward current (Fig. 10*Ai*). This inward current reached a plateau after several hundred milliseconds and showed no inactivation in the time window in which it was recorded. Addition of ZD7288 (40 μ M), a selective blocker of I_h , to the perfusion resulted in a significant reduction in this current (Fig. 10*Aii*). Off-line subtraction of the inward current before and after the perfusion with ZD7288 revealed the I_h , which starts to be activated ~ -70 mV with peak at -130 mV (-335 ± 11 pA; $n = 6$; Fig. 10*Aiii,Bi*), half-activation voltage of -97.3 ± 0.6 mV, and a slope factor of -7.1 ± 0.6 mV (Fig. 10*Bii*). We examined the participation of this I_h in the pacemaker activity of RVLM presympathetic neurons by using ZD7288 in the perfusion. The effect of ZD7288 on the spike discharge frequency of nonmodulated C1 pacemaker neuron is illustrated in the Figure 10, *Ci* and *Cii*. Selective blockade of I_h produced no changes in the pacemaker spike discharge frequency of group I of RVLM presympathetic neurons (7 ± 1.6 vs 6 ± 1.1 Hz; $n = 9$; Fig. 10*Ci,Cii*).

Properties of low-threshold spike and T-type calcium currents in the inspiratory- and postinspiratory-modulated RVLM presympathetic neurons

As shown in Figure 11*Ai*, a burst discharge was evoked at the end of a hyperpolarizing pulse, which was followed by a train of action potentials, only in the groups II and III of RVLM presympathetic neurons. In the presence of TTX, a prominent low-threshold spike (LTS) was displayed at the end of a hyperpolarizing pulse,

Figure 10. Bulbospinal nonmodulated pacemaker RVLM presympathetic neurons express hyperpolarization-activated inward current (I_h). **Ai**, Hyperpolarizing voltage steps (-10 mV increments) from a holding potential of -70 mV evoked a time-dependent inward current in the nonmodulated RVLM presympathetic neuron. **Aii**, The inward current is reduced by addition of ZD7288 (40 μ M), a selective blocker of the I_h , to the perfusion solution. **Aiii**, Off-line subtraction of the traces presented in **Ai** and **Aii** are showing the I_h ZD7288-sensitive current. **Bi**, Current–voltage relationship of the steady-state portion of the averaged I_h . **Bii**, I_h activation curve (Boltzmann fitted; see Materials and Methods) showing the voltage dependence of the inward current. **Ci**, Representative tracing of the pacemaker activity of nonmodulated RVLM presympathetic before and after (**Cii**) perfusion with ZD7288. Addition of ZD7288 produced no changes in the pacemaker spike discharge frequency of nonmodulated RVLM presympathetic neuron.

which was blocked by perfusion of 50 μ M Ni^{2+} , a known T-type calcium channel blocker (Fig. 11*Aii*).

T-type calcium currents were activated using a series of depolarizing command pulses (from -90 to 0 mV, in 10 mV increments). The peak amplitudes of the evoked currents were plotted as a function of the command potential (I/V plots) and the voltage-dependent activation of the evoked currents was assessed. A representative example of one RVLM presympathetic neuron from group III is shown in Figure 11*Aiii*. On average, the peak of activation of T-type calcium current in the groups II and III of RVLM presympathetic neurons was -50 mV, and there was no difference in the T-type calcium currents between these groups of neurons (at -50 mV: -2.05 ± 0.6 vs -2.2 ± 0.7 pA/pF from group II and group III, respectively; $n = 10$; Fig. 11*Bi*). We investigated a possible role of T-type calcium currents in the

Figure 11. Electrophysiological properties of LTS and T-type calcium current in the bulbospinal inspiratory- and postinspiratory-modulated RVLM presympathetic neurons. **Ai**, Examples of electrophysiological recordings obtained from one representative postinspiratory-modulated RVLM presympathetic neuron showing the generation of a burst discharge at the end of a hyperpolarizing pulse (2 s, at different holding currents) followed by a train of action potentials. **Aii**, In the presence of TTX (0.5 μM), the LTS appeared at the end of the hyperpolarizing pulses as a short-lasting depolarizing hump and it was entirely blocked by 50 μM Ni²⁺. **Aiii**, T-type calcium current obtained from another postinspiratory modulated RVLM presympathetic neuron using a series of depolarizing command pulses (from -90 to 0 mV, in 10 mV increments), from a hyperpolarizing conditioning pulse of -90 mV. **Bi**, Average peak amplitudes of the evoked currents as a function of the command potential in the groups II and III of RVLM presympathetic neurons. **Bii**, In current-clamp mode, the pacemaker spike discharge frequency, of the same postinspiratory-modulated RVLM presympathetic neuron shown in **Ai**, increased after application of Ni²⁺. **Biii**, Ni²⁺ reduced the amplitude of after-hyperpolarization potential, causing a shorter duration of the interspike interval. Group II: inspiratory-modulated RVLM presympathetic neurons; Group III: postinspiratory-modulated RVLM presympathetic neurons.

