PATRÍCIA ALINE BOER

HIPERTENSÃO ARTERIAL EM SHR: MODIFICAÇÕES HOMEODINÂMICAS DA INERVAÇÃO RENAL

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Tese de Doutorado apresentada à Pós-Graduação da Faculdade de Ciências Médicas da Universidade Estadual de Campinas para Obtenção do título de Doutor em Clínica Médica, Área de Concentração Básica.

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Ao meu orientador

Prof. Dr. José Antonio Rocha Gontijo

Não há palavras para expressar
a magia dos teus gestos poéticos...

Dedico a você este trabalho.

Os momentos difíceis nos ensinam a distinguir o joio do trigo.

Gabriel Boer

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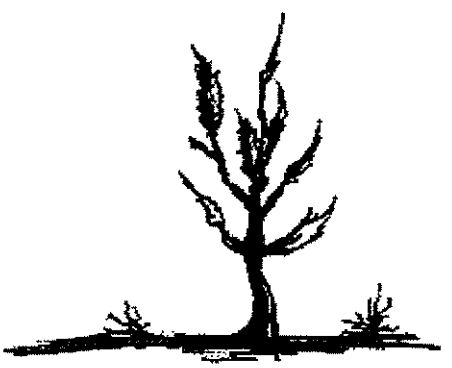


A ciência, a ciência, a ciência...
Ah, como tudo é nulo e vão!

A pobreza da inteligência
Ante a riqueza da emoção!

Aquela mulher que trabalha
Como uma santa em sacrifício,
Com quanto esforço dado ralha!
Contra o pensar, que é o meu vício!

A ciência! Como é pobre e nada!
Rico é o que alma dá e tem.

[...]

Fernando Pessoa, 4-10-1934

SUMÁRIO

LISTA DE ABREVIATURAS

ARNA- Afferent renal nerve activity

AT1- Angiotensin II receptor

BP- Blood pressure

BSA- Bovine serum albumin

C- Renal clearance

CCr- Creatinine clearance

CGRP- Calcitonin gene-related peptide

CLi^+- Lithium clearance

CLSM- Confocal laser scanning microscope

CNa^+- Clearance of sodium

CNS- Central nervous system

COBEA- Brazilian College of Animal Experimentation

CRs- Quimioreceptores

CRE- cAMP response element

CREB- cAMP response element binding protein

DBH- dopamina beta-hidroxilase

DG- 1,2-diacylglycerol

DIC- Differential image contrast

DRG- Dorsal root ganglia

Dx_{SHR}- Bilateral renal denervated SHR

EGF-R- Epidermal growth factor receptor

ERKs- Extracellular signal-regulated kinases

ERSNA- Efferent renal simpathetic nerve activity

FEK^+- Fractional urinary potassium excretion

$FENa^+$- Fractional urinary sodium excretion

$FEPNa^+$- Fractional proximal sodium excretion

$FEPPNa^+$- Fractional post-proximal sodium excretion

GRD- Gânglios da raiz dorsal

IP_3- 1,4,5- inositol trisphosphate

MHS- Milan hypertensive strain

MRs- Mechanoreceptors

NGF- Nerve growth factor

NLS- Nuclear localization signal

NPY- Neuropeptideo Y

NTS- Nucleus tractus solitarius

PBS- Phosphate-buffered saline

PGE- Prostaglandina E

PKC- Protein kinase C

PLC-γ- Phospholipase C-γ

pro-TGF- Pro-form of transforming growth factor

SERCA- Sarco/endoplasmic Ca^{2+}-ATPase

SHR- Spontaneously hypertensive rats

SHRSP- Stroke-prone spontaneously hypertensive rats

SNS- Sistema nervoso simpático

SP- Substance P

trkA- Tyrosine kinase A receptor

VIP- Peptideo intestinal vasoativo

WKy- Wistar-Kyoto

RESUMO

Os rins têm papel central na patogênese da hipertensão arterial primária. Dentre os mecanismos renais envolvidos no desenvolvimento pressórico são descritos àqueles relacionados a alterações hemodinâmicas e na manipulação tubular renal hidro-eletrolítica. No estudo fisiopatológico da hipertensão arterial primária humana têm sido utilizados modelos experimentais análogos dentre os quais destaca-se a linhagem geneticamente hipertensa de Kyoto (SHR). A gênese da hipertensão arterial nestes animais ainda não está inteiramente entendida. Tem sido observado nestes animais um envolvimento etiopatogênico mutifatorial dentre os quais destacam-se as modificações na atividade neural central e periférica, distúrbios neuro-endócrinos e eletrolíticos, acentuada atividade mitogênica da musculatura vascular e significativas alterações funcionais nos rins. Algumas destas alterações antecedem a elevação pressórica. Sabendo-se que a inervação simpática eferente e sensorial aferente renal participam da modulação hemodinâmica, da secreção de renina e do controle da excreção urinária de sódio, o presente trabalho teve como objetivo geral investigar a participação neural (aferente e eferente) renal nestes aspectos etiopatogênicos da hipertensão arterial em SHR. Especificamente, para avaliar a contribuição da atividade nervosa renal sobre a excreção urinária de sódio, investigamos a manipulação tubular deste íon em diferentes segmentos do néfron (proximal e pós-proximal) em SHR antes e após a elevação pressórica (4 e 12 semanas) comparando os resultados a ratos Wistar Kyoto (WKy). Nossos resultados demonstraram elevada reabsorção tubular (proximal e pós-proximal) de sódio em SHR acompanhada de significativa redução na natriurese. Esta menor excreção renal de sódio foi atenuada pela denervação renal bilateral. Estes resultados sugerem aumento da atividade simpática neural associada ou não a menor atividade aferente sensorial (ARNA) neste modelo. Estudos prévios indicam que ratos espontaneamente hipertensos apresentam diminuição da ARNA. Uma vez que a ARNA modula a atividade simpática eferente renal (ERSNA) e, conseqüentemente a natriurese (reflexo renorenal) e, que a resposta aferente renal a estímulos mecano- (MR) e quimioreceptores (CR) e a SP está deprimida em SHR adultos, avaliamos se existe modificações na expressão e localização de neuropeptídeos envolvidos na mediação MR e CR, no gânglio da raiz dorsal (GRD) e na pelve renal (PR), destes animais. O presente estudo avaliou em GRD e na PR a expressão e distribuição sub-celular de CGRP, SP e seu receptor NK_1 (por western blot, microscopia confocal a laser e eletrônica de transmissão) em SHR (de 7 e 14 semanas) e em WKy. Nossos resultados mostram, pela primeira vez, a localizaçao nuclear de CGRP, SP e seu receptor NK_1 (NK_1R) em neurônios de GRD. Os resultados também indicam em WKy, a presença de NK_1R associados à heterocromatina em 30% das sub-populações de neurônios presentes nos GRD. Adicionalmente, a presença nuclear de NK_1R foi também evidenciada em células de Schwann e satélites. A transposição nuclear e os sítios aceptores destes neuropeptídeos junto à heterocromatina ainda deverão ser futuramente investigados. Em SHR, observamos aumento na expressão nuclear de CGRP e SP, associado a menor expressão de NK_1. Tal redução de NK_1R ocorreu na superfície de neurônios do GRD e na pelve renal. O presente estudo indica que alterações na expressão e localização de neuropeptídeos e NK_1R, podem estar relacionados à atenuada ARNA com conseqüente elevação da ERSNA, retenção renal de sódio e hipertensão arterial em SHR.

ABSTRACT

The kidneys play a pivotal role in the pathogenesis of essential hypertension by a primary defect in renal hemodynamics and/or tubule hydro-saline handling that results in the retention of fluid and electrolytes. In the physiopathological studies of human essential hypertension similar experimental models have been used including spontaneously hypertensive rats (SHR). In this lineage the genesis of hypertension is not totally understand. In this animals have been observed multifactorial etiopathogenesis that include changes at central and peripheral neural activity, neuroendocrine and electrolytic alterations, rise in the mitogenic activity muscle vascular cells and kidney functional alterations. Some that alterations, preceding the pressoric rise. The efferent renal sympathetic neural activity (ERSNA) and the sensorial afferent renal neural activity (ARNA) are involved in the homeodynamic modulation, renin secretion and in the control of urinary sodium excretion, thus, this work has a general objective of investigate the neural (afferent and efferent) renal participation in that ethiopathogenic aspects of arterial hypertension in SHR. Specifically, to analyze the renal nervous activity contribution in the sodium urinary excretion, we investigate the manipulation of this ion at the different nephon segments (proximal and post-proximal) in SHR before and after arterial blood pressor rise (4 and 7 weeks) compared to results obtained to Wistar Kyoto (WKy) rats. Our results show an increased tubular (proximal and post-proximal) sodium reabsorption in SHR accompanied to significant reduction in natriuresis. This decreased sodium renal excretion was attenuated by bilateral renal denervation. These results suggests enhancement in the neural sympathetic activity associated or not, to the lesser ARNA in this genetic hypertensive model. Previous studies indicated that SHR presents reduced ARNA. The ARNA modulate ERSNA and, consequently the natriuresis (renorenal reflexes) and the afferent renal responses to mechano- (MR) and chemoreceptors (CR) and to Substance P (SP) are depressed in adult SHR, thus we evaluated the existence of modifications in the expression and localization of neuropeptides involved in the mediation of MR and CR, in the dorsal root ganglia (DRG) and renal pelvis (RP), on these animals. The present study verified in DRG (T_{10} to L_1) and in RP the subcellular expression and distribution of CGRP, SP and NK_1 receptor (by western blot, confocal laser scanning and transmission microscopy) in SHR (7 and 14-old-weeks) compared to WKY. Our results show, by the first time, the nuclear location of CGRP, SP and NK_1 receptors (NK_1R) in DRG neurons. The results also indicate in WKY, the occurrence of NK_1R associated to heterochromatin in neurons sub-populations of DRG. Additionally, the nuclear presence of NK_1R was also showed in satellite and Schwann cells. The nuclear translocation and acceptors sites at the chromatin will be investigated in the future. In SHR we observed elevated nuclear expression of CGRP, SP e NK_1R, associated to minor cellular membrane expression of NK_1R. This reduction was also observed in the RP. The present study indicate that location and altered expression of DRG and pelvic neuropeptides and NK_1R may be related to afferent renal nerve attenuated activity associated with sympathetic hyperactivity, sodium retention and consequently arterial hypertension in SHR.

1. INTRODUÇÃO GERAL

Em 1937, HOMER SMITH afirmou "For the moment it may be said that substantial evidence for the neural control of either the tubular excretion or reabsorption of any urinary constituent is lacking". Esta idéia permaneceu inalterada durante muitos anos, excluindo qualquer ação fisiológica neural sobre o controle da função renal. Entretanto, atualmente sabemos que a inervação renal é o elo de ligação entre os rins e o sistema nervoso central, tendo ação fundamental sobre todos os aspectos funcionais do rim.

Em resposta a múltiplos estímulos centrais e periféricos, a atividade nervosa simpática renal eferente (ERSNA) é alterada no sentido de converter informações, via inervações, aos principais componentes estruturais e funcionais dos rins; vasos, glomérulos e túbulos. A transferência de informações ocorre via interação de neurotransmissores, liberados na junção neuroefetora terminal da inervação simpática, com receptores pós juncionais específicos que estão acoplados a sistemas efetores e vias de sinalização celular definidas. A alteração na atividade nervosa simpática renal, em resposta a estímulos fisiológicos normais, promove a regulação homeostática, freqüência dependente, do transporte tubular de íons, secreção de renina e do fluxo sanguíneo renal.

A inervação aferente renal participa de um sistema de controle renal reflexo através de receptores que possibilitam a manutenção da homeostasia hidro-salina através de modificações na função renal total (DiBONA e KOPP, 1997).

1.1- Distribuição da inervação renal

A distribuição da inervação renal eferente e aferente foi estabelecida usando dopamina beta-hidroxilase (DBH) e neuropeptideo Y (NPY), como marcadores de nervos simpáticos eferentes, e peptídeo relacionado ao gene da calcitonina (CGRP) e substância P (SP) como marcadores de fibras sensoriais aferentes (Fig. 1).

Desta forma, verificou-se que os nervos simpáticos renais eferentes estão localizados na árvore vascular e túbulos renais e nas células justaglomerulares (LIU e BARAJAS, 1993). Os nervos aferentes estão localizados predominantemente na pelve renal. Eles podem ser observados também no tecido conjuntivo corticomedular e, em menor número, na vasculatura renal (LIU e BARAJAS, 1993).

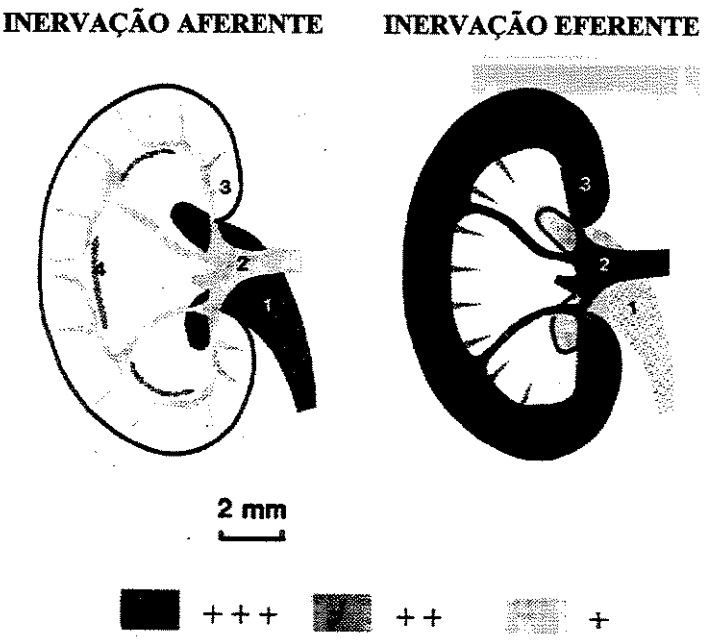


Fig. 1- Diagrama representando a distribuição dos nervos renais aferentes e eferentes. 1, Pelve renal; 2, artéria renal e suas ramificações; 3, região peri-hilar; 4, tecido conjuntivo corticomedular (LIU e BARAJAS, 1993).

Baseado no conteúdo pélvico de SP, podem ser identificadas pelo menos 4 sub-populações de receptores sensoriais renais: dois grandes grupos contendo ou SP ou CGRP isoladamente e dois pequenos grupos de neurônios um deles contendo ambos os peptídeos e outro sem qualquer um deles (FERGUSON e BELL, 1988).

1.2- Inervação renal eferente

Os nervos renais penetram pelo hilo renal, associados à veia e à artéria renais, e são distribuídos ao longo de todos os segmentos da vasculatura arterial tanto no córtex quanto na medula (BARAJAS e WANG, 1979; BARAJAS et al., 1992). A inervação adrenérgica da vasculatura renal é mais densa nas regiões justaglomerulares do córtex interno (GORGAS, 1978). Nos nefrons, terminações sinápticas adrenérgicas estão localizadas, em maior número, em junções neuroefetoras no túbulo proximal (BARAJAS e POWERS, 1989) seguidas, em menor número, pelas presentes na porção fina ascendente da alça de Henle, no túbulo contorcido distal e ducto coletor (BARAJAS et al., 1984). Dopamina está presente em todas as terminações neurais pós-ganglionares simpáticas adrenérgicas, como precursor de norepinefrina. O neuropeptídeo Y ocorre em colocalização com norepinefrina nas terminações simpáticas renais sendo liberado com norepinefrina tanto nos rins quanto na circulação venosa durante estimulo neural (PERNOW e LUNDBERG, 1989). O peptideo intestinal vasoativo (VIP) também é colocalizado com norepinefrina em terminações neurais que inervam a vasculatura renal, predominando nas artérias arqueadas e interlobulares. Nervos contendo óxido nítrico estão presentes nas artérias arqueadas e interlobulares, nas arteríolas aferentes glomerulares e na pelve renal (LIU e BARAJAS, 1993; BACHMANN et al., 1995; LIU et al., 1996).

1.3- Inervação renal aferente

Através de estudos utilizando traçadores retrogrados axonais, verificou-se que os nervos renais aferentes são projetados a partir de gânglios da raiz dorsal (GRD) ipsilaterais, localizados de T_6 a L_2. Após a injeção, em ratos, de tais traçadores no rim direito a maior concentração de neurônios marcados foi observada em GRDs T_{10} e T_{11} e no rim esquerdo em GRDs T_{12} e T_{13} (CIRIELLO e CALARESU, 1983; DONOVAN et al., 1983; FERGUSON et al., 1984).

Aproximadamente 8% dos nervos renais aferentes têm projeção direta na medula (WYSS e DONAVAN, 1984). Nos GRDs estão localizados pericários pequenos (< 20 μm de diâmetro), médios (20-40 μm de diâmetro) e grandes (> 40 μm de diâmetro) (ANEXO I). Através de imunolocalização, foi verificada a presença de CGRP em neurônios pequenos, médios e grandes, sendo tal marcação observada em 30-50% do total de neurônios dos GRDs (SU et al., 1986). Em estudo recente verificou-se a presença de CGRP em neurônios localizados nos GRDs de T_9 a L_1, sendo que estes apresentavam intensidade de marcação variável. Em alguns houve colocalização com SP, porém todos os neurônios imunomarcados para SP também continham CGRP (KOPP et al., 2001).

1.3.1- Neuropeptídeos aferentes

A substância P (SP) é um neuropeptídeo, membro da família das taquicininas, composto por 11 aminoácidos. SP está presente nas porções centrais e periféricas de neurônios sensoriais (KREULEN e PETERS, 1986). Este neuropeptídeo é sintetizado nos corpos celulares dos gânglios da raiz dorsal e distribuído, via axônios, nas terminações espinhais do tecido neural periférico. Concentrações apreciáveis de SP encontram-se também em gânglios autonômicos mesentéricos e celíacos.

Evidências demonstrando concentração acentuadamente maior de SP nas raízes dorsais da medula espinhal, quando comparada às raízes ventrais, sugerem uma função neurotransmissora primariamente sensorial para este neuropeptídeo. Tem sido sugerido que a SP faz também a mediação não-colinérgica de neurônios simpáticos paravertebrais (KREULEN e PETERS, 1986). Por outro lado, foi verificado que a concentração de SP no gânglio cervical superior é regulada pela atividade simpática (SUN et al., 1992). A presença de SP no arco aórtico, seio carotídeo,

vago, gânglio nodoso e no núcleo do trato solitário enfatiza sua função neurotransmissora no reflexo químio e pressoreceptor. Este neuropeptídeo é expresso seletivamente em sistemas sensoriais e se liga preferencialmente ao receptor NK_1, sendo considerado o ligante endógeno deste receptor. NK_1 é uma proteína receptora transmembrana multipasso que apresenta sete domínios transmembrana e está acoplada à proteína G (NAKANISHI, 1991; NAKAJIMA et al., 1992). Este receptor apresenta-se amplamente distribuído no cérebro e na medula espinhal NAKAYA et al., 1994; BROWN et al., 1995). A sensibilização, e subseqüentes respostas celulares mediadas por NK_1 após exposições repetidas à SP, é rapidamente perdida e a ressensibilização ocorre gradualmente. Este mecanismo de dessensibilização e ressensibilização previne o estímulo descontrolado das células e, supõe-se que tal mecanismo envolva fosforilação do receptor, levando ao desacoplamento da proteína G, e subseqüente endocitose e reciclagem do receptor. Em células transfectadas e *in vivo* verificou-se que após a ligação da SP ao receptor NK_1, ocorre rápida internalização e reciclagem gradual (Mantyh et al., 1995a; 1995b; 1997).

O *splicing* alternativo de transcritos primários do gene da calcitonina resulta na síntese do peptídeo relacionado ao gene da calcitonina (CGRP), que é composto por 33 aminoácidos. CGRP é um neuropeptídeo amplamente distribuído no sistema nervoso central e periférico (GIBSON et al., 1984). Este neuropeptídeo está colocalizado freqüentemente com outros peptídeos ou neurotransmissores clássicos (GIBSON et al., 1984; SKOFITSCH e JACOBOWITZ, 1985; HOKFELT et al., 1992). CGRP está colocalizado com SP e somatostatina em neurônios sensoriais aferentes primários (GIBSON et al., 1984; SKOFITSCH e JACOBOWITZ, 1985) e com acetilcolina em neurônios motores (TAKAMI et al., 1985). No sistema nervoso periférico, CGRP está presente em nervos do trato gastrointestinal e dos sistemas cardiovascular e urogenital (TAKAMI et al., 1985; MULDERRY et al.,1985; ZAIDI et al., 1987). As ações de CGRP são, aparentemente, mediadas por receptores altamente específicos ligados à adenilato ciclase.

Através de estudos metabólicos verificou-se que CGRP inibe a endopeptidase de SP, isolada do fluido cérebro-espinhal, sugerindo que CGRP inibe a degradação de SP dentro e fora das terminações (LE GRAVES et al., 1985).

A perfusão da pelve renal, com concentrações crescentes de SP e CGRP, causa aumento da ARNA ipsilateral com elevação contralateral da excreção renal de sódio e água (GONTIJO e KOPP, 1994; GONTIJO et al., 1999), isto é, uma resposta reflexa renorenal similar àquela produzida pelo aumento da pressão ureteral. O tratamento crônico dos animais com altas doses de capsaicína, causando depleção destes neuropeptídeos nas terminações neuro-sensoriais renais, abole a ARNA em resposta ao aumento da pressão ureteral (KOPP e SMITH, 1991). Estes achados sugerem que a resposta reflexa renorenal à elevação da pressão pélvica seja mediada por receptores para SP e/ou CGRP (KOPP e SMITH, 1991). Entretanto, estudos recentes utilizando bloqueadores específicos para receptores de SP (CP-96345) ou CGRP ($h\text{-}CGRP_{8\text{-}37}$), demonstraram que a resposta reflexa renorenal promovida pela ativação de mecanoreceptores é mediada apenas pela SP, uma vez que a resposta aferente neural a elevação pressórica pélvica é bloqueada por antagonistas de SP mas não de CGRP (GONTIJO e KOPP, 1994; GONTIJO et al., 1999).

Existem evidências, em outros tecidos neurais, que CGRP atua como neuromodulador. Resultados recentes demonstram potencialização da resposta neural aferente renal desencadeada pelo aumento da pressão ureteral ou injeção concomitante de SP + CGRP (GONTIJO et al., 1999). Este aumento na resposta é abolido pela perfusão pélvica concomitante com thiorphan (um inibidor de peptidases), o que sugere que esta resposta seja mediada pela diminuição na degradação enzimática de SP (GONTIJO et al., 1999).

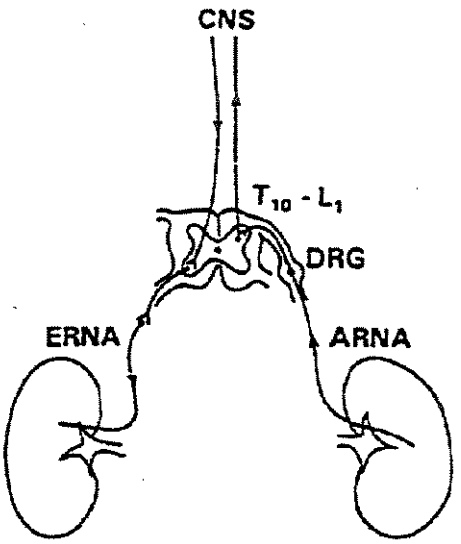
1.4- Reflexo renorenal

O reflexo renorenal é definido como uma resposta, mediada por neurônios periféricos e centrais, que ocorre em um rim em resposta a intervenções no mesmo rim (ipsilateral) ou no rim oposto (contralateral) que é mediada por mecanismos neuro-humorais (KOPP et al., 1984).

O reflexo renorenal é mediado por duas classes de receptores sensoriais renais: Os mecanoreceptores (MR) que respondem ao aumento na pressão intrarenal (elevação da pressão pélvico-ureteral ou da pressão venosa) (Ueda et al., 1967) e os e quimioreceptores (CR) que respondem a isquemia e/ou a perfusão pélvica renal com soluções hipertônicas de NaCl (RECORDATI et al., 1978; 1980).

Desta forma, a obstrução do fluxo urinário aumenta a pressão pélvica renal e ativa os MRs renais e mudanças no conteúdo de solutos medulo-papilar ativam os CRs renais, resultando em aumento ipsilateral da ARNA (KOPP et al., 1995; KOPP e SMITH, 1991a; 1991b; KOPP et al., 1996). O aumento da ARNA induz à diminuição da ERSNA contralateral levando a diurese e natriurese contralateral, o que é conhecido como reflexo renorenal inibitório contralateral que ocorre em situações fisiológicas (KOPP et al., 1984) (Fig. 2).

A denervação renal ipsilateral abole a diminuição da ERNA e o aumento natriurético e diurético contralateral. Em suma, o reflexo renorenal representa um mecanismo através do qual cada rim inibe tonicamente a atividade eferente do rim oposto, interferindo na excreção renal de Na^+/água. Desta forma, anormalidades no mecanismo reflexo renorenal poderiam promover aumento na ERNA e, em conseqüência, maior retenção renal de sódio. Isto poderia ocorrer em uma situação hipotética na qual houvesse disfunção da atividade aferente sensorial renal.



ESTÍMULO DE MECANORECEPTORES RENAIS

⇓

↑ ARNA IPSILATERAL

⇓

↓ ERNA CONTRALATERAL

⇓

↑ FLUXO URINÁRIO CONTRALATERAL

E

↑ EXCREÇÃO URINÁRIA DE SÓDIO CONTRALATERAL

Fig. 2- Esquema representativo da resposta reflexa renorenal ao estímulo de mecanoreceptores por elevação da pressão ureteral (este esquema também se aplica ao estímulo de quimioreceptores pélvicos renais após perfusão com NaCl 0.9M) em ratos anestesiados. Na esquerda, um diagrama simplificada da inervação renal ipsilateral e contralateral. A maioria dos nervos renais aferentes projeta-se do gânglio da raiz dorsal (GRD) ipsilateral para regiões entre T_{10}-L_1. CNS: sistema nervoso central; ERNA: atividade nervosa (simpática) renal eferente; ARNA: atividade nervosa renal aferente (DiBONA e KOPP, 1997)

1.5- Hipertensão arterial e inervação renal

Os rins têm papel chave na patogênese da hipertensão essencial em conseqüência de um defeito funcional renal primário e/ou na hemodinâmica renal promovendo retenção de sódio (FREY et al., 2000; STRAZZULLO et al., 2003). O controle no balanço hidro-eletrolítico tem ação preponderante no controle da pressão arterial. Estudos nos quais foram realizados transplantes renais cruzados, entre ratos geneticamente hipertensos e sua linhagem normotensa (SHR e WKY, respectivamente), demonstraram que os rins estão envolvidos na patogênese da hipertensão (BIANCHI et al., 1974).

Evidências indicam que os nervos renais contribuem para a patogênese da hipertensão arterial, em vários modelos experimentais de hipertensão (WISS, 1992; XAVIER et al., 2000; STRAZZULLO et al., 2003). A atividade nervosa simpática periférica e, em particular, a ERSNA é aumentada em SHR (JUDY e FARREL, 1980). Sabendo-se que o reflexo renorenal gera aumento na diurese e natriurese associado ao decréscimo na ERSNA, tem sido sugerido que a atenuação deste reflexo resultaria na retenção elevada de sódio e água, fatores que contribuem para o processo hipertensivo (DIBONA, 1992). Estudos em ratos SHR demonstraram que o aumento da pressão pélvica renal não produziu aumento na ARNA e o conseqüente reflexo renorenal contralateral (KOPP et al., 1987). Tem sido levantada a hipótese de que esta perda do reflexo renorenal em ratos SHR seja proveniente, pelo menos em parte, de um defeito periférico nos receptores sensoriais da pelve renal. Esta alteração na resposta destes receptores foi também verificada após perfusão pélvica renal com bradicinina em SHR (KOPP e SMITH, 1996). A bradicinina é conhecida por ativar os receptores sensoriais em vários tecidos, através do estímulo da fosfoinositidase C, levando ao aumento do cálcio intracelular e ativação da PKC (CHUANG, 1989; PHEILSCHIFTER, 1989). A ativação da PKC estimula a formação de prostaglandinas dependente de fosfolipase A_2 (CHUANG, 1989; PHEILSCHIFTER, 1989). Estudos *in vitro* de neurônios ganglionares da raiz dorsal mostraram que a ativação da PKC também aumenta a liberação de SP (BARBER, 1996). Na pelve renal, a ativação de PKC aumentou a ARNA e o bloqueio da atividade PKC reduziu a resposta da ARNA à bradicinina em ratos normotensos (KOPP e SMITH, 1995), sugerindo importante papel para PKC na ativação de receptores sensores renais.

Em ratos normotensos a administração de bradicinina na pelve renal ou o aumento da pressão pélvica renal estimularam a liberação de prostaglandina E (PGE), elevando a liberação de substância P e ao aumento na ARNA (KOPP e SMITH, 1991a; 1991b; KOPP e SMITH, 1993a;

1993b; KOPP et al., 1995; 1996; 1997). Estes estudos indicam que o mecanismo celular desencadeado pela ativação de receptores sensoriais renais envolve a ativação da PKC, levando a liberação de PGE e SP. Entretanto constatou-se que a diminuição da ARNA em resposta ao aumento da pressão pélvica renal, em ratos SHR, está associada à diminuição na liberação de SP na pelve renal sem que fossem observadas alterações na liberação de PGE na pelve renal destes animais quando comparados aos WKY (KOPP et al., 1998). Porém, quando estes autores administraram SP na pelve renal de ratos SHR, não foi verificado aumento na ARNA utilizando concentrações que provocaram aumento significativo na ARNA em ratos WKy.

3. OBJETIVO

A partir dos resultados existentes na literatura podemos supor que a diminuição na ARNA, em resposta ao aumento da pressão pélvica renal ou à administração de bradicinina, em ratos SHR pode estar relacionada: 1) ao decréscimo no conteúdo de SP nas terminações aferentes, 2) as alterações na expressão ou função dos receptores NK_1 e 3) ao decréscimo no conteúdo de CGRP nas terminações aferentes. Tais alterações hipotéticas podem ocorrer isoladas ou simultaneamente. Além disso, sabemos que a influência da atividade neural renal, sobre a manipulação tubular de sódio, tem papel preponderante no balanço hidro-salino. Cabe ainda, a análise da expressão e localização de SP, NK_1 e CGRP em GRDs as quais poderiam estar modificadas como fatores causais ou como conseqüência da elevação pressórica em SHR. No presente projeto pretendemos estudar o papel da inervação renal durante o desenvolvimento da hipertensão arterial em ratos SHR. Desta forma avaliaremos, tanto em ratos SHR quanto em ratos WKY, a expressão, distribuição e possível translocação celular de CGRP, SP e seu receptor NK_1 através de técnicas de Western Blot e imunocitoquímica em cortes de tecido renal e de GRDs, com utilização de microscopia de luz e confocal a laser e de imunocitoquímica ultraestrutural.

Com a finalidade de avaliar a possível relação entre a atividade neural renal e o balanço hidro-eletrolítico, o presente trabalho propõe:

- Avaliar a influência da atividade neural renal sobre a manipulação tubular de sódio em ratos Okamoto-Aoki jovens e adultos.
- Investigar a participação de CGRP, SP e seu receptor NK_1 na gênese e manutenção da hipertensão arterial.

3. ARTIGOS

3.1. ARTIGO I

Early altered renal sodium handling determined by lithium clearance in spontaneously hypertensive rats (SHR): Role of renal nerves

Running Title: Urinary Sodium Excretion and Kidney Denervation in SHR

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ABSTRACT

The mechanism by which blood pressure rises in the SHR strain remains to be elucidated. Since the long-term changes in renal sodium tubule handling associated with genetic hypertension have not been examined in detail, we hypothesized that SHR hypertension development may result from sustained renal sympathetic nerve overactivity and consequently decreased urinary sodium excretion. To test this hypothesis, we assessed renal sodium handling and cumulative sodium balance for 10 consecutive weeks in unanesthetized renal-denervated SHR and their age-matched normotensive and hypertensive controls. The present investigation shows that SHR excreted less sodium than Wistar-Kyoto (WKy) rats during the initial 3-week observation period ($p < 0.05$). This tendency was reversed when SHR were 10-wk old. Fractional urinary sodium excretion (FE_{Na}) was significantly lower in 3 and 6-wk-old SHR when compared with the WKy age-matched group, as follows: $SHR_{3\text{-wk-old}}$: 0.33 ± 0.09% and $WKy_{3\text{-wk-old}}$: 0.75 ± 0.1% ($P < 0.05$); $SHR_{6\text{-wk-old}}$: 0.52 ± 0.12% and $WKy_{6\text{-wk-old}}$: 0.83 ± 0.11%. The decreased FE_{Na} in young SHR was accompanied by a significant increase in proximal sodium reabsorption (FEP_{Na}) compared with the normotensive age-matched control group ($P < 0.01$). This increase occurred despite unchanged creatinine clearance (CCr) and fractional post-proximal sodium excretion ($FEPP_{Na}$)in all groups studied. The decreased urinary sodium excretion response in SHR up to the age of 6 weeks was significantly eradicated by bilateral renal denervation of $SHR_{3\text{-wk-old}}$: 0.33 ± 0.09% and $SHR_{6\text{-wk-old}}$: 0.52 ± 0.12% to $Dx_{SHR\ 3\text{-wk-old}}$: 1.02 ± 0.2% and $Dx_{SHR\ 6\text{-wk-old}}$: 0.94 ± 0.2% ($P < 0.01$), in renal denervated rats. The current data suggest that neural pathways may play an instrumental role on renal sodium reabsorption as result of sustained sympathetic nervous system overexcitability.

Key words: Arterial hypertension, SHR, kidney denervation, natriuresis and lithium clearance.

INTRODUCTION

It has been postulated that the kidneys play a pivotal role in the pathogenesis of essential hypertension as consequence of a primary defect in kidney function and/or renal hemodynamics that promote retention of sodium (Strazzullo et al., 2003; Frey et al., 2000). Although the precise mechanism by which blood pressure rises in the SHR strain remains to be elucidated, renal control of the fluid and electrolyte balance is thought to play a dominant role in the long-term control of arterial blood pressure. Sodium metabolism disturbances appear to be important in the pathogenic process in the Okamoto-Aoki strain of spontaneously hypertensive rats (SHR), since chronic consumption of excess sodium increases, whereas sodium restriction generally attenuates the hypertension in this species (Louis et al., 1971; Aoki et al., 1972). In addition, cross-transplantation studies indicate that a defect in renal function plays an important role in determining the level of arterial pressure (Bianchi et al., 1974). Previous balance studies (Oparil, 1987) examining urinary sodium excretion throughout 3 to 7-week periods provide evidence of renal dysfunction in young genetically hypertensive rats of the Milan strain (MHS), SHR and stroke-prone sub-strain of SHR. These studies show decreased urinary salt and water excretion than in pair-fed age-matched normotensive Wistar Kyoto (WKy) rats. Similar results were reported by Herlitz et al. (1979) for 7-week-old SHR as compared with normotensive Wistar rats; sparse data were presented for the appropriate genetic control, WKy.

The role of CNS in the control of blood pressure and hydrosaline homeostasis has been remarkably demonstrated by several studies (Brody and Johnson, 1980; Gontijo et al., 1992; McCann et al., 1997; DiBona, 2000). The mechanisms underlying this phenomenon are currently

still not well understood but may be related to renal sodium handling. There is evidence implicating an important role of renal nerves in the pathogenesis of many experimental models of hypertension (Oparil, 1987; Kline,1987; Xavier et al., 2000; Strazzullo et al., 2003). We have recently shown a role of sympathetic nerve activity in arterial blood pressure and renal function changes caused by the chronic administration of nitric oxide synthase inhibitors (Xavier et al., 2000). There are reports that neural stimulation elicits enhanced renal release of norepinephrine (Collis et al., 1980) and that renal norepinephrine turnover is elevated in young SHR (Patel et al., 1981). SHR kidneys exhibit a rightward shift of their pressure-natriuresis curve compared with normotensive WKy rats (Kubota et al., 1993). The larger depressor and natriuretic response in young SHR compared with WKy may reflect differences in efferent renal nerve activity, which is reported to be higher in SHR as young as 5 weeks (Judy and Farrel, 1980). Further evidence suggestive of strain differences derives from pharmacological studies demonstrating that isolated perfused kidneys of 4 to 10-week old SHR exhibit exaggerated vascular response to electric stimulation of the renal nerves and infusion of vasoconstricting substances (Collis et al., 1980; Berecek et al., 1980).

In the present study, we assessed renal sodium handling and cumulative sodium balance in SHR (aged 3 to 12 weeks after weaning) for 10 consecutive weeks, comparing them with age-matched WKy. Since the long-term changes in renal sodium tubule handling associated with genetic hypertension have not been examined in detail, we hypothesized that SHR hypertension development may result, at least in part, from sustained renal sympathetic nerve overactivity, consequently promoting an age-dependent decreased segmental tubule sodium excretion. To test this hypothesis, we investigated during 10 weeks (3 to 12 weeks after weaning) tubular sodium

handling effects, evaluated by lithium clearance, in unanesthetized, unrestrained renal-denervated rats and their sham-operated appropriate WKy controls.

MATERIALS AND METHODS

The general guidelines established by the Brazilian College of Animal Experimentation (COBEA) were followed throughout the investigation. Two long-term metabolic balance studies were conducted on age-matched, male offspring of sibling-mated SHR and randomly outbred WKy. Our local colonies originated from breeding stock supplied by CEMIB/Unicamp, Campinas, SP, Brazil. Immediately after weaning at 3 weeks of age, two groups of SHR (n=10) and WKy (n=10) were maintained under controlled temperature and lighting conditions, with free access to tap water and standard rat laboratory chow (Purina rat chow: Na content: 135 ± 3 μEq/g; K content: 293 ± 5 μEq/g) and studied from 3 to 12 weeks of age. In the renal bilaterally-denervated group (Dx_{SHR}, n=8), performed prior to the start of the entire 10-week metabolic studies, the animals were anesthetized and both kidneys were exposed through dorsal abdominal incisions and surgically denervated with the aid of a stereomicroscope. Denervation was accomplished by cutting all visible nerves along the renal artery and by stripping the connective tissue passing next to and along the course of the renal artery and vein. Immediately thereafter, the renal vessels were surrounded with cotton swabs previously soaked in 10% (v/v) phenol diluted in absolute ethanol (Xavier et al., 2000; Furlan et al., 2003). Observations were made of rats individually housed in metabolic cages located in a room with controlled temperature, 12-h photoperiod and humidity. Rats in each group were fed a uniform amount of pellet chow on a given day. The animals had free access to tap water throughout the 10-week observation period. Food and water consumption were determined daily and twice-weekly (subsequently normalizing for body weight), respectively, and body weight was recorded once a week. Urine was collected

twice-weekly from each metabolic cage, drained into a volumetric cylinder containing mineral oil. Tail arterial pressure was estimated each week, one day before the renal test, in conscious restrained rats by the tail-cuff method, using an electrosphygmomanometer (Narco Bio-System, Austin, TX, USA). This indirect approach permits repeated measurements with a close correlation (correlation coefficient = 0.975) compared to direct intra-arterial recording (Lovenberg, 1987). Sodium intake (mM/week) was determined from the daily consumption of chow and sodium content. Fractional sodium excretion (%) was computed as a percentage of dietary sodium excreted in the twice-weekly collected urine. Urinary sodium excretion (mM/week. 100-g body weight) was determined as the average of two collected samples per week and then normalized for body weight.