pacemaker activity with Ni²⁺ in the perfusion in the groups II and III of RVLM presympathetic neurons. In the example of one RVLM presympathetic neuron from group III (Fig. 11Bii,Biii), Ni²⁺ did not block the pacemaker activity, but induced a depolarization [(group II: -46 ± 0.8 vs -51 ± 2.3 mV; $n = 12$; $p < 0.05$) (group III: -45 ± 0.5 vs -52 ± 3.2 mV; $n = 12$; $p = 0.04$)] and increased spike discharge frequency [(group II: 36 ± 5.5 vs 22 ± 3 Hz; $n = 12$; $p = 0.03$) (group III: 25 ± 3.5 vs 13 ± 2 Hz; $n = 12$; $p = 0.007$)].

Role of voltage-activated outward conductance in the pacemaker activity of inspiratory-inhibited RVLM presympathetic neurons

The transient A-type voltage-dependent IA, only expressed in the group IV of RVLM presympathetic neurons (Fig. 9Di,Dii), is characterized by a strong voltage dependence and rapid activation and inactivation kinetics, resulting in a transient outward current (Fig. 12Ai). Depolarizing steps of increasing amplitudes (-80 to 60 mV, in 10 mV increments) were used to activate IA. The average IA peak current density at 60 mV was 71 ± 5.2 pA/pF ($n = 6$; Fig. 12Aii). Plots of conductance versus membrane potential show a half-activation voltage of -39.1 ± 1.6 mV, with a slope factor of 12 ± 1.4 mV (Fig. 12Bi). The voltage dependence of inactivation was then studied (Fig. 12Aiii). The average IA half-inactivation potential was -68 ± 0.6 mV with a slope factor

of -11.7 ± 0.6 (Fig. 12Bi). When the voltage-dependent activation and inactivation curves of IA were plotted together, a small overlapping area between these two curves (i.e., “window current”) at potentials between -70 mV to -35 mV was observed (Fig. 12Bi).

Since the IA was active in the voltage range traversed by pacemaker potentials (“window current”), we examined the modulatory effect of IA on the pacemaker activity of group IV of RVLM presympathetic neurons by using a blocker of this conductance, 4-aminopyridine (4-AP; 5 mM; Sigma-Aldrich), in the perfusion solution. Application of 4-AP resulted in an increase in the pacemaker spike discharge frequency, from 8 ± 1.9 Hz to 13 ± 0.9 Hz ($n = 10$; $p = 0.03$; Fig. 12Bii,Biii).

Persistent sodium current is essential for pacemaker activity of nonmodulated and respiratory-modulated RVLM presympathetic neurons

To further address the pacemaker activity of respiratory-modulated RVLM presympathetic neurons, the presence of I_{NaP} was tested directly in the voltage-clamp mode. An example of one inspiratory-modulated RVLM presympathetic neuron is illustrated in Figure 13A. To isolate I_{NaP} and study its current-voltage relationship without concomitant activation of fast sodium currents, we used slow depolarizing voltage ramps (30 mV/s) from a holding potential of -85 to 15 mV. The activation of I_{NaP} was evident ~ -55 mV and reached its peak amplitude

between -40 and -35 mV. Application of riluzole (10 μM) substantially reduced the inward deflection indicating that riluzole-sensitive sodium current underlies this conductance (Fig. 13A).