The renal function tests were performed on the last day of 3, 6 and 12 weeks of age in unanesthetized, unrestrained SHR and WKy rats. In order to evaluate the influence of renal denervation on tubular sodium handling, the rats were randomly assigned to one of three separate groups (SHR, WKy and Dx_{SHR}). Fourteen hours before the renal test, 60-mmol LiCl/100 g body weight was given by means of gavage. The rats were subsequently individually housed in metabolic cages with free access to tap water but no food. After an overnight fast, each animal received a load of tap water by gavage (5% of the body weight), followed by a second load of the same volume one-hour later. Thirty minutes after the second load, spontaneously voided urine was collected over 120 min into a graduated centrifuge tube. At the end of the experiment, blood samples were drawn through the tail vein or cardiac puncture in anesthetized rats and urine and plasma samples were collected for analysis.

Plasma and urine sodium, potassium and lithium concentrations were measured by flame photometry (Micronal, B262, São Paulo, Brazil), while creatinine concentration was determined

spectrophotometrically (Instruments Laboratory, Genesys V, USA). The results are reported as means ± SEM per 100-g body weight. Renal clearance (C) was calculated by a standard formula (C= UV/P) using the plasma creatinine and lithium levels for each period. Creatinine clearance was used to estimate the glomerular filtration rate and lithium clearance (Cli^+) was used to assess proximal tubule output. Fractional sodium ($FENa^+$) and potassium (FEK^+) excretion was calculated as CNa^+/CCr and CK^+/CCr, respectively, where CNa^+ and CK^+ are the ion clearances and CCr is the creatinine clearance. Fractional proximal ($FEPNa^+$) and post-proximal ($FEPPNa^+$) sodium excretion was calculated as CLi^+/CCr x 100 and CNa^+/CLi^+ x 100, respectively (Gontijo et al., 1992; Xavier et al., 2000; Furlan et al., 2003). All data are reported as means ± SEM. Data obtained over time were analyzed using appropriate ANOVA. *Post hoc* comparisons between selected means were done with Bonferroni's contrast test when initial ANOVA indicated statistical differences between experimental groups. Comparisons envolving only two means within or between groups were done using a Student'*t* test. A p value < 0.05 was considered to indicate significance.

RESULTS

Metabolic balance data for SHR and WKy fed the standard chow are summarized in Fig. 1. As shown in Fig. 1, tail arterial pressure (mmHg) in SHR was significantly higher than in WKy from 5 to 12 weeks of age. During the study, SHR pressure increased from 118 ± 11 mmHg to 192 ± 9 mmHg as compared with a slower rise from 106 ± 7 mmHg to 119 ± 12 mmHg in WKy. The tail arterial pressure in SHR appeared to reach a plateau after 11 weeks of age. Between 10 and 12 weeks, tail arterial pressure averaged 180.6 ± 8 mmHg in SHR and 117 ± 10 mmHg in WKy ($p < 0.01$). Initial body weight was slightly but significantly lower in SHR (table

1). The continuous increased blood pressure in SHR was blunted and significantly reduced by bilateral renal denervation ($P < 0,025$) (see Fig 1) over a 5-wk period (between the age of 3-wk and 8-wk old). This attenuated blood pressure was associated with significant increase in urinary sodium excretion and fall in proximal sodium reabsorption (see bellow). SHR grew less rapidly over the observation period, and significant differences were observed after the age of 12 weeks, though weight gain was uniform for each strain.

Data on sodium balance are presented also in Fig. 1 and Table 1. In general, food intake and therefore sodium intake were similar, when normalize by body weight, in SHR and WKy, during the investigation. The present study shows that SHR excreted less sodium than WKy during the initial 3-wk observation period ($p < 0.05$). This tendency was reversed when SHR were 10, 11 and 12 weeks old. Of particular interest is the observation that the reduced excretion by SHR during the initial 3-wk occurred while sodium ingestion did not appreciably differ between strains. This is further highlighted by the significant differences in fractional sodium excretion during the same time period (Fig. 1). As a result of the relative retention of sodium, SHR inhibited a greater cumulative sodium balance through the age of 7 weeks. These differences were statistically significant whether expressed in absolute terms or per 100 g of body weight. The fractional sodium excretion in SHR and WKy appeared to plateau after a 7-wk follow-up. Cumulative sodium balance did not statistically differ between strains after 9 weeks of age.

Figure 1 also shows the results for SHR subjected to bilateral renal denervation at 3 weeks of age. Renal bilateral phenol denervation significantly prevented tail arterial blood pressure increase up to the 8^{th} week of observation when compared with the non-denervated SHR group ($p < 0.01$). There were no significant differences between the daily solid rat chow, body weights,

plasma sodium, potassium and lithium levels (Table 1) and, sodium intake (Fig. 1) in the SHR renal bilaterally denervated rats compared with the other groups. Kidney denervation in SHR also resulted in a transient (up to the 6th week) enhanced absolute and fractional urinary sodium excretion compared with WKy and SHR non-denervated animals.

The data for renal function over the 12-wk period are summarized in Fig. 2. Urinary flow rates (data not included) and the glomerular filtration rate estimated by CC_r did not significantly differ among the groups during the renal tubule sodium handling studies (Fig. 2). Fractional urinary sodium excretion (FE_{Na}) was significantly lower in 3 and 6-wk-old SHR when compared with the WKy age-matched group, as follows: $SHR_{3\text{-wk-old}}$: 0.33 ± 0.09% and $WKy_{3\text{-wk-old}}$: 0.75 ± 0.1% ($P < 0.05$); $SHR_{6\text{-wk-old}}$: 0.52 ± 0.12% and $WKy_{6\text{-wk-old}}$: 0.83 ± 0.11% ($P < 0.2$). The decreased FE_{Na} in young SHR was accompanied by significant decrease in proximal sodium excretion (FEP_{Na}) compared with the normotensive age-paired control group ($P < 0.01$). This increase occurred despite unchanged C_{Cr} and $FEPP_{Na}$ (Fig. 2). The decreased urinary sodium excretion response in SHR up to 6-wk of age was significantly eradicated by previous bilateral renal denervation of $SHR_{3\text{-wk-old}}$: 0.33 ± 0.09% and $SHR_{6\text{-wk-old}}$: 0.52 ± 0.12% to $Dx_{SHR\ 3\text{-wk-old}}$: 1.02 ± 0.2% and $Dx_{SHR\ 6\text{-wk-old}}$: 0.94 ± 0.2% ($P < 0.01$), in renal denervated rats (see Fig.2). This consistently enhanced FE_{Na} produced by renal denervation was followed by significant increase in proximal sodium rejection compared with the undenervated rats. The renal natriuretic responses for both strains, confirming previous research, were not altered and similar in all studied groups at 12 weeks of age. Likewise, CCr, post-proximal sodium handling and kaliuresis were unaffected at any time of the present investigation (Fig. 2).

DISCUSSION

The present study was designed to evaluate the influence of bilateral renal denervation activity on renal urinary sodium handling determined by lithium clearance in young and adult Okamoto-Aoki rats compared with appropriate age-matched WKy control subjects. Also, our findings demonstrate that young SHR developing hypertension while maintained on standard normal sodium diet retain, up to the 6th week of life, more sodium than age-matched WKy rats. The present investigation also confirmed previous research showing pronounced differences in the renal natriuretic response between 3 and 6-wk-old bilateral renal denervated and non-denervated SHR during the development of hypertension. Renal denervation in SHR produced natriuresis and significant increases in proximal fractional urinary sodium rejection, indicating a direct tubular effect. These results suggest that a neural activity effect on renal proximal tubule sodium reabsorption may be important in the pathogenesis of hypertension in SHR strain. Moreover, the significant differences in urinary sodium excretion and higher retained sodium in these hypertensive animals between 3 and 7-wk of age than age-matched WKy strain can be attributed to renal abnormal mechanisms. In contrast, as the animals matured and arterial blood pressure rose above 150 mmHg after 7 weeks of age, urinary sodium excretion normalized in 12-week-old SHR.

Similarly, in the balance study by Bianchi et al. (1975), young MHS rats exhibited a period of greater positive sodium balance early during the development of hypertension than normotensive controls. As in our study, after the MHS were 8 weeks old, no differences in sodium balance were evident. Young Stroke-prone Spontaneously Hypertensive Rats (SHRSP) also have reduced fractional urinary sodium excretion (Dietz et al., 1978). Previously, Herlitz et al. (1979) reported that 7-week-old SHR excrete less sodium in the urine than normotensive

Wistar rats. In that research, although the authors state that WKy, the appropriate genetic control, behaved as normotensive Wistar rats, no data were presented for WKy.

Interpretation of research on urinary sodium excretion in adult hypertensive rats is puzzling by the interdependency of renal salt excretion and increased arterial pressure. Several reports indicate that basal rates of ion excretion are similar in WKy and SHR older than 12-wk of age with established hypertension (Roman and Cowley, 1985; Harrap, 1986; Hall et al., 1996). However, when renal perfusion pressure was reduced to the range observed in the normotensive strain, urinary sodium excretion in SHR was reduced (Roman and Cowley, 1985). The present and previous studies indicate that SHR kidneys require higher arterial pressure than kidneys of normotensive rats to excrete the same amount of salt under basal conditions. The present findings were consistent with this view. When young SHR had relatively low blood pressure, they excreted less sodium than age-matched WKy rats; after the arterial pressure rose, beyond 12th weeks of age, the sodium balance did not differ between strains. Thus, sodium retention could contribute to the development of hypertension by interacting with a variety of mechanisms such as vasoconstriction (Sofola et al., 2002), effective extracellular volume expansion (Schafer, 2002) or hyperactivity of the sympathetic nervous system (Strazzullo et al., 2001). However, the mechanisms by which sodium retention occurs in young genetically hypertensive rats remain unknown. Evidence supporting functional impairment of glomerular vasculature associated with reduced glomerular filtration rate and renal blood flow as well as in potassium metabolism is provided by other studies (Dilley et al., 1984; Zhou and Frohlich, 2001). However, we did not observe in the present investigation significant changes in the CCr, plasma K levels and urinary potassium excretion, suggesting that enhanced blood pressure has a more direct tubule

antinatriuretic effect in the proximal segment than at the sodium-potassium exchange site, such as confirmed in the present sodium handling study.

Over the last 2 decades, genetic studies have provided important clues about the nature of inherited functional defects in renal sodium handling that cause an increase in blood pressure. Monogenic forms of hypertension have been described that are caused by well-characterized mutations, most often associated with major alterations in the rate of renal tubular sodium reabsorption (Wilson et al., 2001). All of these mutations together, however, probably account for less than 1% of the prevalence of human hypertension. The bulk of evidence suggests that most often hypertension is the result of multiple lifestyles, metabolic and genetic interaction rather than the consequence of an isolated single gene abnormality. Several allelic variants of candidate genes for hypertension have been detected that are associated with higher blood pressure levels, and the number of these susceptibility genes is expected to grow considerably in the future. It is important to note that the great majority of these genes encode for proteins either directly involved with sodium transport through the renal tubular epithelia or with the endocrine and paracrine regulation of renal tubular sodium handling (Zhang et al., 1998).

The influence of the sympathetic nervous system on renal function in animals during maturation is a relatively unexplored field of research. Results of several investigations suggest that the involvement of the sympathetic nervous system in the pathogenesis of genetic hypertension may be mediated, at least in part, by its influence on renal function. Thus, an elevated efferent renal adrenergic tone could promote urinary retention of salt by eliciting arteriolar constriction and/or enhancing tubular reabsorption. Electric stimulation of the renal nerves in acute experiments enhances sodium reabsorption, particularly in the proximal convoluted tubule (DiBona, 2002), and efferent renal nerve traffic as assessed by multifiber and

single unit activity (Judy and Farrel, 1980) is elevated in SHR beyond the age of 8 weeks. Unfortunately, such recordings have not been made with younger SHR. Electrical stimulation of the renal nerves at low frequency or intrarenal infusion of norepinephrine is capable of producing hypertension (Collis et al., 1980; Patel et al., 1981) by increasing sodium reabsorption in the proximal tubule and the loop of Henle, effects that are independent of changes in renal hemodynamics (DiBona and Kopp, 1997; DiBona, 2000). Conversely, the authors and others (Rudd et al., 1986; Oparil, 1987; Kline,1987) have shown that bilateral renal denervation in 3- to 8-week-old SHR delays the development of hypertension for approximately 3 weeks, associated with reduced sodium reabsorption by the proximal tubule, loop of Henle, and distal convolution. The most interesting, and novel, finding of the present research shows that natriuresis is associated with an increased fluid delivery from the proximal tubule and incompletely compensated by more distal nephron segments. The enhanced fractional sodium excretion during this 3-week period provides evidence of a functional relation between the renal nerve activity and attenuated sodium excretion in SHR developing hypertension. Furthermore, the subsequent rise in arterial pressure up to the 8^{th} week was associated with renal re-innervation and increases in norepinephrine content, an index of renal reinnervation (Oparil, 1987; Kline,1987).

The present study confirms that natriuretic response to bilateral renal denervation during 3-weeks in young euvolemic SHR is related to a decreased proximal tubular reabsorption. To explain these findings we may refer to previous studies showing that nerve fiber distribution may differ between hypertensive and normotensive Okamoto-Aoki strains. Although renal cortical tissue is more completely innervated at an early age in SHR, the anatomical density is similar in SHR and WKy by 2-wk of age (Gattone et al., 1990). Another explanation for the strain difference in renal Dx_{SHR} response may concern intrarenal prostaglandin metabolism. It has been

reported that WKy kidneys have diminished prostaglandin synthesis activity and enhanced 15-hydroxyprostaglandin dehydrogenase activity relative to SHR (Limas and Limas, 1977). Barber et al. (1986) observed that inhibition of prostaglandin synthesis blunts natriuresis following renal denervation in adult rats.

The remarkable findings of the present investigation suggest that renal nerve (Fig. 2) associated with a rise of proximal sodium reabsorption at least partly mediate the increased arterial blood pressure in genetic hypertensive strain. Although the rationale for renal denervation has generally been to interrupt sympathetic nerve activity directed to the kidney, denervation of the renal plexus also deprives the kidney of its sensory enervation. Selective renal afferent nerves may have markedly widespread effects on the renorenal sympathetic reflexes and urinary sodium excretion (DiBona and Kopp, 1997; Gontijo et al., 1999). Since our experiments were not specifically designed to distinguish between the effects of efferent or afferent renal denervation, we cannot discount an influence of afferent renal nerves on our results. In addition, we also cannot rule out the possibility that several humoral factors may be involved in mediating the decreased natriuresis observed in the present research

Perspectives

Although the precise mechanism responsible for the subsequent attenuated natriuretic response observed in young SHR is still unclear, the current data led us to hypothesize that efferent neural pathways may play an instrumental role on renal sodium reabsorption as a result of significant and sustained sympathetic nervous system overexcitability. Speculatively, it seems interesting to suggest that perhaps one of the efferent nerve signal defects may result in inability of renal tubules to handle the hydro-electrolyte balance, consequently causing development of arterial hypertension.

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Table 1 – Body weight as related to age, sodium intake, serum sodium, potassium and lithium levels in spontaneously hypertensive (SHR) and strain normotensive (WKy) rats compared with bilateral renal denervated SHR (Dx_{SHR}) fed a standard diet. The data are reported as the means ± SEM. * P≤ 0.05 *vs* WKy (Student'*t* test).

Groups	Na^+ (mM)	K^+ (mM)	Li^+ (μM)	Body weight (g) 3-wk	12-wk	Sodium Intake (mmol/wk/100 g)
WKy (n=10)	138 ± 2.6	4.3± 0,6	85 ± 19	32 ± 3.8	262 ± 15	12.7 ± 2.3
SHR (n=11)	142 ± 3.1	4.1 ± 0,4	79 ± 15	26 ± 2.6*	180 ± 13*	11.8 ± 1.9
Dx_{SHR} (n= 8)	141 ± 3.3	4.1 ± 0,9	92 ± 22	25 ± 2.7*	186 ± 15*	13.1 ± 2.7

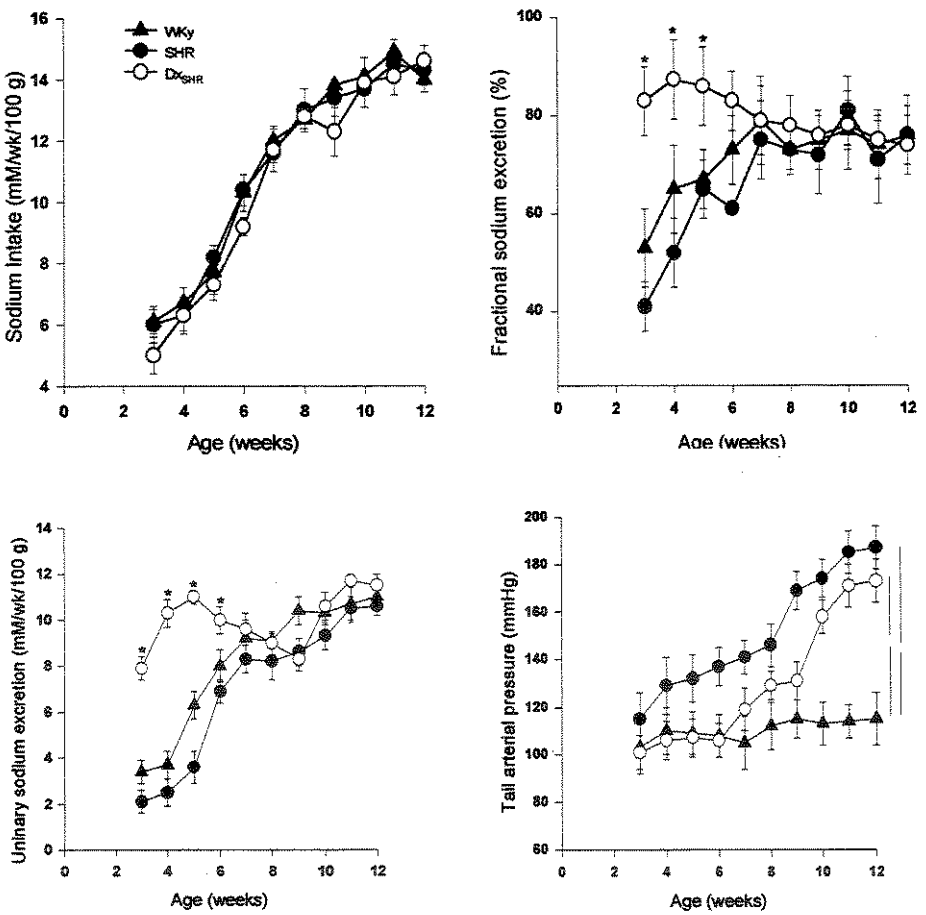

Figure 1- Sodium intake, urinary sodium excretion, fractional sodium excretion and tail arterial pressure related to age in SHR, WKy and Dx_{SHR}. The data are reported as the means ± SEM. * P≤ 0.05 *vs* WKy over time data (ANOVA and Bonferroni's contrast test). See Results for statistical analysis details.

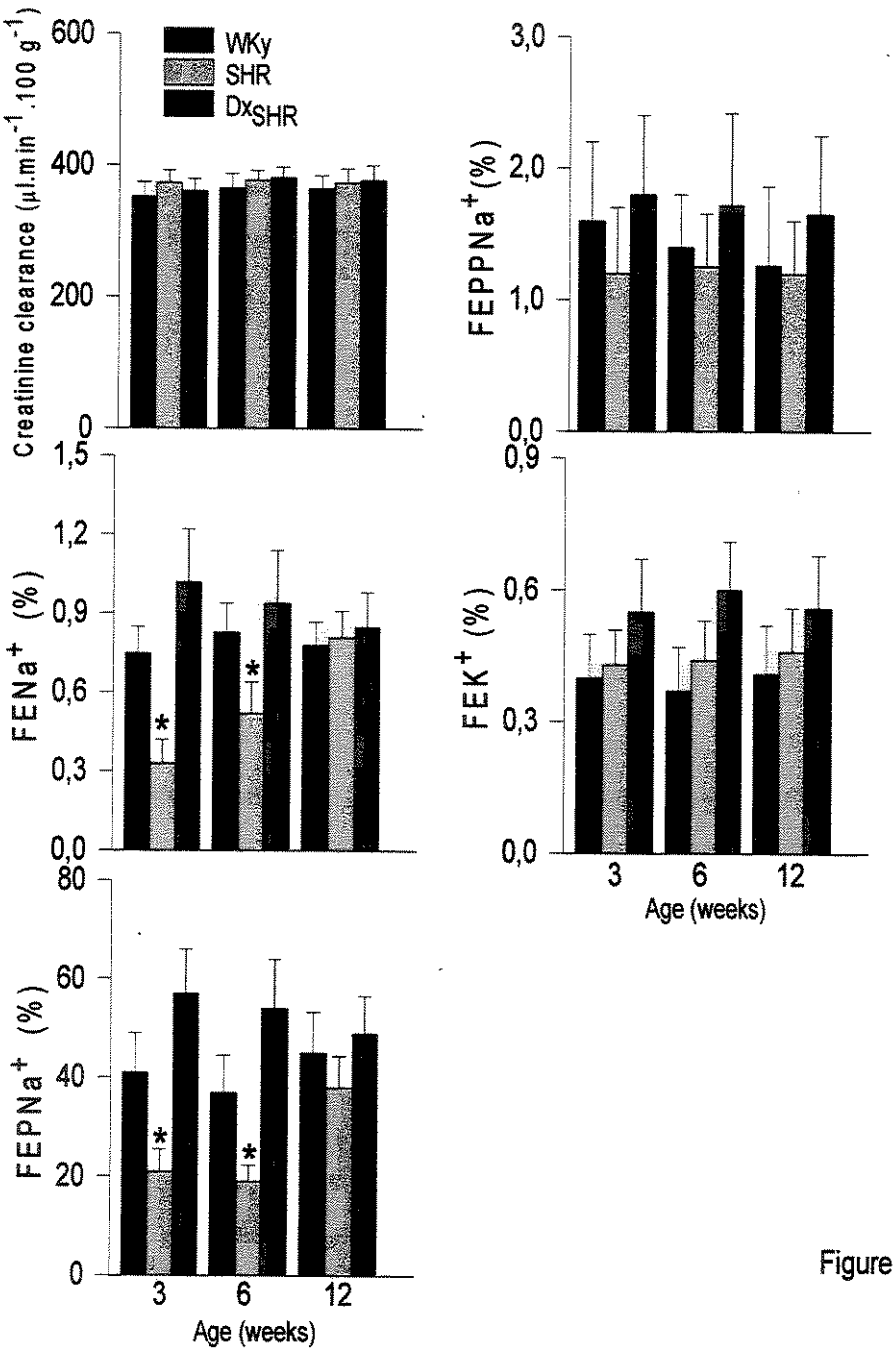

Figure 2

Figure 2 - Creatinine clearance (C_{Cr}), fractional sodium excretion (FE_{Na}), proximal (FEP_{Na}) and post-proximal ($FEPP_{Na}$) fractional sodium excretion and fractional potassium excretion (FE_K) related to age in SHR, WKy and Dx_{SHR}. The data are reported as the means ± SEM. * $P \leq 0.05$ *vs* WKy (Student'*t* test). See Results for statistical analysis details.

3.2. ARTIGO II

NUCLEAR LOCALIZATION OF SP, CGRP AND NK_1R IN THE DORSAL ROOT GANGLIA SUBPOPULATION NEURONS IN RATS

Short Running Title: Sub-cellular location of NK_1R, SP and CGRP.

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SUMMARY

Signals generated by pelvic afferent nerves in response to chemo- or mechanoreceptor stimulation are transmitted from peripheral processes of dorsal root ganglia (DRG) neurons to their central terminals in the dorsal horn of the spinal cord to cause the release of neuropeptides, including SP and CGRP. Although SP and CGRP are co-localized in afferent neurons of DRG where they are synthesized, the precise role of SP and CGRP in many sensorial cells including possible interaction during their synthesis and in their neuromodulation remain unclear. All of the cellular activities of SP are considered to be mediated through interaction with NK_1R located on the cell surface. However, neuropeptides may undergo translocation to the nucleus after internalization. Since SP and CGRP can interact to affect the expression and activity each other, we have investigated the co-localization and subcellular organelles distribution of NK_1R, SP and CGRP in different subpopulations of primary DGR neurons that innervate renal tissue. Our findings therefore provide the first evidence for the presence of NK_1R, SP and CGRP in the nuclei of DGR neural cells. The nuclear localization of NK_1R may reflect translocation from the cell surface or *de novo* synthesis. Since these cells are exposed to circulating SP, ligand-mediated receptor internalization and nuclear translocation is a possibility. The physiological significance of this interaction remains unknown. One possibility is that pelvic sensory neurons may regulate their responses to different stimuli (or to the same stimuli in different activated neuronal subpopulations) by modulating the ratio of CGRP and SP release and/or nuclear NK_1R expression.

Keywords: Tachykinin NK1 receptor; Substance P; CGRP; Dorsal Root Ganglia

INTRODUCTION

Substance P (SP) and calcitonin gene-related peptide (CGRP) are present in the central and peripheral regions of primary afferent sensory neurons (FERGUSON e BELL, 1988; KNIGHT et al., 1991; TAMAKI et al., 1992). The presence of SP- and CGRP-immunoreactive nerves in the aortic arch, carotid sinus region, vagus nerve, nodose ganglion, and nucleus tractus solitarius (NTS) suggests a role for these peptides in chemo- and baroreceptor reflexes (PERNOW, 1983; KUMMER et al., 1989). Sensory and postganglionic autonomic nerves in which several neuroactive substances have been identified innervate the kidney. As with to sensory neurons in several areas of the central and peripheral nervous systems, the renal afferent nerves also contain SP and CGRP, and have been these peptides co-localized in many neurons (HELKE et al., 1980; DONOVAN et al., 1983; KUMMER et al., 1989; KNIGHT et al., 1991; TAMAKI et al., 1992). Based on the neuropeptide content, there are at least four populations of sensory neurons present in renal tissue: two large groups containing either SP or CGRP alone and two small groups containing either both peptides or neither (NIIJIMA, 1975; KNUEPFER e SCHRAMM, 1987). The renal afferent neurons, most of which are unmyelinated, project to the ipsilateral dorsal root ganglia (DRG), mainly T_{10} to L_3 (DONOVAN et al., 1983). Previous studies in normotensive rats have shown that the activation of renal mechanoreceptors (MR) by increasing renal pelvic pressure and of renal chemoreceptors (CR) by renal pelvic perfusion with KCl or hypertonic saline solutions results in increased ipsilateral afferent renal nerve activity (ARNA) associated with a decrease in contralateral efferent renal nerve activity (ERNA), and an increase in urinary flow rate and natriuresis. These findings indicate a contralateral inhibitory renorenal reflex response (MOSS, 1989; KOPP, 1989; KOPP et al 1991; GONTIJO e KOPP, 1994; KOPP et al., 1996; GONTIJO et al., 1999; KOPP et al., 2000).

Substance P, a tachykinin with nanomolar affinity, interacts with specific membrane receptors belonging to the family of G protein-coupled receptors (GPCRs) (PENNEFATHER et al., 2004). Currently, three distinct tachykinin receptors, NK1, NK2 and NK3, have been cloned in different species (YOKOTA et al., 1989; HERSHEY e KRAUSE, 1990; GERARD et al., 1991; TAKAHASHI et al., 1992). NK_1 receptors (NK_1R) are widely distributed in the renal pelvis and brain sites and, have been implicated in the signaling of nociception to the spinal cord (DE KONINCK e HENRY, 1991; PENNEFATHER et al., 2004) and in the modulation of cortical and striatal cell functions (MANTYH et al., 1984). NK_1R mediate most of the inflammatory, immune and mitogenic effects of SP. In addition, SP has been defined as a potent mitogen for several cell types, including smooth muscle (YANG et al., 2002), fibroblasts (KAHLER et al., 1993) and endothelial cells (DANIEL et al., 1986).

CGRP-containing neurons are at least as abundant, if not more so, than substance P-containing neurons in the renal pelvic wall (DONOVAN et al., 1983; KUMMER et al., 1989; KNIGHT et al., 1991; TAMAKI et al., 1992). Approximately 90% of neurons in T_{10}-L_3 of the DRG that are retrogradely labeled by dye injection into the hilus of the rat kidney are immunoreactive for CGRP (SU et al., 1986), whereas only 24% of cat dye-labeled renal afferent neurons are immunoreactive for SP (KUO et al., 1984). CGRP regulates the expression of NK_1R by rat spinal neurons (SEYBOLD et al., 2003) and retards the metabolism of SP (GONTIJO et al., 1999), thereby increasing the amount of SP available for stimulation of SP receptors.

Although these neuropeptides are localized in afferent neurons of DRG where they are synthesized, the precise role of SP and CGRP in many sensorial neurons including possible interaction during their synthesis and in their neuromodulation remains unclear. All of the cellular activities of SP are considered to be mediated through interaction with NK_1R located on the cell surface. After binding to SP, the NK_1R is internalized in the endosomal compartment and

then recycled to the membrane after dissociation from SP (MANTYH et al., 1995). However, neuropeptides and growth factors can undergo translocation to the nucleus after internalization (YANKER e SHOOTER, 1979; JOHNSON et al., 1980; MARCHISIO et al., 1980; RAKOWICZ-SZULCZYNSK et al., 1986; BOUCHE et al., 1987; CHABOT et al., 1988a; CHABOT et al., 1988b; BOIVIN et al., 2003). Since SP and CGRP can interact to affect the expression and activity each other, we have investigated the co-localization and subcellular organelles distribution of NK_1R, SP and CGRP in different subpopulations of primary DRG neurons that innervate renal tissue.

MATERIALS AND METHODS

Wistar-Kyoto rats (WK_Y) 7 weeks old and weighing 100-150g were used in this study. The general guidelines established by the Brazilian College of Animal Experimentation (COBEA) were followed throughout the work. The rats were anesthetized with sodium pentobarbital (30-50 mg kg^{-1}body weight, i.p.) and the level of anesthesia was controlled by monitoring the corneal reflex. The carotid artery was cannulated and the rats were perfused as described below. The rats were not perfused when the tissues were required for western blot and dot-blot experiments.

Immunofluorescence cystochemistry for NK1, SP and CGRP – The rats were anesthetized and perfused by the left carotid with saline containing heparin (2%) for 5 min under constant pressure. This was followed by perfusion with 0.1 M-phosphate buffer (PB; pH 7.4) containing 4% (w/v) paraformaldehyde and 0.1 M sucrose. After the perfusion, the left DRG (T_{13} and L_1) were immediately removed and placed in the same fixative for 1 h, followed by PBS containing 0.1% glycine for 1 h and PBS containing 15% (w/v) sucrose overnight at 4°C. The following day, the DGR were placed in OCT compound cryoprotector (Tissue-tech®), freeze-thawed in liquid

nitrogen and the cut in serial sections (10 μm thick) on a Leica cryostat at –25°C and mounted on silane-coated slides. For immunohistochemistry, the sections were incubated sequentially with: (1) phosphate-buffered saline (PBS) containing 8% fetal calf serum, 2% normal goat serum, 2% milk and 2.5% bovine serum albumin (BSA) for 45 min to minimize nonspecific reactions; (2) rabbit anti-NK_1R antiserum (1:50 dilution; Sigma) or rabbit anti-CGRP antiserum (1:250 dilution; Sigma) or goat anti-SP antiserum (1:600; Santa Cruz), at 4°C overnight; (3) goat anti-rabbit CY2-labeled antibody (1:600 dilution; Jackson ImmunoResearch) or rabbit anti-goat CY3-labeled antibody (1:1000 dilution; Jackson ImmunoResearch) for 2 h at room temperature. After incubation, the sections were rinsed in 0.1 M PBS and cover-slipped with Vectashield anti-fading medium containing DAPI (Vector). The sections were examined with a confocal laser scanning microscope (CLSM, LSM510 ZEISS) using laser beams of 543 and 488 nm (and UV) and appropriate emission filters for CY3 (590-610 nm) and CY2 (510-525 nm) (and DAPI). Digital images were captured using specific software (LSM; Zeiss) and were printed on a color printer. No immunoreactivity was seen in control experiments in which one of the primary antibodies was omitted.

Immunogold transmission electron microscopy for NK1, SP and CGRP - In these experiments, anesthetized rats were perfused via a carotid catheter with 0.1 M PB (pH 7.4) containing 4% (w/v) paraformaldehyde, 0.2% glutaraldehyde and 0.1 M sucrose. After the perfusion, the left DGR (T_{13} and L_1) were immediately removed and immersed in the same fixative for 2 h at room temperature. The samples were rinsed in PBS and placed in PBS containing 0.1% glycine for 1 h. This steps was followed by dehydrating in a graded ethanol series at –20°C and infiltration with Lowicryl K4M in a 1:1 (v/v) mixture with 100% ethanol and then pure resin (60 min each). The samples were stored in pure lowicryl K4M overnight at the same temperature. The resin was

polymerized under indirect, diffuse UV light for 24 h at –20°C, and for a further 24 h at room temperature.

Ultrathin sections (80-90 nm) mounted on nickel grids were treated with 1% BSA in PBS for 10 min to prevent non-specific binding, followed by incubation with primary antibodies overnight. After washing with PBS, the grids were incubated for 2 h with goat anti-rabbit IgG conjugated to 10 nm colloidal gold (1:40; Aurion) or with rabbit anti-goat IgG conjugated with 15 nm colloidal gold (1:40; Aurion) diluted in PBS with 1% BSA. After washing and drying, the sections were double-stained with uranyl acetate and lead citrate for 3 min and 40 s, respectively and were observed and photographed with a LEO 906 transmission electron microscope operated at 60 kV. No immunoreactivity was seen in control experiments in which one of the primary antibodies was omitted.

Quantification and data analysis - Cell counts - The sub-cellular pattern and localization of staining was assessed in ultrathin sections (80-90 nm) processed for immunohistochemistry and observed with a confocal laser scanning microscope (CLSM). In these sections, the immuno-stained cell profiles were easily recognized and counted. The sections were divided into five regions and the profiles within each region were counted. The percentage of cells that were positive with NK_1R, SP and CGRP antiserum in each region was then averaged to give the overall percentage of immuno-stained cells in each section (BOWIE, 1994). All of the sections used for quantification were from experiments in which the immunostaining was considered optimal.

Cell size distribution – To determine the cell size distribution, five DRG sections immuno-stained with NK_1R, SP and CGRP antiserum were selected from three T_{13} DRGs of different rats.

The images were stored and analyzed using the Bio-Rad software specifically designed for CLSM. Briefly, the boundaries of immuno-stained cell profiles were traced manually using a computer mouse and the length, breath and area of each cell calculated automatically. To compare our results with previous quantitative studies on DRG, the cell areas were transformed into cell diameters by assuming that ganglion cells are circular and that the ranges of diameters in the different subpopulations were (in μm): 10-25 μm for small cells; 25-37.5 μm for intermediate cells and 37.5-60 μm for large cells (ALVAREZ, 1991).

Where appropriate, the results were expressed as the mean ± SEM. The percentage data from NK_1R, SP and CGRP-immunoreactive neurons were compared by the Kruskal-Wallis test for repeated measurements. p value < 0.05 indicated significance.

RESULTS

Qualitative analysis of NK_1R, SP and CGRP immunostaining

In preliminary experiments using an indirect immunocytochemical approach and fluorescence microscopy, we sought to determine the sites of NK_1R, SP and CGRP immunestaining in the neuronal subcellular structures. However, since overlapping of the nucleus by the Golgi apparatus could interfere with the analysis, and in order to determine the correct ultrastructural site, we examined ultrathin (< 100nm) optical sections using CLSM. Numerous NK_1R, SP and CGRP immunoreactive cells structures were seen coursing through the ganglia and sometimes emerging from cell bodies.

NK_1R, SP and CGRP immunoreactivity was present in a large number of DRG neuronal subpopulations (Figure 1). Small, intermediate and large neurons showed NK_1 immunoreactivity in the membrane and cytosol, with strong staining in the nuclei (Figure 3). Immunogold

transmission electron microscopy confirmed the localization of NK_1R in the perikaryon and nucleus. In the nuclei, NK_1R were preferentially distributed in clusters associated with heterochromatin. In the cytosol, the receptors were associated with vesicular profiles and ribosomes. As expected, NK_1R were also present in the cell membrane (Figure 3).

In many of the smallest immunoreactive cells (small and intermediate cells) the NK_1R-immunostaining was homogeneous and practically filled the entire perikaryon and nuclei (Figures 2 and 3). This differed from the largest immunoreactive cells, in which, despite intense nuclear staining, there was a sparse and mild granular staining of the cytosol and cell surface (Figure 3). The same pattern of distribution was seen in the satellite and Schwann cells. Myelin and neural fibers also stained for NK_1R (Figures 4 and 5).

In contrast to NK_1R, SP was difficult to detect in a number of DRG neuronal nuclei, and was seen exclusively in cells of intermediate size. In most neurons this tachykinin was located exclusively in the perikaryon (Figures 2, 3, 6A and 7A). Although, the SP immunoreactivity was most clearly seen in the cell perikaryon, SP co-localized with NK_1R in the perikaryon or on the nuclear surface, or periphery in all neuronal body cells (Figures 2, 3 and 6B and C). Thus, the sub-cellular localization and distribution of SP were more heterogeneous than for NK_1R.

CGRP immunoreactivity was also, difficult to detect in a number of DRG cell nuclei (Figure 2), but was found in intermediate and large size cells. Cells with CGRP-positive nuclei were heterogeneously distributed with CGRP-negative cells and with cells showing a very high content of this neuropeptide (Figure 7B, C and D). In most neuronal body cells of the three subpopulations, CGRP immunoreactivity was located exclusively in the cytosol (Figures 2, 3, 6A and 7A). The subcellular co-localization of NK_1R, SP and CGRP showed that DRG neurons had distinct patterns of localization for these neuropeptides relative to SP receptors (Figures 7D and E). Immunogold transmission electron microscopy showed clusters of CGRP crossing the nuclear

envelop, and also showed that in the nucleus CGRP was associated with heterochromatin (Figure 7E).

Quantitative analysis and cell size distribution of NK_1R-, SP- and CGRP-positive cells

The size distribution of NK_1R, SP and CGRP positive cells was determined in 100 nm thick sections. Figure 1 shows an unimodal subpopulation distribution of NK_1R, SP and CGRP immunoreactivity cells that was skewed towards larger cells (intermediate and large diameters). The skewed distribution and the different patterns of immunestaining in the subcellular structures suggested that DGR neurons consist of various subpopulations. The precise relationship between primary afferent function and neurochemically characterized DRG is still unclear (Figures 1A, B and C).

Of the total number of cells stained cells, a small percentage of small neurons were immunoreactive for NK_1R (12.8 ± 1,2%). This percentage increased in large cells (25.7 ± 2.5%) and was significantly ($p=0.01$) higher in intermediate size neurons (61.5 ± 4,2%) (Figure 1A). The percentage of NK_1R-, SP- and CGRP-positive cells distribution did not vary significantly in the different DRG subpopulations. In five ultrathin sections of the DRG, the mean percentage of DRG neuron nuclei with NK_1R, SP and CGRP immunoreactivity was 61.9 ± 5.3%, 5.1 ± 3.4% and 11.4 ± 4.5% respectively (Fig. 1B). As shown in Figure 1C, in subpopulations of rat DGR cells subpopulations there was no correlation between the homogeneous, heavy subcellular distribution of NK_1R immunoreactivity and weak nuclear and surface reactivity and predominantly cytosolic neuropeptide contents.