To test the hypothesis that I_{NaP} may be differentially expressed in the different groups of RVLM presympathetic neurons, we compared the amplitude of their riluzole-sensitive current. As illustrated in the Figure 13B, the peak current density of I_{NaP} was significantly different between some of the RVLM presympathetic neurons and all RVLM presympathetic pacemaker neurons expressed I_{NaP} [(group I: 0.5 ± 0.06 pA/pF; $n = 5$) (group II: 3.3 ± 0.2 pA/pF; $n = 5$) (group III: 0.99 ± 0.07 pA/pF; $n = 6$) (group IV: 0.96 ± 0.06 pA/pF; $n = 5$)]. The voltage-clamp data are consistent with the observed inhibition of pacemaker spike discharge frequency by blocking I_{NaP} with riluzole (Fig. 13C), by hyperpolarization to voltages higher than -55 mV, and clearly implicate I_{NaP} as the main conductance underlying pacemaker potentials in the nonmodulated and respiratory-modulated RVLM presympathetic neurons after synaptic blockade.

Discussion

We are showing that bulbospinal respiratory-modulated RVLM presympathetic neurons constitute a heterogeneous neuronal population regarding their electrophysiological properties, synaptic inputs, and expression of ionic current, albeit all presympathetic neurons presented intrinsic pacemaker activity after

synaptic isolation. This pacemaker activity of C1 and adjacent non-C1 respiratory-modulated presympathetic neurons, in *in situ* preparations, is due to an intrinsic cellular mechanism dependent on I_{NaP} . We are also showing that the increase in the spike discharge frequency of the respiratory-modulated presympathetic neurons, in a condition of metabolic challenges in rats produced by CIH, is restricted to a specific subpopulation of neurons (non-C1 postinspiratory-modulated), which presents an enhancement of the excitatory synaptic inputs from the respiratory network.

In the present study we identified three types of respiratory-modulated RVLM presympathetic neurons, as well as a fourth type showing no respiratory-related modulation. All these subpopulations of neurons correspond qualitatively to the patterns reported by other laboratories using *in vivo* anesthetized animals (McAllen, 1987; Haselton and Guyenet, 1989; Miyawaki et al., 1995). The firing pattern of group II of RVLM presympathetic neurons is quite similar to the pattern of activity recorded from cardiac, splanchnic, and adrenal sympathetic nerves (Numao et al., 1987). The discharge pattern of the group III of RVLM presympathetic neurons closely matched the pattern seen in the lumbar sympathetic nerve activity (Haselton and Guyenet, 1989) and in postganglionic neurons innervating the vasoconstrictor muscle (Häbler et al., 1993). Therefore, we are showing that the different patterns of the respiratory-modulated RVLM presympathetic neurons recorded are due to direct synaptic inputs from distinct respiratory neurons, and it may explain the regional differences in sympathetic nerve activity innervating different vascular beds (Numao et al., 1987).

Previous studies showed that the RVLM presympathetic C1 neurons are implicated in cardiovascular control (Allen and Guyenet, 1993; Lipski et al., 1995a). We used single-cell qRT-PCR and immunofluorescence to study the expression of TH and VGLUT₂ within functionally identified respiratory-modulated RVLM presympathetic neurons. Despite the fact that all respiratory-modulated RVLM presympathetic neurons investigated with qRT-PCR were glutamatergic neurons, we detected the TH mRNA only in the groups I and II of presympathetic neurons. The efficiency of our TH primer to detect mRNA at the single cell level is supported by our findings of TH protein expression only in RVLM neurons from groups I and II.

We documented that C1 respiratory-modulated RVLM presympathetic neurons send direct projections to sympathetic

Figure 12. Modulatory effect of IA on the pacemaker frequency of bulbospinal inspiratory-inhibited RVLM presympathetic neurons. **Ai**, Transient IA current and a sustained outward current of a pacemaker nonmodulated RVLM presympathetic neuron elicited by depolarizing voltage steps (from -80 to 60 mV) with a holding voltage either of -110 mV or -40 mV. With the off-line subtraction of the currents evoked with holding voltage of -110 and -40 mV, the IA current was isolated. **Aii**, Average amplitude of IA current measured at the peak values of the subtracted current showed in **Ai**. **Aiii**, Voltage-dependent inactivation was measured as the decrease in IA during a step to -30 mV after 1 s of preholding potentials from -130 to -30 mV. **Bi**, When the average normalized IA amplitude obtained with the voltage-dependent activation (■) and inactivation (□) protocols are plotted together (Boltzmann fitted—see Materials and Methods) as a function of the command potential, a region of overlap between -70 mV and -35 mV was observed. **Bii**, In current-clamp mode, the pacemaker spike discharge frequency of the inspiratory-inhibited RVLM presympathetic neuron increased after application of 4-AP (5 mM). **Biii**, Broadening of a pacemaker action potential and reduction of after-hyperpolarization potential in the presence of 4-AP was observed.