DISCUSSION

Signals generated by pelvic afferent nerves in response to chemo- or mechanoreceptor stimulation are transmitted from peripheral processes of small DRG neurons to their central terminals in the dorsal horn of the spinal cord to cause the release of neuropeptides, including SP and CGRP, which are often stored in the same large and, dense vesicles (NIIJIMA, 1975; DONOVAN et al., 1983; KNUEPFER e SCHRAMM, 1987; KUMMER et al., 1989; KNIGHT et al., 1991; TAMAKI et al., 1992). The actions of SP are normally mediated via G protein-coupled receptors located in the plasma membrane (PENNEFATHER, 2004). So far, the intracellular existence and subcellular location of NK_1R, SP and CGRP have not been demonstrated. Our findings therefore provide the first evidence for the presence of NK_1R, SP and CGRP in the nuclei of DRG neural cells. The nuclear localization of NK_1R may reflect translocation from the cell surface or *de novo* synthesis. Since these cells are exposed to circulating SP, ligand-mediated receptor internalization and nuclear translocation is a possibility. In the rat central nervous system (CNS), NK_1R localize primarily in the plasma membrane. After internalization of the receptor-peptide complex, the peptide SP is degraded in an acidified cellular compartment and the receptor is recycled to the cell membrane (MANTYH et al., 1995). The endosomal internalization of NK_1R occurs in all parts of the cell where this receptor is normally present, including the dendrites. The pseudo-unipolar neurons in DGR have no dendrites, but our results indicated the presence of this receptor in the membrane projections of the cell body of satellite cells and neurons. Thus, the internalization and structural reorganization that occurs in response to an agonist can alter the interrelations of these cells and the electrophysiological properties of the perikaryon.

There is increasing evidence that growth factor receptors and G protein coupled receptors (GPCR) are present in the nuclear or perinuclear membranes. In addition, receptors for

angiotensin II (AT1) (BOOZ et al., 1992), epidermal growth factor (EGF-R) (CARPENTIER et al., 1986), insulin (VIGNERI et al., 1978), interferon β (KUSHNARYOV et al., 1985), acetylcholine (muscarine receptors) (LIND e CAVANAGH, 1993), nerve growth factor (YANKNER e SHOOTER, 1979), prostaglandin E_2 (PGE_2) (BHATTACHARYA et al., 1999), and opioids (BELCHEVA et al., 1993) have been found in the nucleus. Ligands for these receptors may be derived from the extracellular milieu or synthesized within the cell. Ligand-mediated receptor internalization and translocation to the nucleus has been demonstrated for AT1 (LU et al., 1998) and EGF-R (MARTI et al., 2001). Conversely, the pro-form of transforming growth factor (pro-TGF) may exert a mitogenic effect by interacting with nuclear EGF-R (GRASL-KRAUPP et al., 2002). In contrast, PGE_2 is taken up via prostaglandin transporters after which it acts upon nuclear receptors (GOBEIL et al, 2002). For intracellular NK_1R to be of functional relevance there must also be a source of intracellular ligand. Studies have detected two isoforms of human NK_1R indicating that differential activation of intracellular effectors may be involved in generating the complex biological effects of SP. Additionally, NK_1R of different tissues do not always show the same profile of agonist selectivity (ELLIOTT e IVERSEN, 1987), suggesting the possibility of differential binding sites and/or second messenger pathways for this receptor.

Potential roles for nuclear signaling pathways include the regulation of nuclear transport, gene expression, and nuclear envelope formation. Key components of various signaling pathways are present at the nuclear envelope or within the nucleus itself, supporting the presence of nuclear signaling cascade. Evidence suggests the presence of nuclear heterotrimeric G-proteins (WILLARD e CROUCH, 2000). Nuclear AT1 receptors display guanine nucleotide-dependent ligand binding, indicating the presence of and coupling to heterotrimeric G-proteins (BOOZ et al., 1992). Biophysical and microscopy studies have shown the presence of effectors, including

adenyl cyclase (YAMAMOTO et al., 1998), phosphodiesterase (LUGNIER et al., 1999), diacylglycerol kinase (TOPHAM et al., 1998), phospholipase A_2 (FATIMA et al., 2003), phospholipase C (FAENZA et al., 2000), phospholipase D (BALDASSARE et al., 1997), and phosphatidylinositol 3-kinase (DIDICHENKO e THELEN, 2001). The nuclear envelope consists of inner and outer nuclear membranes; with the luminal space between these membranes forming the nuclear cisterna or perinuclear space (STEHNO-BITTEL et al., 1995). Nuclear membranes contain sarco/endoplasmic Ca^{2+}-ATPase (SERCA)-pumps (ABRENICA e GILCHRIST, 2000) as well as ryanodine-sensitive Ca^{2+}-channels and IP_3-sensitive (GUIHARD et al, 1997) Ca^{2+}-channels. The signaling processes associated with the nuclear envelope include the products of nuclear lipid metabolism, such as 1,2-diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3) (IRVINE, 2002). DG recruits and/or activates protein kinase C to phosphorylate intranuclear proteins while the generation of IP_3 releases Ca^{2+} stored within the nuclear cisterna to increase the nucleoplasmic Ca^{2+} level. Stimulation of PGE2 receptors (BHATTACHARYA et al., 1998) causes an influx of Ca^{2+} into the nuclear cisterna or nucleoplasm. Nucleoplasmic Ca^{2+} regulates key nuclear functions, including gene transcription, apoptosis, gene repair, topoisomerase activation, and polymerase unfolding.

In addition to the regulatory effects mediated by the release of Ca^{2+} into nucleoplasm, the filling status of the nuclear cisterna alters the conformational state of the nuclear pore complex, inhibiting diffusion across the nuclear envelope and hence controlling the transport of molecules between the cytosol and the nucleoplasm (STEHNO-BITTEL et al., 1995). SP and CGRP are 1.5 and 3.8 kDa in size, respectively and, when synthesized by neurons may be translocated across nuclear pore complexes by Ca^{2+}-mediated process. The nuclear localization of NK_1R may reflect the importance of receptors in the plasma membrane for the early response to SP and for long-term interaction with the nucleus. The biological response to insulin is also characterized by early

effects at the cell surface followed by more profound long-term changes in cellular metabolism (LOSTROH e KRAHL, 1974).

Recent studies have evidenced that increased endogenous levels of SP and CGRP and over-expression of NK_1R in the spinal cord occur after peripheral nociceptive receptor activation in conjunction with increased levels of mRNA in primary afferent neurons (SEYBOLD et al., 2003). Thus, gene expression may be modulated by sensory receptors and may contribute to the changes in cellular protein levels (MENARD et al., 1996; POWELL et al., 2000). However, little is known about the transmembrane signals that initiate intracellular signaling that leads to changes in gene expression in DRG neurons. The induction and activation of transcription factors that regulate gene expression occur in DRG as a consequence of peripheral nociceptor stimulation. Changes in the activity of transcription factors indicate that intracellular signaling pathways can mediate changes in protein expression. A potential signaling molecule for regulating the expression of NK_1R by DRG could be released after peripheral afferent nerve activation. Our own studies and other reports showing that CGRP receptors activate an intracellular pathway that increases the expression of NK_1R extend our understanding of the significance of SP and CGRP cotransmission. Both peptides coexist in primary afferent neurons (chemo- and mechanoreceptors) (HELKE et al., 1980; DONOVAN et al., 1983; KUMMER et al., 1989; KNIGHT et al., 1991; TAMAKI et al., 1992). In addition, CGRP can enhance the bioavailability of SP (GONTIJO et al., 1999) and may increase the expression of receptors activated by SP (SEYBOLD et al., 2003). The extent of this interaction is likely to vary in subpopulations of neurons that express NK_1R, SP and CGRP in DRG. The possible regulation of gene expression by CGRP in different subpopulations of DRG neurons also suggests a specific function for these different subgroups of cells and, may extend to other proteins expressed in DRG.

To date, SP-related effects have been thought to be mediated via specific NK_1R located in the plasma membrane. As shown here, this receptor may also be directly bound to the chromatin of some neurons, satellite and Schwann cells in DRG. The mechanisms involved in the of nuclear uptake and binding of SP to chromatin acceptor sites and the events possibly activated by them remain to be determined. For nuclear NK_1R to be of functional relevance, there must also be an intracellular source of ligand. This source may be via uptake of SP from the extracellular milieu and/or production of this neuropeptide by neuronal cells. In this case, the peptide may act intracellularly as an "intracrine" mediator.

Perspectives

The nuclear co-localization of NK_1R, SP and CGRP in DGR sensory neurons provides an anatomical basis for a possible functional interaction between these two neuropeptides. However, the physiological significance of this interaction remains unknown. One possibility is that pelvic sensory neurons may regulate their responses to different stimuli (or to the same stimuli in different activated neuronal subpopulations) by modulating the ratio of CGRP and SP release and/or nuclear NK_1R expression.

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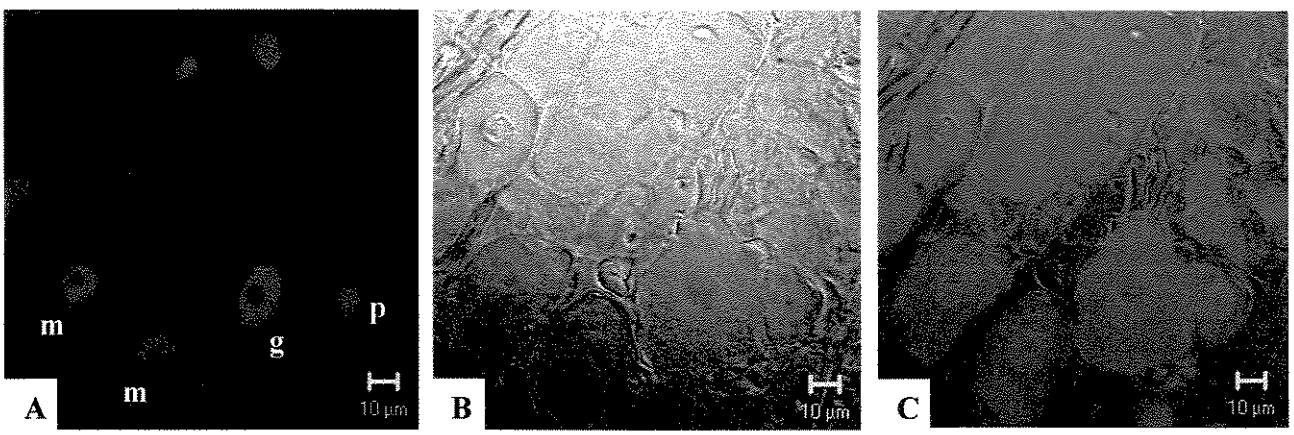
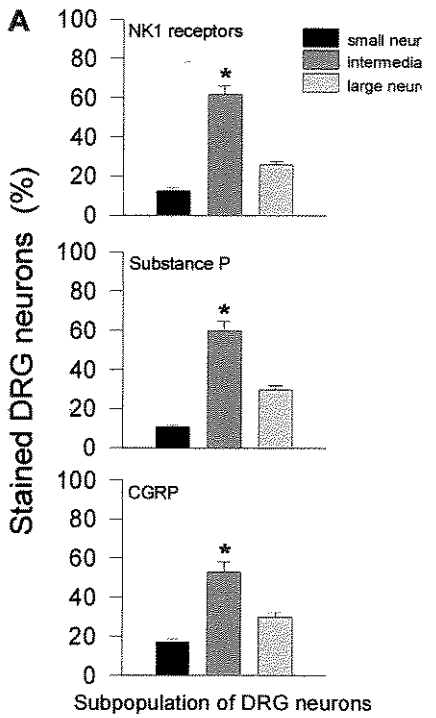
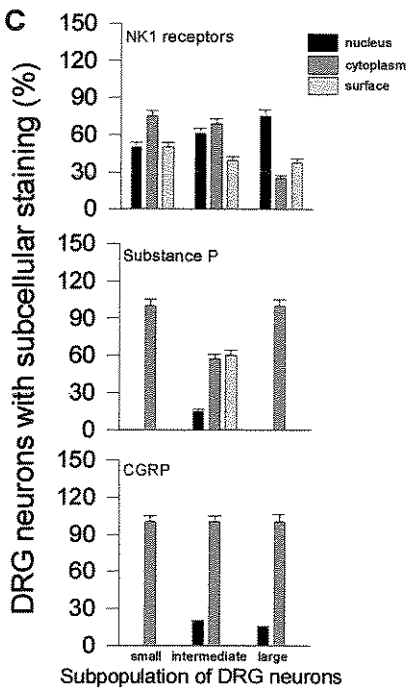


Figure 1. Distribution of NK_1R immunoreactivity in DRG T_{13} neurons as shown by CLSM. (A) shows small (p), intermediate (m) and large (g) perikaryon with predominantly nuclear NK1 immunoreactivity. (B) The same image as in (A) obtained by DIC (differential image contrast) of transmitted light. (C) Images A and B combined.





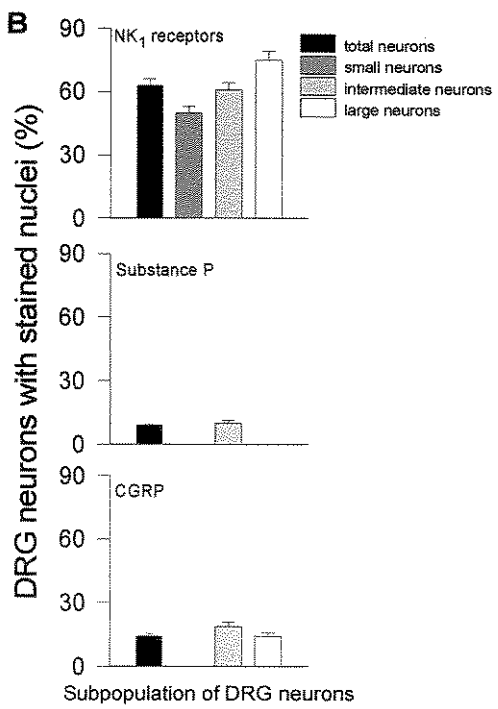


Figure 2. Total number of NK_1-positive neuronal cells and distribution of immunoreactivity according to cell size.

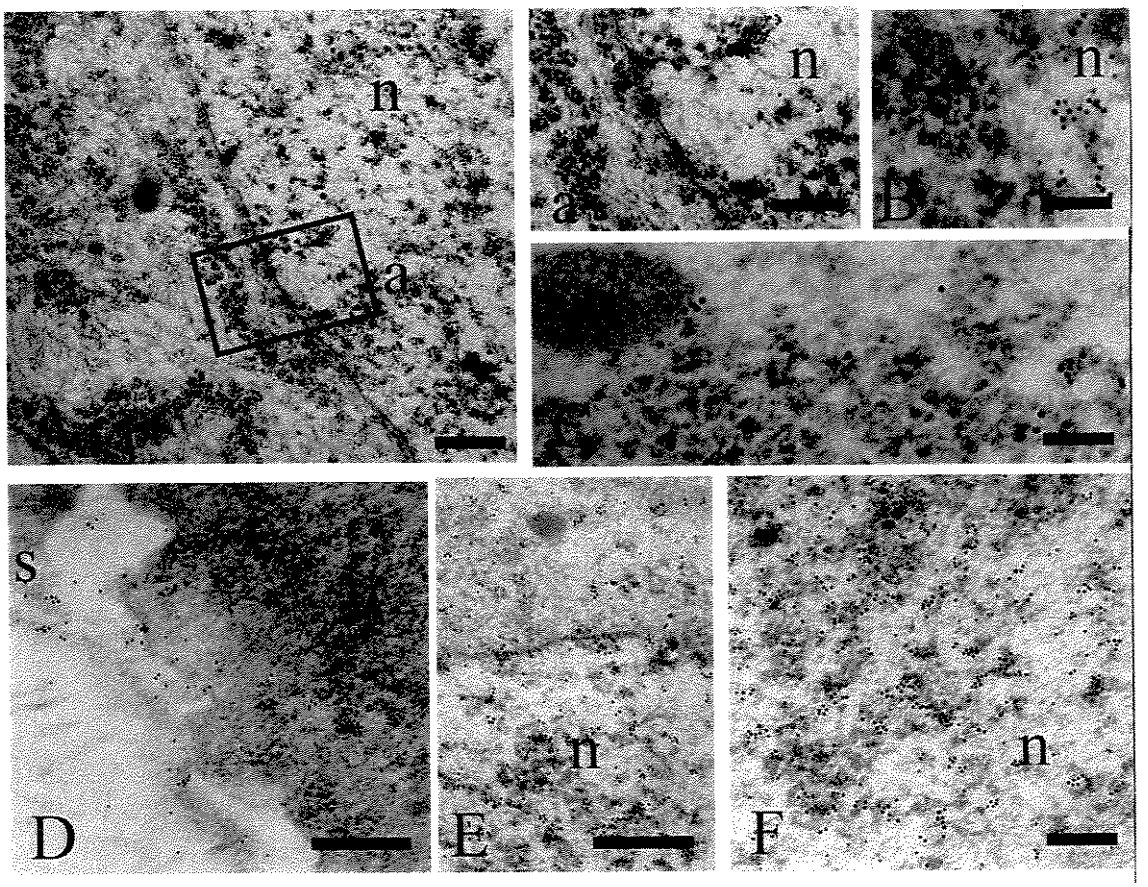


Figure 3. (A) Image of an intermediate size neuron showing cytosolic and nuclear (n) pattern of NK_1R distribution. (a) A detail of the interface between the nucleus (n) and cytosol. (B) Higher magnification of nucleus with NK_1R associated with heterochomatin. (C) Higher magnification of cytosol showing NK_1R localization in vesicles and in association with ribosomes. (D) Detail of a region of contact between a satellite cell and neurons. The NK_1R occur in isolation or in clusters (E and F). (F) A detail of the nucleoplasmic regions (n) shows that NK_1R are associated with regions of heterochromatin. Bars: A, D and E = 0,5 μm; B and C = 0,1 μm; F = 0,2 μm.

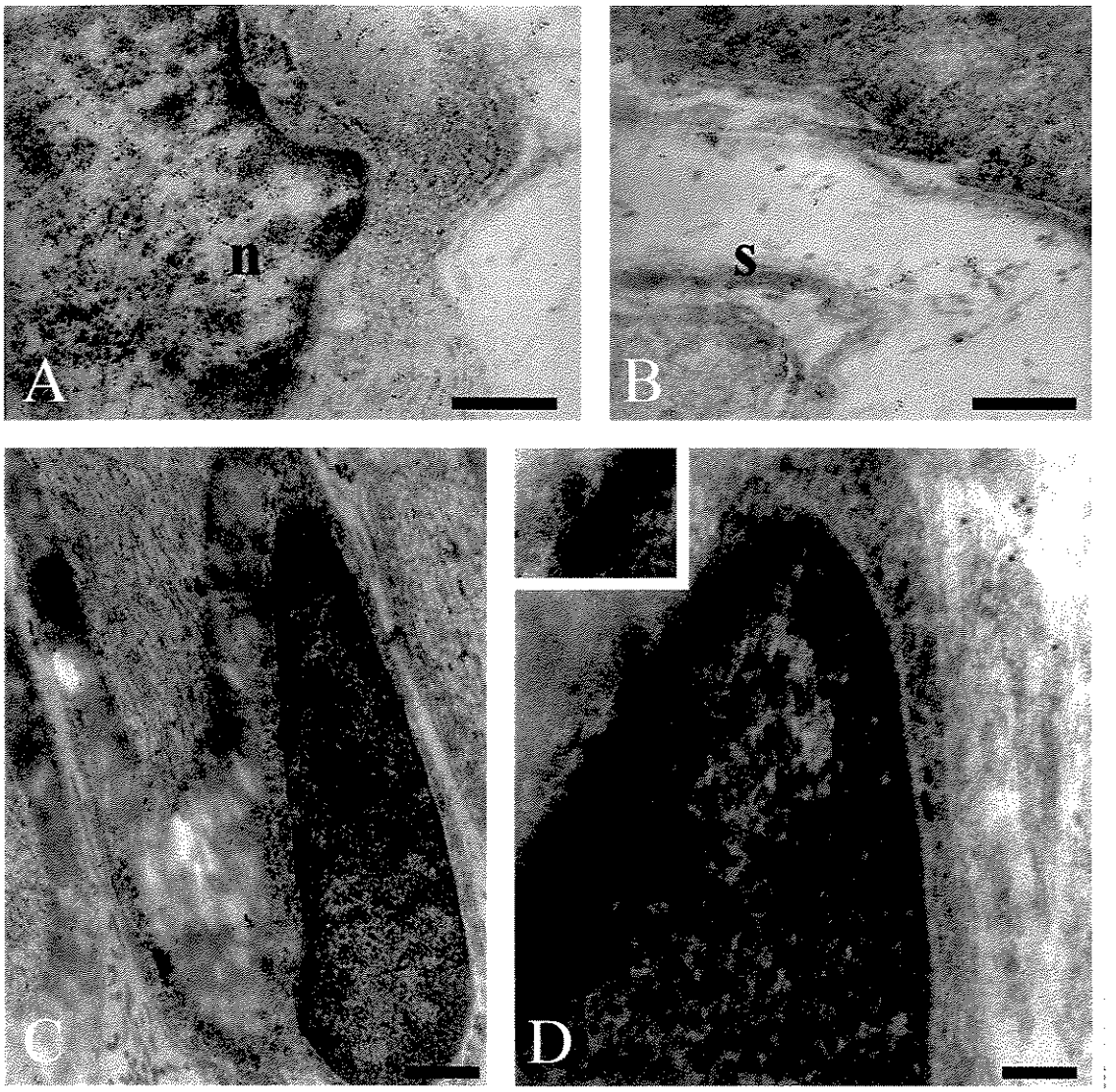


Figure 4. Immunogold transmission electron microscopy showing NK_1R localization in T_{13} DRG cells. (A) A satellite cell showing nuclear and cytosolic localization of NK_1R. Note that in the nucleus (n) this receptor localizes preferentially with heterochromatin. (B) Linear distribution of receptors in a cytosolic projection of a satellite cell (s) between two neurons. (C and D) Schwann cells also content NK_1R as seen in the fibers, myelin, cytosol and nucleus (n) of this cell. The inset (higher magnification) shows a cluster of NK_1R crossing the nuclear envelope. Bars:A, B and C = 0,5 μm; D = 0,1 μm.

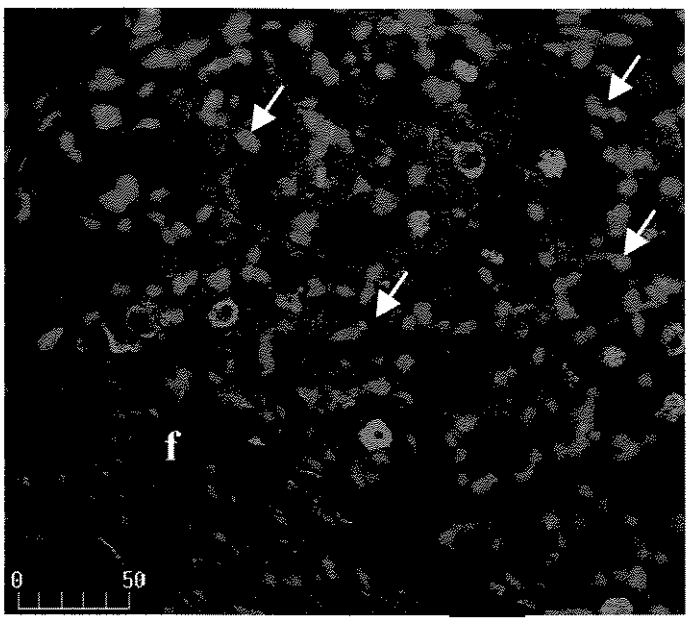


Figure 5. Distribution of NK_1R immunoreactivity in T_{13} DRG cells as show by CLSM. The receptor (green) shows distinct patterns in the three populations of DRG neurons. DNA is labeled with DAPI (red). The receptor is also present in neuron nuclei. The nuclei of satellite cells display yellow fluorescence (arrows) caused by the colocalization of DNA and NK_1R. Yellow spots between the fibers (f) indicate a similar colocalization to that seen are present in satellite cells and the nuclei of Schwann cells.

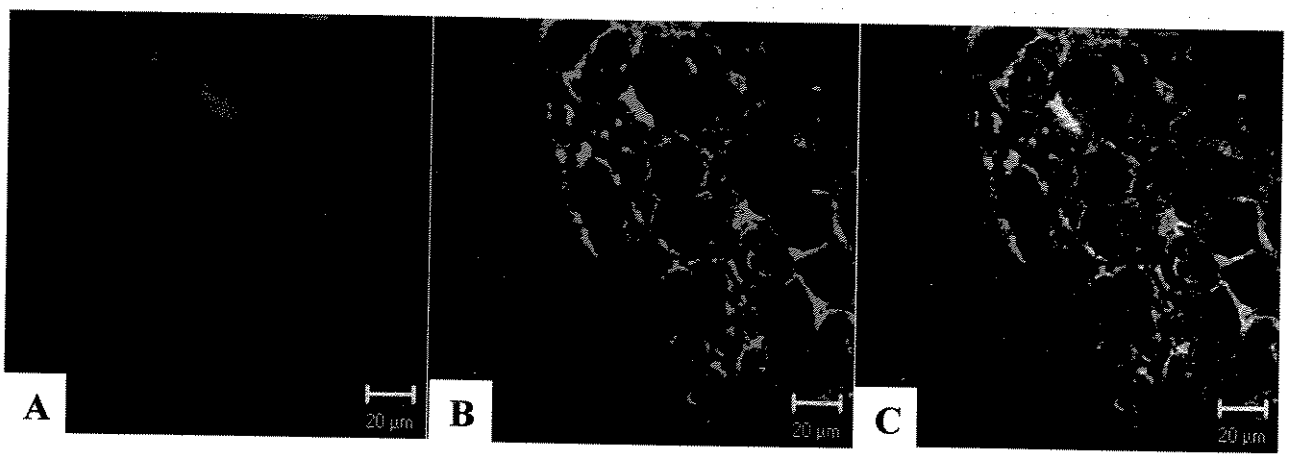


Figure 6. Distribution of NK_1R (green) and SP (red) immunoreactivity in T_{13} DRG cells as shown by CLSM. (A) Occurrence of SP. Note that the nuclear localization of this neuropeptide is not clear. (B) NK_1R in the same DRG region. (C) Note the colocalization of SP and its receptor (yellow) in the cell and nuclear membranes.

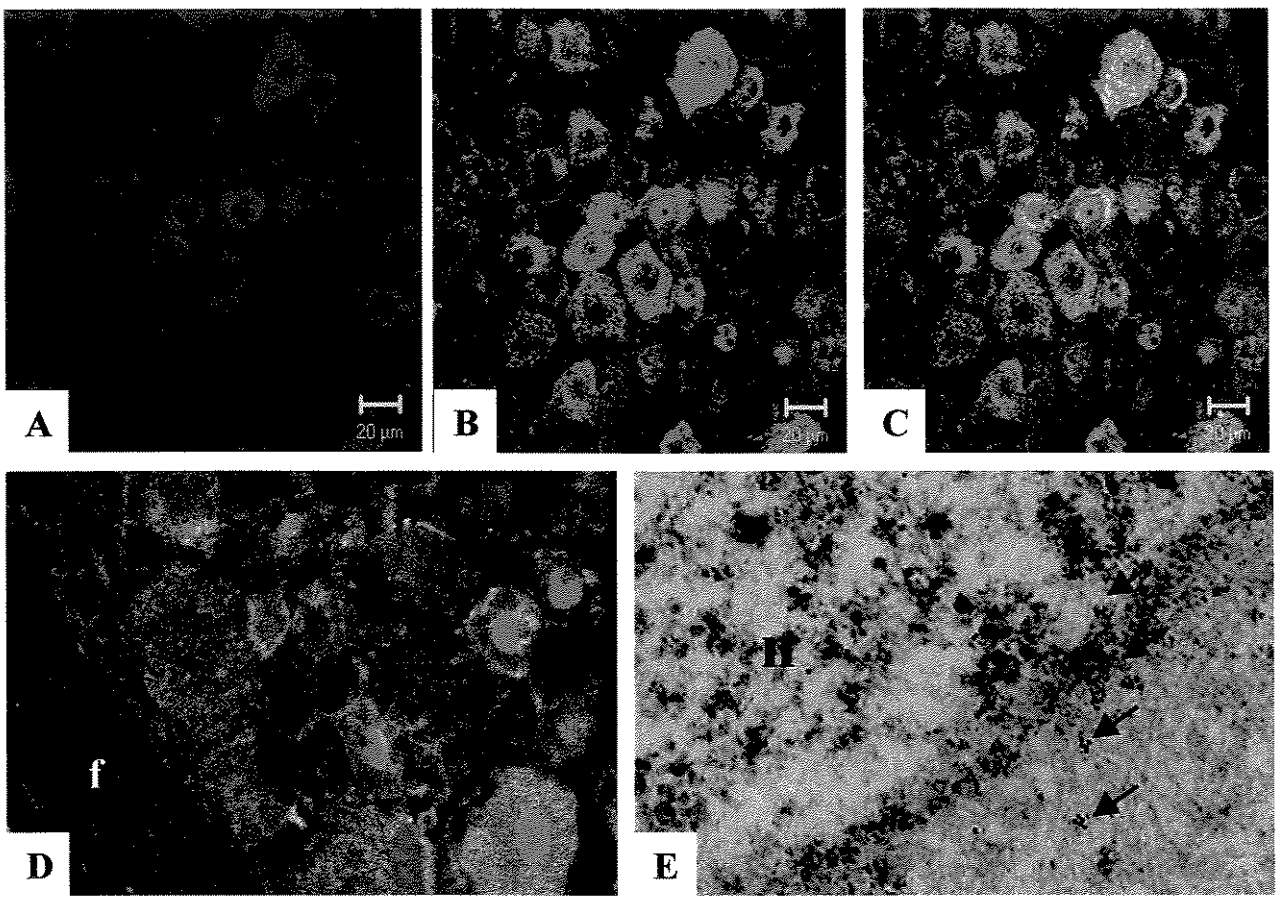


Figure 7. Distribution of SP and CGRP immunoreactivity in T_{13} DGR cells as shown by CLSM. (A and B) Immunolocalization of SP (red) and CGRP (green). Red spots in some neurons indicate nuclear SP localization. In some neurons, the CGRP immunoreactivity is mainly cytosolic and/or nuclear. (C) The colocalization of these neuropeptides in the nuclei of neurons shows populations containing no peptides, CGRP alone, or both peptides. (D) A higher magnification showing the localization of neuropeptides. CGRP can be seen in the fibers (f). (E) Immunogold staining of a region of the nucleus (n) and cytosol interface. Clusters of CGRP can be seen crossing a pore complex of the nuclear envelope (arrows). In the nucleus, CGRP is associated with heterochromatin (Bar: 0,5 µm).

3.3. ARTIGO III

EXPRESSION AND LOCALIZATION OF NK_1R, SUBSTANCE P AND CGRP ARE ALTERED IN DORSAL GANGLIA ROOT NEURONS OF SPONTANEOUSLY HYPERTENSIVE RATS (SHR)

Short Running Title: SP, NK_1 and CGRP expression in SHR.

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SUMMARY

The kidneys play a pivotal role in the pathogenesis of essential hypertension because of a primary defect in renal hemodynamics and/or tubule hydro-saline handling that results in the retention of fluid and electrolytes. Previous studies have shown that increasing the renal pelvic pressure increased ipsilateral ARNA, the ipsilateral renal pelvic release of substance P, and the contralateral urinary sodium excretion in WKy. However, increasing the renal sensory nerve stimuli to the same extent in SHR failed to the increase ipsilateral ARNA and the contralateral urinary sodium excretion. An impaired renorenal reflex activity in SHR is associated, partly, with a peripheral defect at the level of the sensory receptors in the renal pelvis. Furthermore, the renal pelvic administration of substance P failed to increase ARNA in most of SHR at concentrations that produced marked increases in WKy. Since all these findings suggest that interactions between CGRP and SP may modulate their expression and function, we have assessed the co-existence, expression and localization of NK_1R, SP and CGRP in different DRG cell subtypes associated with renal pelvic sensory receptors in SHR (aged 7 and 12 weeks after weaning). In the present study, indirect immunocytochemical screening by fluorescence microscopy was used to locate NK_1R, SP and CGRP in subcellular structures of DGR neurons. The results of this study show increased SP and CGRP expression in the dorsal ganglia root cells of SHR compared to WKy rats. Additionally, there was a progressive, significant, age-dependent, decrease in NK_1R expression on the membrane surface of SHR cells, with no detectable NK_1R-immunoreactivity in 14-week-old SHR. In conclusion, the results of the present study suggest that the impaired activation of renal sensory neurons in SHR may be related to changes in the expression of neuropeptides and/or to a decreased activation of substance P receptors in DGR cells. This decreased activation of substance P receptors may reflect a decrease in the number of substance P receptors or a defect beyond the

substance P receptor pathways. Our findings also provide evidence that NK_1R may control gene expression in the CNS. We speculate that impaired renal responses to substance P and CGRP and the activation of substance P receptors in SHR may be related to altered rates of axonal transport and occurs previously establishment of the high blood pressure (7-week-old rats). Such abnormalities could contribute to the enhanced sodium retention and elevation of blood pressure (BP) seen in SHR.

Keywords: Tachykinin NK1 receptor; Substance P; CGRP; SHR (genetically hypertensive rat); Dorsal Root Ganglia

INTRODUCTION

The kidneys play a pivotal role in the pathogenesis of essential hypertension because of a primary defect in renal hemodynamics and/or tubule hydro-saline handling that results in the retention of fluid and electrolytes (BEIERWALTERS, 1982; DIBONA, 1992; FREY, 2000; STRAZZULLO, 2001; 2003). Hydro-saline balance studies have shown that young (3-7 weeks old) spontaneously hypertensive rats (SHR) excrete less salt and water than pair-fed, age-matched normotensive Wistar-Kyoto rats (WKy) (BEIERWALTERS, 1982; OPARIL, 1987; WYSS, 1992). The renal nerves contribute to the pathogenesis of hypertension in SHR (OPARIL, 1987; KOPP, 1987; DIBONA & KOPP, 1997; DiBona, 1992). Efferent renal sympathetic nerve activity (ERSNA) is enhanced in SHR and may be related to water and sodium retention, both of which contribute to the hypertensive process (OPARIL, 1987; KOPP, 1987; DIBONA, 1992).

The afferent renal nerve activity (ARNA) from sensory receptors located in the renal veins, arteries and pelvic area participates in the reflex control system via renorenal reflexes that enable

total renal function to be self-regulated and balanced between the two kidneys (KOPP et al., 1984; MOSS, 1989; KOPP AND SMITH, 1991a; 1991b;1996; KOPP et al., 1996). KOPP et al., (1987; 1998) demonstrated that increasing the renal pelvic pressure in SHR failed to increase ARNA and thus failed to elicit a contralateral renorenal reflex in these rats, partly because of a peripheral defect at the level of the sensory receptors in the renal pelvis (KOPP et al., 1998). In an other study, the renal pelvic administration of SP failed to increase ARNA in SHR at concentrations that produced marked increases in WKy rats (Kopp et al., 1998).

Substance P, a tachykinin with nanomolar affinity, interacts with specific membrane receptors belonging to the family of G protein-coupled receptors (GPCRs) (PENNEFATHER, 2004). Currently, three tachykinin receptors, NK_1, NK_2 and NK_3, have been cloned in different species (YOKOTA et al., 1989; HERSHEY and KRAUSE, 1990; GERARD et al., 1991; TAKAHASHI et al., 1992). NK_1 receptors (NK_1R) are widely distributed in the renal pelvis and brain, have been implicated in the signaling of nociception to the spinal cord (De KONINCK and HENRY, 1991) and in the modulation of cortical and striatal cell functions (MANTYH et al., 1984; 1995). NK_1R mediate most of the inflammatory, immune and mitogenic effects of SP. In addition, SP has been defined as a potent mitogen for several cell types, including smooth muscle (YANG et al., 2002), fibroblasts (KAHLER et al., 1993) and endothelial (DANIEL et al., 1986) cells. The activation of substance P pelvic receptors plays an essential role in the activation of renal chemo- and mechanosensitive neurons (KOPP and SMITH, 1991b; 1996; KOPP et al., 1996; 1998).

Calcitonin gene–related peptide (CGRP), a 37–amino-acid neuropeptide, is produced by the tissue-specific alternative splicing of the primary transcript from the calcitonin/CGRP gene (KUO et al., 1984; WIMALAWANSA, 1996). This peptide is distributed throughout the central

and peripheral nervous systems and is located in areas involved in cardiovascular function (BRAIN, 1985; DIPETTE, 1989). CGRP-containing neurons are at least as abundant, if not more so, than substance P-containing neurons in the renal pelvic wall (SU et al., 1986). Approximately 90% of neurons in T_{10}-L_3 of the dorsal ganglia root (DGR) that are retrogradely labeled by dye injection into the hilus of the rat kidney are immunoreactive for CGRP (KUO et al., 1984; SU et al., 1986), whereas only 24% of cat dye-labeled renal afferent neurons are immunoreactive for SP (KUO et al., 1984). CGRP regulates the expression of NK_1R by rat spinal neurons (SEYBOLD, 2003) and retards the metabolism of SP (GONTIJO et al., 1999), thereby increasing the amount of SP available for stimulation of SP receptors.

Although these neuropeptides are located in afferent neurons of DGR where they are synthesized, the precise role of SP and CGRP in many sensorial neurons, including possible interactions during their synthesis and in their neuromodulation, remain unclear. All of the cellular activities of SP are considered to be mediated through interaction with NK_1R located on the cell surface. Following binding to SP, the NK_1R is internalized in the endosomal compartment and then recycled to the membrane after dissociation from SP (MANTYH et al., 1995). However, neuropeptides and growth factors can undergo translocation to the nucleus after internalization (YANKER and SHOOTER, 1979; MARCHISIO et al., 1980; JONSON et al., 1980; RAKOWICZ-SZULCZYNSK et al., 1986; BOUCHE et al., 1987; CHABOT et al., 1988a; CHABOT et al., 1988b; BOIVIN et al., 2003). Since all these findings suggest that interactions between CGRP and SP may modulate their expression and function, we have assessed the co-existence, expression and localization of NK_1R, SP and CGRP in different DRG cell subtypes associated with renal pelvic sensory receptors in SHR (aged 7 and 12 weeks after weaning).

MATERIALS AND METHODS

Seven and 14-week-old male Wistar-Kyoto (WKy) rats and SHR, weighing between 100 and 350 g were obtained from the University's breeding colony. The general guidelines established by the Brazilian College for Animal Experimentation (COBEA) were followed throughout this work. The rats were anesthetized with sodium pentobarbital (30-50 mg kg^{-1}body weight, i.p.) and the level of anesthesia was controlled by monitoring the corneal reflex. The carotid artery was cannulated and the rats were perfused as described below. The rats were not perfused when the tissues were required for western blot experiments. The systemic arterial pressure was measured, before the experiments, in conscious rats by an indirect tail-cuff method using an electrosphygmomanometer (Narco Bio-Systems, Austin, TX) combined with a pneumatic pulse transducer/amplifier that provided output signals proportional to the cuff pressure and amplified Korotkoff sound. This indirect approach allowed repeated measurements with a close correlation (correlation coefficient = 0.975) compared to direct intra-arterial recording (LOVENBERG, 1987). The mean of three consecutive readings represented the blood pressure of SHR and WKY.