Figure 13. Persistent sodium current (I_{NaP}) underlies the pacemaker activity in bulbospinal nonmodulated and respiratory-modulated RVLM presympathetic neurons. **A**, Representative current response of one inspiratory-modulated RVLM presympathetic neuron from control rats voltage-clamped to a slow depolarizing voltage ramp (30 mV/s) applied from a holding potential of -85 mV to -25 mV. A developing slow inward current was evident as a negative slope conductance on the current–voltage plot with activation voltage ($\uparrow$) ~ -55 mV. Riluzole (10 μ M) completely suppressed the inward current. **B**, Comparison of the riluzole-sensitive I_{NaP} at -30 mV, expressed as peak current density, between all pacemaker RVLM presympathetic neurons. Note that the group II of RVLM presympathetic neurons presented a higher I_{NaP} current density in relation to other groups of neurons. **C**, Representative voltage traces of the pacemaker activity of same inspiratory-modulated RVLM presympathetic neuron showed in **A**. Riluzole blocked the spontaneous spike discharge frequency of the bulbospinal pacemaker inspiratory-modulated RVLM presympathetic neuron. Group I: nonmodulated RVLM presympathetic neurons; Group II: inspiratory-modulated RVLM presympathetic neurons; Group III: postinspiratory-modulated RVLM presympathetic neurons; Group IV: inspiratory-inhibited RVLM presympathetic neurons; $*p < 0.05$ in relation to the groups I, III, and IV of RVLM presympathetic neurons.

preganglionic neurons in the spinal cord, indicating that they are undoubtedly part of the neural substrate that regulates the cardiovascular system as showed before for cardiac-modulated neurons (Reis et al., 1989; Ruggiero et al., 1989; Allen and Guyenet, 1993). We also documented that C1 neurons are not the only group of respiratory-modulated presympathetic neurons that drive the sympathetic outflow. The increased sympathetic activity observed in *in situ* preparations of CIH rats was due to changes in the spike discharge frequency of non-C1 postinspiratory-modulated presympathetic neurons during the late-E phase, whose mechanisms are dependent on glutamatergic synaptic inputs from respiratory network. These results corroborate the hypothesis that elevations in sympathetic activity, in different models of hypertension, might involve exaggerated respiratory–sympathetic coupling (Czyzyk-Krzaska and Trzebski, 1990; Toney et al., 2010; Moraes et al., 2012a). The data of the present study provide a mechanistic insight to explain why CIH awake rats, which are also hypertensive, presented active expiration in the abdominal muscle in the same phase of increased spike discharge frequency of presympathetic neurons observed in *in situ* preparations.

We identified differences in some of the electrophysiological properties of respiratory-modulated RVLM presympathetic neurons, such as intrinsic excitability, which may be related to differences in ionic conductances operating at or close to the resting membrane potential, albeit all neurons presented pacemaker activity after synaptic blockade. However, before the synaptic blockade when these cells were receiving multiple synaptic inputs, the pattern of spike discharge frequency was probably determined by the balance of excitatory and inhibitory inputs, as also observed in *in vivo* anesthetized rats (Lipski et al., 1995b), in which experiments the synaptic blockade was not performed. Although these cells, as part of a complex network, are in charge of the generation of sympathetic activity, it does not imply that under this condition their spike discharge frequency is driven by their intrinsic pacemaker properties.

The ionic currents underlying the hyperpolarization activated inward conductance in the group I of presympathetic neurons resembles I_h , a well characterized current in a variety of neurons in the CNS (McCormick and Pape, 1990; Sirois et al., 2002) and in heart pacemaker cells (Brown et al., 1984). The I_h can accelerate pacemaker frequency of presympathetic neurons by increasing the slope of the depolarizing phase of the membrane potential. However, this current is not essential for pacemaker activity of the group I of presympathetic neurons. This is indicated by the results showing that the I_h blockade by ZD7288 produced no effect on the pacemaker activity. In this case we suggest that the membrane potential of these neurons after synaptic blockade was more depolarized than the voltage necessary to activate the I_h .