Immunofluorescence cytochemistry for NK_1, SP and CGRP – Seven-week-old male WKy (n=5) rats and 7 and 14-week-old SHR (n=10) were used. The rats were anesthetized and perfused by the left carotid artery with saline containing heparin (2%) for 5 min under constant pressure. This was followed by perfusion with 0.1M phosphate buffer (PB; pH 7.4) containing 4% (w/v) paraformaldehyde and 0.1 M sucrose. After the perfusion, the left DGR (T_{13} and L_1) were immediately removed and placed in the same fixative for 1 h, followed by PBS containing 0.1% glycine for 1 h and PBS containing 15% (w/v) sucrose overnight a placed in OCT compound cryoprotector (Tissue-tech®), freeze-thawed in liquid nitrogen and the cut in serial sections (10 μ m thick) on a Leica cryostat at –25°C and mounted on silane-coated slides. For

immunohistochemistry, the sections were incubated sequentially with: (1) phosphate-buffered saline (PBS) containing 8% fetal calf serum, 2% normal goat serum, 2% milk and 2.5% bovine serum albumin (BSA) for 45 min to minimize nonspecific reactions, (2) rabbit anti-NK_1R antiserum (1:50 dilution; Sigma), rabbit anti-CGRP antiserum (1:250 dilution; Sigma) or goat anti-SP antiserum (1:600; Santa Cruz), at 4°C overnight; and (3) goat anti-rabbit CY2-labeled antibody (1:600 dilution; Jackson ImmunoResearch) or rabbit anti-goat CY3-labeled antibody (1:1000 dilution; Jackson ImmunoResearch) for 2 h at room temperature. After incubation, the sections were rinsed in 0.1 M PBS and cover-slipped with Vectashield anti-fading medium containing DAPI (Vector). The sections were examined with a confocal laser scanning microscope (CLSM, LSM510 ZEISS) using laser beams of 543 and 488 nm (and UV) and appropriate emission filters for CY3 (590-610 nm) and CY2 (510-525 nm) (and DAPI). Digital images were captured using specific software (LSM; Zeiss) and were printed on a color printer. No immunoreactivity was seen in control experiments in which one of the primary antibodies was omitted.

Immunogold transmission electron microscopy for NK_1, SP and CGRP - In these experiments 14-week-old WKy rats (n=2) and SHR (n=2) were used. Anesthetized rats were perfused via a carotid catheter with 0.1 M PB (pH 7.4) containing 4% (w/v) paraformaldehyde, 0.2% glutaraldehyde and 0.1 M sucrose. After the perfusion, the left DGR (T_{13}) were immediately removed and immersed in the same fixative for 2 h at room temperature. The samples were rinsed in PBS and placed in PBS containing 0.1% glycine for 1 h. This step was followed by dehydration in a graded ethanol series at –20°C and infiltration with Lowicryl K4M in a 1:1 (v/v) mixture with 100% ethanol and then pure resin (60 min each). The samples were stored in pure lowicryl K4M overnight at the same temperature. The resin was polymerized under indirect, diffuse UV light for 24 h at –20°C, and for a further 24 h at room temperature.

Ultrathin sections (80-90 nm) mounted on nickel grids were treated with 1% BSA in PBS for 10 min to prevent non-specific binding, followed by incubation with primary antibodies overnight. After washing with PBS, the grids were incubated for 2 h with goat anti-rabbit IgG conjugated to 10 nm colloidal gold (1:40; Aurion) or with rabbit anti-goat IgG conjugated with 15 nm colloidal gold (1:40; Aurion) diluted in PBS with 1% BSA. After washing and drying, the sections were double-stained with uranyl acetate and lead citrate for 3 min and 40 s, respectively, and were observed and photographed with a LEO 906 transmission electron microscope operated at 60 kV. No immunoreactivity was seen in control experiments in which one of the primary antibodies was omitted.

Tissue extracts - Seven-week-old and 7 (n = 12) and 14-week-old male (n= 12) WKy and SHR rats, weighing between 100 and 350 g were used. After anesthetized, the abdominal cavity was opened, five DRGs (T_{10}-L_1) and renal pelvis were removed, minced coarsely and homogenized immediately in 10 volumes of solubilization buffer (10 ml/l Triton-X 100, 100 mmol/l Tris[hydroxymethyl]amino-methane (Tris) pH 7.4, 10 mmol/l sodium pyrophosphate, 100 mmol/l sodium fluoride, 10 mmol/l ethylendiaminetetracetic acid (EDTA), 10 mmol/l sodium vanadate, 2 mmol phenylmethylsulfonyl fluoride (PSMF) and 0.1 mg/ml aprotinin at 4°C, using a polytron PTA 20S generator (model PT 10/35, Brinkmann Instruments, Westbury, N.Y., USA) operated at maximum speed for 20 s. The tissue extracts were centrifuged at 12,000 rpm at 4°C for 20 min, and the supernatants used as sample.

Protein analysis by immunoblotting - Protein quantification was performed using the Bradford method (BRADFORD, 1976). Quantitation in either tissue, total extract samples (250µg protein)

were subjected to SDS-PAGE. After electrophoretic separation, proteins were transferred to nitrocellulose membranes and then blotted with specific antibody. The samples were treated with Laemmli buffer (LAEMMLI, 1970) containing 100 mmol/l dithiothreitol (DTT), heated in a boiling water bath for 4 min and subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a Bio-Rad minigel apparatus (Mini-Protean, Bio-Rad). The prestained molecular mass standards used were myosin (205 kDa), -galactosidase (116 kDa), BSA (80 kDa) and ovalbumin (49.5 kDa). Electrotransfer of proteins from the gel to the nitrocellulose membranes was performed for 90 min at 120 V (constant) in a Bio-Rad miniature transfer apparatus (Mini-Protean) as described by (TOWBIN, 1979), but with 0.02% SDS and β-mercaptoethanol added to the transfer buffer to enhance the elution of high-molecular-mass proteins. The non-specific protein binding to the nitrocellulose was reduced by preincubating the filter for 2 h at 22°C in blocking buffer (5% non-fat dry milk, 10 mmol/l Tris, 150 mmol/l NaCl, and 0.02% Tween 20). The nitrocellulose blots were incubated for 4 h at 22°C with antibodies against NK_1R diluted in blocking buffer (3% non-fat dry milk, 10 mmol/l Tris, 150 mmol/l NaCl, and 0.02% Tween 20), followed by washing for 30 min in blocking buffer without milk. The blots were incubated with 2 μCi of [^{125}I]protein A (30 μCi/μg) in 10 ml of blocking buffer (1% non-fat dry milk) for 1 h at 22°C and washed again as described above. [^{125}I]protein A bound to the antibodies was detected by autoradiography using preflashed Kodak XAR film (Eastman Kodak, Rochester, NY) with Cronex Lightning Plus intensifying screen (DuPont, Wilmington, DE) at -80°C for 12-48 h. Images of the developed autoradiographs were scanned (Hewlett Packard Scanjet 5P) and band intensities were quantitated by optical densitometry (Scion Image Corporation) of the developed autoradiographs that were used at exposures in the linear range.

Statistical analysis - Where appropriate, the results were expressed as the means ± SEM accompanied by the indicated number of rats used in experiments. Comparisons among groups were made using parametric two-way ANOVA. Further comparisons were made using Newman-Keuls test. A p value of less than 0.05 was considered statistically significant.

Cell counts - Quantification and data analysis - The sub-cellular pattern and localization of staining was assessed in optical sections obtained by confocal laser scanning microscope (CLSM) In these sections, the immunostained cell profiles were easily recognized and counted. The T_{13} DRG sections were divided into five regions and the profiles within each region were counted. The percentage of cells that were positive with NK_1R, SP and CGRP antisera, in each region, was then averaged to give the overall percentage of immunostained cells in each section (Bowie, 1994). All of the sections used for quantification were from experiments in which the immunostaining was considered optimal.

Cell size distribution – To determine the cell size distribution, five DRG sections immunostained with NK_1R, SP and CGRP antisera were selected from three T_{13} DRGs of different rats. The images were stored and analyzed using the Bio-Rad software specifically designed for CLSM. Briefly, the boundaries of immunotained cell profiles were traced manually using a computer mouse and the length, breath and area of each cell was calculated automatically. To compare our results with previous quantitative studies on DRG, the cell areas were transformed into cell diameters by assuming that ganglion cells are circular and that the ranges of diameters in the different subpopulations were: 10-25 μm for small cells, 25-37.5 μm for intermediate cells and 37.5-60 μm for large cells (ALVAREZ, 1991).

Statistical analysis –Where appropriate, the results were expressed as the mean ± SEM. The percentage data from NK_1R, SP and CGRP-immunoreactive neurons were compared by the Kruskal-Wallis test for repeated measurements. A p value < 0.05 indicated significance.

RESULTS

The tail arterial pressure (mmHg) in SHR was significantly higher than in WKy from 7 to 12 weeks of age. During the study, SHR pressure increased from 118 ± 11 mmHg to 192 ± 9 mmHg as compared with a slower rise from 106 ± 7 mmHg to 119 ± 12 mmHg in WKy. The tail arterial pressure in SHR appeared to reach a plateau after 11 weeks of age. Between 10 and 12 weeks, tail arterial pressure averaged 180.6 ± 8 mmHg in SHR and 117 ± 10 mmHg in WKy ($p < 0.01$). In this study, preliminary, indirect immunocytochemical screening by fluorescence microscopy was used to locate NK_1R, SP and CGRP in subcellular structures of DRG neurons. Since overlap of the Golgi apparatus with nuclear structures may interfere with this observations, and in order to identify the correct site of localization, we analyzed ultrathin optical sections obtained by CLSM. Intense migration of SP and CGRP towards the nuclei was seen in some subpopulations of cells in DGR neurons in 7- and 14-week-old SHR (Figure 1D and K). The NK_1R immunoreactivity showed in SHR that receptors were preferentially located in the nuclei of DRG neurons (Figure 1). This pattern of distribution was assessed quantitatively (see below). The renal pelvis of SHR showed poor NK_1R immunoreactivity when compared to that of WKy (Figure 2B). Immunogold staining confirmed the strong content of SP in the nuclei of cell bodies in DRG (Figure 3). Additionally, the immunogold reaction for CGRP showed a higher percentage of these neuropeptide in perikaryon vesicles and in association with nuclear heterochromatin, with poor,

weak staining integrated on the cell surface in SHR compared to WKy rats (Figure 4). The same pattern of nuclear localization was seen for NK_1R (Figure 5 and 6).

Western blot analysis of NK_1R expression - Although different immunoreactivity for NK_1R was seen in DRG neurons and pelvis of SHR compared with WKY rats, artifacts may be introduced by different laser settings and by the depth of sectioning. To exclude this possibility, we used western blot to assess the quantification of the differences in receptor content. The protein expressions of NK_1R in the DRGs from WKy and SHR rats was quantified by immunoblotting with anti-NK_1R antibody and, in the 7-week-old rats, there was no significant difference in the NK_1R protein expression (WKy: 100 ± 10% *vs.* SHR: 105 ± 9%, p=0.230; n = 6, Figure 2C). However, compared to basal levels of DRGs protein expressions, obtained from WKy, in 14-week-old SHR there was a significantly lower expression (WKy: 100 ± 1% *vs.* SHR: 59 ± 2%, p=0.0001; n = 6, Figure 2C). The pelvic NK_1R immunoblotting expression confirms our immunofluorescence cytochemistry results. The expression for this receptor was stronger in WKy rats than in SHR at both periods observed. The 7-week-old SHR shows significant lower expression of NK_1 pelvic receptors when compared to WKy (WKy: 100 ± 28% *vs.* SHR: 75 ± 18%, p=0.0176; n = 12, Figure 2D) and this reduction was also verified in14-week-old SHR (WKy: 100 ± 7% *vs.* SHR: 69 ± 14%, p=0.049; n = 6, Figure 2D)

Qualitative and quantitative analysis of NK_1R, SP and CGRP immunoreactivity- In WKy DRG NK_1R-immnunoreactive cells were homogeneously distributed in all subcellular structures (nuclei, cytosol and surface), especially in intermediate and large neurons. However, in all cases the immunoreactivety for NK_1R was smaller in SHR than in WKy rats. No NK_1R immunoreactivity was seen within the smallest DRG neurons of 7-week-old SHR. On the other hand, a marked

reduction in NK_1R immunoreactivity was seen on the cell surface of progressively larger neuronal subpopulations, with no NK_1R immunoreactivity on the surface of the largest cells.

The substance P immunoreactivity was stronger in SHR compared to WKy rats and was independent of age. In contrast to the subcellular distribution of NK_1R, SP was homogeneously and heavily distributed in all subcellular organelles and on the cell surface. In T_{13} DRG of SHR, SP immunoreactivity was more homogeneous and greater in subcellular structures including the cytosol, than in WKy DGR neurons. In WKy rats, SP immunoreactivity was observed predominantly in the perikaryon, with sparse and weak nuclear and membrane staining exclusively in intermediate DRG cells.

CGRP neuronal immunoreactivity distribution was detected in a number of DRG cells in SHR compared to WKy DRG cells. In most WKy neuronal body cells of the three DRG subpopulations, CGRP was located predominantly in the cytosol, except in intermediate immunoreactive cells where CGRP was also detected on the cell surface and, to a lesser extent in nuclei. DRG from 7- and 14-week-old SHR, CGRP increased significantly in the perikaryon, with greater immunoreactivity in larger cells. The subcellular co-existence of NK_1R and SP revealed that DRG neuronal immunoreactivities had distinct patterns of localization with little overlap.

Size distribution of NK_1R-, SP- and CGRP-positive subpopulation of cells

Figure 7 (A, B and C) shows a unimodal distribution for the size of NK_1R-, SP- and CGRP-positive cells based on CLSM optical sections, with no significant skewing of the curve. No NK_1R, SP and CGRP immunoreactivity was seen in the smallest cells in 7-week-old SHR.

Only optimally immunostained sections were used for cell quantification. A minor percentage of small neurons in DRG were immunoreactive for NK_1R in WKy (12.8 ± 1,2%)

compared to 7-week-old (0%) and 14-week-old (30.0 ± 2.2%) SHR. This percentage increased with the largest immunoreactive cells (WKy: 25.7 ± 2.1%; 7-week-old SHR: 30.0 ± 1.9%; but not in 14-wk-old SHR: 12.9 ± 1.4%) and was significantly (p=0.001) higher than in intermediate size neurons (WKy: 61.5 ± 4.6%; 7-week-old SHR: 70.0 ± 4.8% and 14-week-old SHR: 57.1 ± 5.0%) (Figure 7A). The distribution of NK_1R, SP and CGRP immunoreactivity did not vary significantly in different subpopulations of DRG neurons in WKy rats or in SHR. In five DRG sections, the average percentage of DRG cells nuclei immunoreactivity for NK_1R, SP and CGRP was extremely weak and low for WKy (NK_1R: 63.1 ± 3.6%; SP: 8.95 ± 0.65% and CGRP: 14.3 ± 1.5%, respectively) compared to the significantly (p=0.0001) higher nuclear immunoreactivity in SHR (7-week-old SHR NK_1R: 97.1 ± 5.4%; SP: 92.0 ± 5.6% and CGRP: 89.3 ± 4.6%) and (14-wk-old SHR, NK_1R: 95.7 ± 4.7%; SP: 98.4 ± 6.0% and CGRP: 95.3 ± 4.3%) (Figure 7D, E and F). As described above, none of the smallest cells in DRG sections from 7-week-old SHR was immunoreactive for NK_1R. Also, there was no significant difference between the SP and CGRP immunoreactive nuclei in 7-week-old and 14-week-old SHR. There was no correlation between the homogeneous, extensive subcellular distribution of NK_1R imunoreactivity and the sparse surface content of SP in DRG cell subpopulations (practically absent in 14-week-old SHR)(Figure 7G, H and I).

DISCUSSION

The results of the present study show increased NK_1R, SP and CGRP expression in the dorsal ganglia root cells of SHR compared to WKy rats. There was a progressive, significant, age-dependent, decrease in NK_1R expression on the membrane surface of SHR cells, with no detectable NK_1R-immunoreactivity in 14-week-old SHR. Previous studies (KOPP et al., 1984; 1996; KOPP and SMITH, 1993) have shown that increasing the renal pelvic pressure increased ipsilateral ARNA, the ipsilateral renal pelvic release of substance P, and the contralateral urinary sodium excretion in WKy. However, increasing the renal sensory nerve stimuli to the same extent in SHR failed to the increase ipsilateral ARNA and the contralateral urinary sodium excretion. An impaired renorenal reflex activity in SHR is associated with a decreased release of substance P into the renal pelvic effluent (KOPP et al., 1987; 1998). Furthermore, the renal pelvic administration of substance P failed to increase ARNA in most of SHR at concentrations that produced marked increases in WKy (KOPP et al., 1998). Previous studies (MOSS, 1984; KOPP et al., 1987; GONTIJO and KOPP, 1994; GONTIJO et al., 1999) in normotensive rats demonstrated that substance P and CGRP elicit a similar renorenal reflex response, including an increase in renal pelvic pressure. Moreover, treatment with substance P and *h-CGRP*$_{8-37}$ receptor antagonists or capsaicin, which depletes sensory neurons of substance P, blocked the ARNA response to increased renal pelvic pressure (KOPP and SMITH, 1991b; 1993; GONTIJO and KOPP, 1994; GONTIJO et al., 1999). Taken together, these results suggest that the impaired responsiveness of renal sensory receptors in SHR is related not only to a decreased renal pelvic release of substance P, but may be also associated with increased axonal neuropeptides and with the presence of few or no NK_1R, as revealed by immunofluorescence cytochemistry and immunoblotting in 14-week-old SHR. These observations are relevant because they may provide a fresh framework for

understanding the etiology of the impaired symmetrical renal sensory responses commonly associated with the development of hypertension in SHR.

Possible mechanisms involved in renal sensory receptor activation include activation of calcium-mediated stimuli in response to the release of substance P and CGRP from synaptic nerve-endings. Recent studies *in vitro* in cultured DRG neurons have demonstrated that PDBu causes a calcium-mediated release of substance P from these sensory neurons (BARBER and VASKO, 1996). Signaling by the phosphoinositidase C-PKC pathways is altered in SHR (TAKATA and KATO, 1996), since treatment with the PKC activator PDBu failed to increase ARNA in SHR (KOPP and SMITH, 1996; KOPP et al., 1998). These results agree with previous studies in this model demonstrating a failure of substance P to increase in response to feeding (MORI et al., 1993). Mental stress can result in a reduced increase in the plasma concentration of substance P in hypertensive compared to normotensive subjects (FAULHABER et al., 1987), and several studies have shown an increased pain threshold in hypertensive men and rats (ZAMIR and SHUBER, 1980; VIRUS et al., 1981; SITSEN and De JONG, 1983; GUASTI et al., 1995; SCHOBEL et al., 1996; IRVINE and WHITE, 1997). These studies suggest that the impaired responsiveness of renal sensory receptors in SHR is, partly, related to a defect in SP synthesis and release by renal sensory neurons. However, the significantly higher SP immunoreactivity seen here in SHR does not agree with such studies. Rather, our findings support the suggestion that the impaired ARNA response to renal sensory activation in SHR is related to decreased NK_1R expression in renal sensory nerve membrane and/or altered distribution of the substance P–containing neurons and their receptors in the renal pelvic area. Observations in non-neural vascular tissue have also led to the conclusion that there is a defect in the level of substance P receptors in the axonal membrane of

hypertensive subjects (VIRUS et al., 1981; POMPEI et al., 1993; PANZA et al., 1994; EGASHIRA et al., 1995; HUANG and KOLLER, 1996; QUYYUMI et al., 1997).