One of the most prominent intrinsic properties observed in the groups II and III of RVLM presympathetic was the presence of calcium-dependent LTS. The concept that the expression of the LTS in presympathetic neurons is mediated by the activation of T-type calcium current is supported by the voltage-dependent properties and sensitivity to micromolar concentrations of Ni^{2+} (Lee et al., 2008). Similarly, the involvement of T-type calcium current underlying the LTS has been shown previously in presympathetic neurons (Sun and Reis, 1994) and in other neuronal types (Luther and Tasker, 2000; Stern, 2001). The LTS has been demonstrated to play a major role in brain areas showing neuronal oscillations and burst firing (Llinás and Yarom, 1981). We suggest that in the groups II and III of presympathetic neurons, calcium influx is not the main conductance underlying pacemaker activity, but plays a minor role probably acting indirectly

by inducing a calcium-activated potassium channel $[(\text{BK}_{\text{Ca}})]$ (Pierrefiche et al., 1995). This conductance underlies AHP in neurons and appears to be a potent modulator of their spike discharge frequency, which can explain the increase in the spike discharge frequency and depolarization after the blockade of T-type calcium channels in the groups II and III of presympathetic neurons.

Depolarizing steps of sufficient amplitude activated a transient outward current in the group IV of RVLM presympathetic neurons. Along with studies by Kangrga and Loewy (1995) using brainstem slices, we observed that this current resembles IA, which is involved in the generation of delay excitation. In agreement with previous reports in other neuronal types (Belugin and Mifflin, 2005; Wang and Schreurs, 2006; Sonner and Stern, 2007), our data support the concept that a tonically active IA (i.e., “window” current) is present in group IV of presympathetic neurons. This window current was found to be active in a narrow, although physiologically relevant, range of membrane potential, and it may play a role in setting resting membrane potential in pacemaker presympathetic neurons.

The voltage dependence of the I_{Nap} recorded in respiratory-modulated RVLM presympathetic neurons resemble that described in other pacemaker neurons (Koizumi and Smith, 2008; Liu and Shipley, 2008). Our results suggest that I_{Nap} drives the pacemaker activity of nonmodulated and respiratory-modulated RVLM presympathetic neurons because: (1) the I_{Nap} is activated at voltages ~ -55 mV and reach its peak amplitude at -35 mV. This is consistent with the observation that pacemaker spike discharge frequency is generated only when membrane potential is ~ -55 mV; (2) the pacemaker activity is blocked by addition of riluzole, a selective blocker of I_{Nap} , or by hyperpolarization. Studies by Kangrga and Loewy (1995) showed the presence of an inward sodium current in C1 bulbospinal neurons, and that this current was significantly larger in pacemaker than in nonpacemaker C1 neurons in brainstem slices. A similar current is present in locus ceruleus cells and may contribute to the pacemaker activity of these cells as well (Williams et al., 1991). However, this current is also present in other cell types (hippocampal neurons) that do not fire spontaneously (French et al., 1990). This may indicate that not only is I_{Nap} essential for expressing pacemaker activity of presympathetic neurons, but the expression of other currents, such as leak currents that by interacting with the I_{Nap} may affect the excitability of these neurons and be a functional set of subthreshold conductances as a potential targets for neuromodulatory control of sympathetic outflow.

In conclusion, our electrophysiological experiments provide new and important data about the electrophysiological properties of nonmodulated and respiratory-modulated RVLM presympathetic neurons in the intact sympathetic and respiratory networks and after synaptic blockade. Based on the *in situ* patch-clamp recordings, the results show that the respiratory-modulated RVLM presympathetic neurons constitute a heterogeneous neuronal population, in regards to the intrinsic electrophysiological properties, synaptic inputs, relation to C1 group and in the control of sympathetic outflow in response to a metabolic challenge produced by CIH. There is experimental evidence pointing out to the involvement of RVLM presympathetic neurons in cardiovascular disorders (Han et al., 1998; Toney et al., 2010), including hypertension, but the cellular mechanisms underlying the possible changes in the excitability of presympathetic neurons are unknown. We are showing here for the first time that the respiratory-modulated RVLM presympathetic neurons (non-C1 neurons with post-I modulation) may contribute to the increased sympathetic outflow in CIH rats, which mecha-

nisms involve enhanced respiratory synaptic inputs, probably from expiratory neurons. Therefore, the study of the cellular mechanisms involved in the central generation of respiratory-sympathetic coupling will have important implications for a better understanding of the medullary control of sympathetic activity in physiological and pathophysiological conditions.

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