NK_1R is widely distributed in various tissues and organs. The high concentration of NK_1R in the sensory nervous system tissues suggests that NK_1R plays an important role in the control of neuronal activity. The activation of NK_1R contributes to the activation of renal sensory receptors by stimuli such as increased renal pelvic pressure in normotensive rats (KOPP and SMITH, 1991a; 1991b; 1993; KOPP et al., 1997). The present findings showing decreased NK_1R both immunoreactivity and protein content in DRG neuron membranes may explain the blunted renal sensory receptor activity in SHR. We therefore hypothesize that low expression or impaired activation of NK_1 receptors could be an additional mechanism contributing to the decreased responsiveness of renal sensory receptors in SHR.

We can not exclude the possibility that impaired ARNA responses to renal sensory receptor stimulation in SHR could be related to a defect in the substance P receptor–membrane coupling mechanisms. In the resting state, NK_1R is present mainly in the perikaryon, but after sensory nerve activation there is translocation of NK_1R from the perikaryon to the cell membrane surface.

A higher immunogold reaction to SP was seen in the perikaryon vesicles and in association with nuclear heterochromatin. In contrast, immunocytochemistry showed little or no translocation of neuropeptides to the cell membrane surface in 14-week-old SHR compared to WKy rats (Figure 3). The cell surface immunoreactivity may correspond to neuropeptide stores located close to submembrane sites on vesicles. Our results raise the possibility that the impaired responsiveness of renal sensory receptors in SHR (KOPP et al., 1987; 1998), could be the result of a defect in NK_1R translocation from the cytosol to the neuron membrane or to an altered NK_1R-SP interaction in the signaling pathway in SHR (SOLOMON et al., 1999). Renal sensory nerve fibers

are activated by increases in renal pelvic pressure of <5 mm Hg (KOPP, 1987; 1998, GONTIJO et al., 1999). Because spontaneous renal pelvic contractions can produce increases in renal pelvic pressure of >5 mmHg, it is conceivable that substance P released by the activation of afferent renal nerves plays a physiological role in the renal control of water and sodium excretion. An impaired NK_1R translocation from the cytosol to the neuronal membrane and an associated decrease in the responsiveness of the substance P receptors during the activation of DRG sensory receptors in SHR may lead to increased water and sodium retention, factors known to contribute to hypertension (DIBONA, 1992).

Substance P and CGRP are potent vasodilator neuropeptide and a prominent site of neuropeptides synthesis is the DRG. CGRP mRNA levels are increased in DRG and the immunoreactive CGRP content is elevated in the spinal cord in mineralocorticoid-salt hypertension (SUPOWIT et al., 1994; 1995; 1997). Dorsal root ganglia neuronal cell bodies synthesize CGRP and send axons peripherally to blood vessels and centrally to spinal cord sites involved in blood pressure regulation. This increased synthesis of a potent vasodilator is a compensatory response to attenuate the increase in BP. However, it is not known whether neuronal CGRP production is upregulated simply by the elevated blood pressure or by changes in other parameters. The marked increase in the level of SP and CGRP in DGR prior to the elevation in blood pressure in 7-week-old SHR suggests that hypertension alone does not contribute to the increase in neuronal neuropeptide contents. Our results demonstrate that the enhanced neuronal immuno-expression of CGRP in SHR is a compensatory vasodilator mechanism to attenuate the elevated BP. We suggest that in SHR there are specific changes in as yet unidentified factors that modulate the neuronal synthesis and release of SP and CGRP, independently of an increase in blood pressure. Since we did not measure the CGRP mRNA levels in DRG, we cannot exclude the possibility that an

increase in BP could modulate CGRP levels at the translational level. However, *in vivo* (KOPP et al., 1994; GONTIJO et al., 1999) and primary cultures of adult DRG neurons (SUPOWIT et al., 1995), a direct correlation between the changes in CGRP mRNA content and the level of CGRP immunoreactivity has been reported. Data on the circulating levels of immunoreactive CGRP in hypertensive humans vary considerably, with investigators reporting increased (MASUDA et al., 1992), unchanged (SCHIFTER et al., 1991), or decreased (EDVINSSON et al., 1989) levels. This variability has been attributed to the heterogeneous nature of hypertension or to differences in the assays used. Contradictory results concerning the circulating levels of CGRP in rodent models of hypertension have also been reported (SUPOWIT et al., 1995; 2001).

One possible explanation for these results includes altered rates of axonal transport of CGRP and SP in SHR compared to normotensive rats. Another possibility is an asymmetrical axonal transport of CGRP between central and peripheral sensory nerve terminals (TOMLINSON et al., 1988) as observed in experimental diabetes models.

Although we have not yet identified the factors that mediate the upregulation of neuronal CGRP expression in SHR, possible candidates include neurotrophins, bradykinin, prostaglandins, and the sympathetic nervous system. Neurotrophin levels are significantly higher in SHR during the first 4 postnatal weeks, and in the heart and mesenteric artery from 2 to 10 weeks of age, compared with levels in WKy rats. The elevated neurotrophin levels in the sympathetic ganglia and hyperinnervated organs of SHR indicates that neurotrophin may have an important role in the development of hyperinnervation, possibly by enhancing the survival and/or nerve sprouting of sympathetic neurons (ZHANG & RUSH, 2001). Nerve growth factor (NGF) acts via a specific tyrosine kinase receptor (trkA) to control the synthesis of several neuropeptides and regulate membrane excitability (EHLERS et al., 1995). NGF can stimulate CGRP expression in mature

sensory neurons *in vivo*. Thus, using primary cultures of adult DRG neurons, LINDSAY (1988) demonstrated that NGF can directly upregulate CGRP synthesis and release. NGF initially signals by binding to the high affinity trkA receptor in nociceptive sensory nerve terminals, with involvement of the low affinity $p75^{NTR}$ receptor (CHAO and HEMPSTEAD, 1995; KAPLAN and MILLER, 1997). The ligand and receptor are then internalized and transported retrogradely down the axon to the cell body (EHLERS et al., 1995). Neurotrophins can induce rapid elevations in tyrosine phosphorylation of trk receptors that are propagated in a retrograde fashion along axons. These events may be mediated via fast axonal transport of the trk receptor-ligand complex but may also involve an undefined process traveling at speeds in excess of those seen for fast axonal transport (EHLERS et al., 1995; BHATTACHARYA et al., 1997; SENGER and CAMPENOT, 1997). Second messengers downstream to trk receptors, such as G proteins, extracellular signal-regulated kinases (ERKs), and phospholipase C-γ (PLC-γ), undergo fast retrograde axonal transport, but the significance of this is unknown (JOHANSSON et al., 1995). Neurons may also be capable of the axonal transport of transcriptional factors (SCHMIED et al., 1993; CURTIS and DiSTEFANO, 1994; O'NEILL and KALTSCHMIDT, 1997). Studies in *Aplysia* have shown that a nuclear localization signal (NLS) can be recognized by the retrograde transport-nuclear import pathway that conveys proteins along the axon to the soma and into the nucleus (SCHMIED et al., 1993). Thus, NGF may maintain the phenotype of a subpopulation of nociceptive sensory neurons in adult vertebrates, partly, through the regulation of substance P and CGRP expression (SNIDER and McMAHON, 1998).

Although we have demonstrated that NK_1R expression is decreased in the DGR of SHR, little is known about the signaling molecules and the second messenger pathways that are activated in regulating the expression of the NK_1 receptor gene. Since the promoter region of the NK_1

receptor contains a cAMP response element (CRE), we hypothesize that the elevated levels of CGRP in DRG nuclei regulates the expression of NK_1 receptors via a pathway involving activation of the transcription factor cAMP response element binding protein (CREB). Collectively our data define a role for CGRP and SP as signaling molecules that induce the expression of NK_1 receptors in DRG sensory neurons. The data also provide evidence that a neuropeptide receptor may control gene expression in the CNS and add another dimension to understanding the cotransmission of substance P and CGRP by primary afferent neurons (SEYBOLD et al., 2003).

In summary, the results of the present study suggest that the impaired activation of renal sensory neurons in SHR may be related to changes in the expression of neuropeptides and/or to a decreased activation of substance P receptors in DRG cells. This decreased activation of substance P receptors may reflect a decrease in the number of substance P receptors or a defect beyond the substance P receptor pathways. Our findings also provide evidence that NK_1R may control gene expression in the CNS. We speculate that impaired renal responses to substance P and CGRP and the activation of substance P receptors in SHR may be related to altered rates of axonal transport and occurs previously establishment of the high blood pressure. Such abnormalities could contribute to the enhanced sodium retention and elevation of BP seen in SHR.

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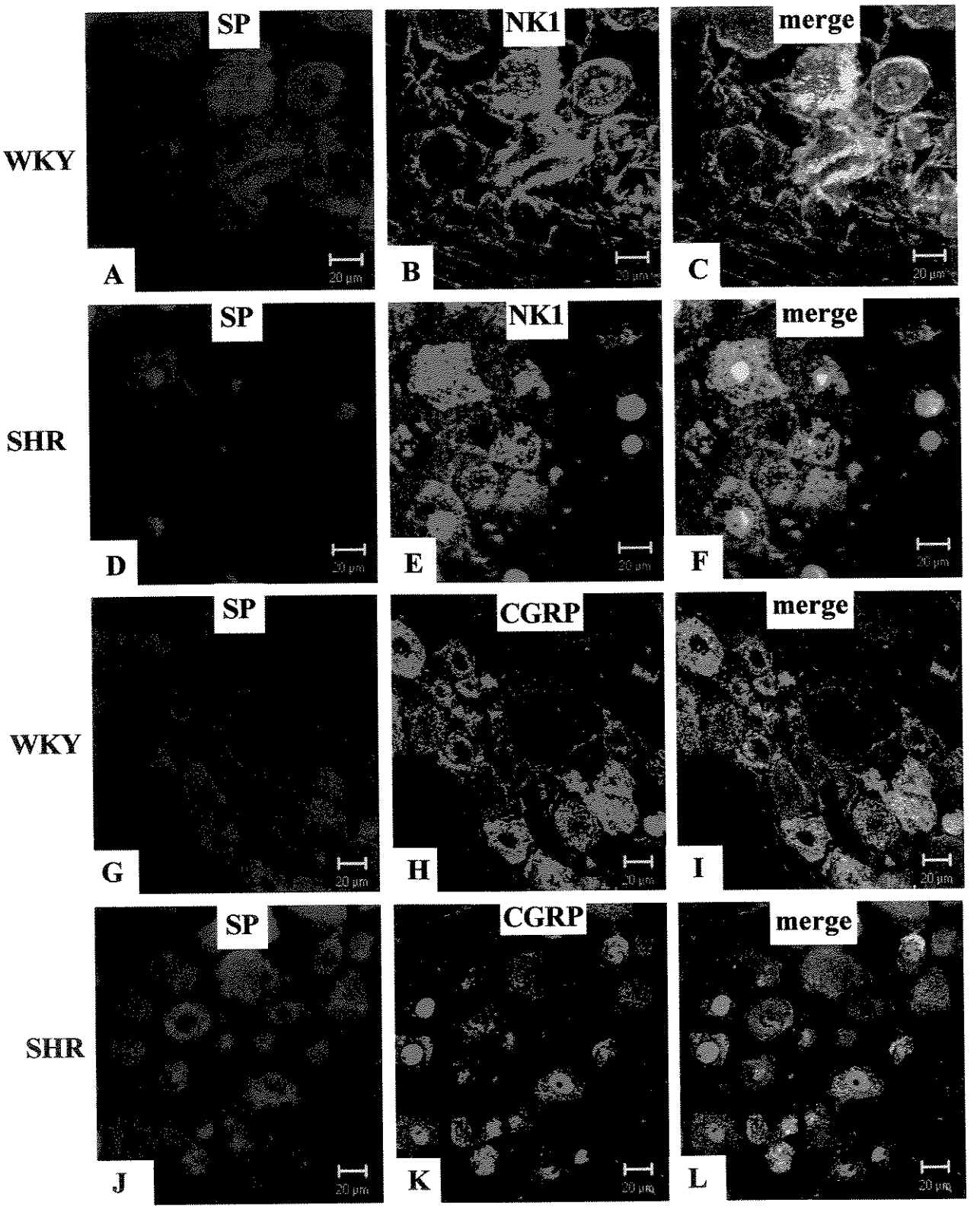

Figure 1. CLSM showing the distribution and colocalization of SP, CGRP and NK_1R in T_{13} DRG cells of 7 week-old rats WKy and SHR.

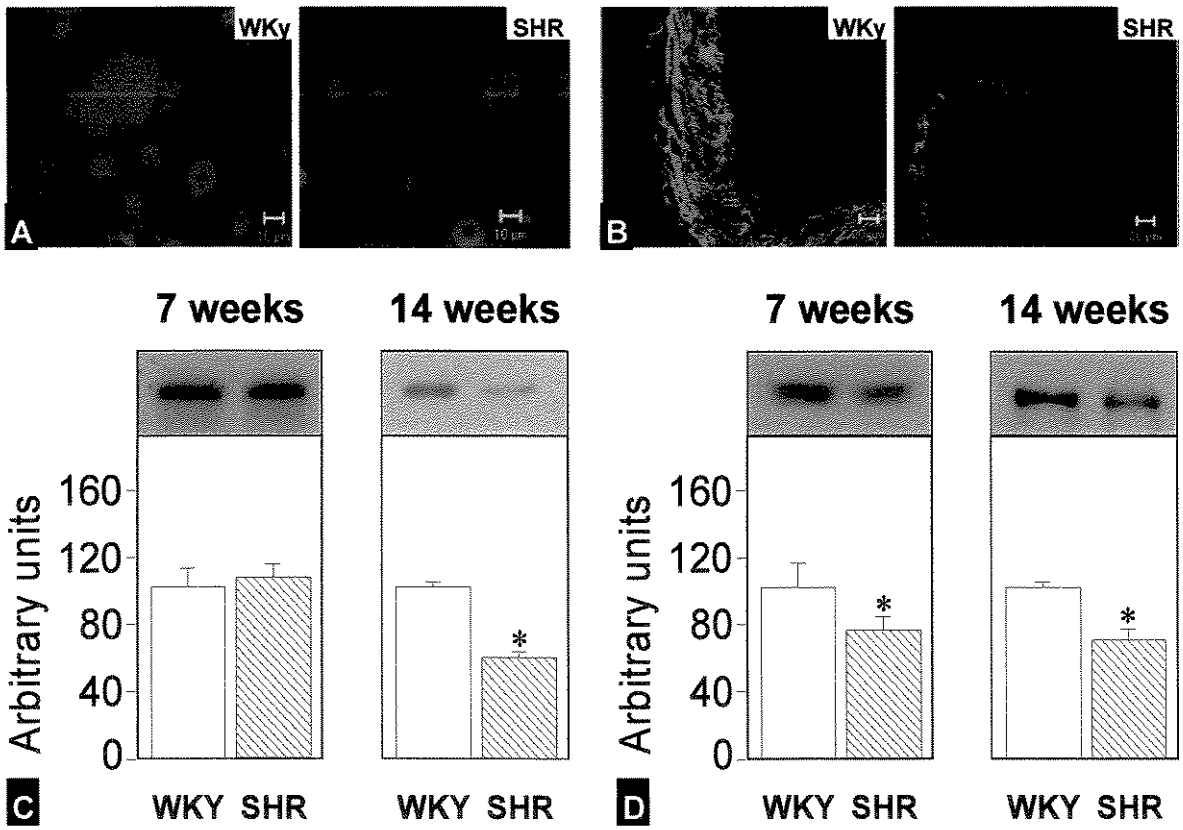

Figure 2. Comparative expression of NK_1R. In A and B, CLSM showing the imunolocalization of NK_1R in the T_{13} DRG and renal pelvis of 14 week-old rats. C ad D shows the results obtained, in whole-tissue extracts that were immunoblotted for NK_1R (46kDa), for this protein content verification in the DRGs and pelvis, respectively. The results of scanning densitometry were expressed as relative to control, assigning a value of 100% to the WKy rats. *Columns* and *bars* represent the mean ± SEM. $^{*}p<0.05$, WKy *vs.* SHR.

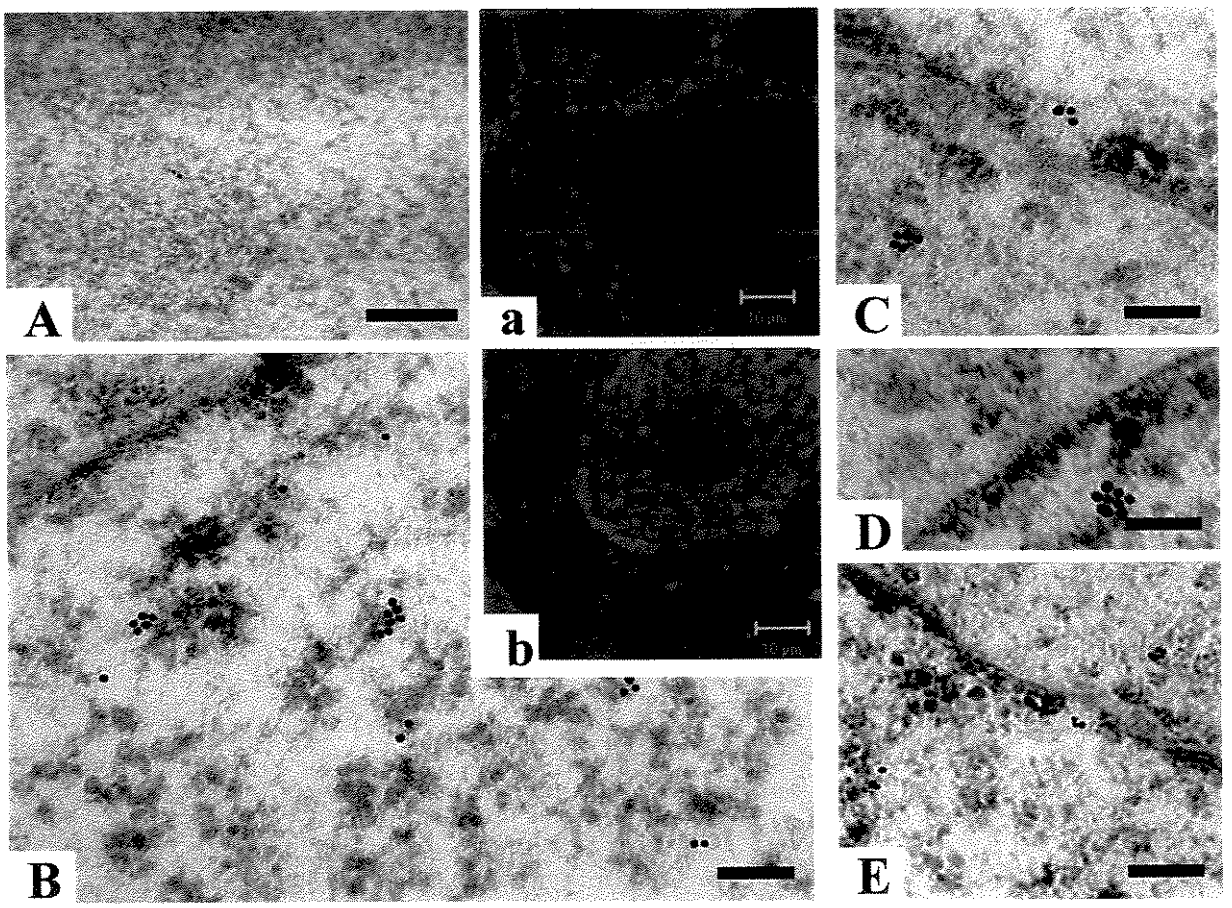

Figure 3. Immunogold electron microscopy or immunofluorescence CLSM showing the weak nuclear localization of SP in DRG neurons of WKy rats (A and a). DGR neurons of SHR show nuclear clusters of these neuropeptides (B and b), that are preferentially associated with heterochromatin. C and E show regions of the nuclear and cytosolic interface. Clusters of SP can be seen moving through a pore complex of the nuclear envelope. (D) Shows a cluster before and after crossing the pore complex in association with heterochromatin. Bars: A = 0,3 μm; B and C = 0,14 μm; D = 1 μm; E = 0,2 μm.

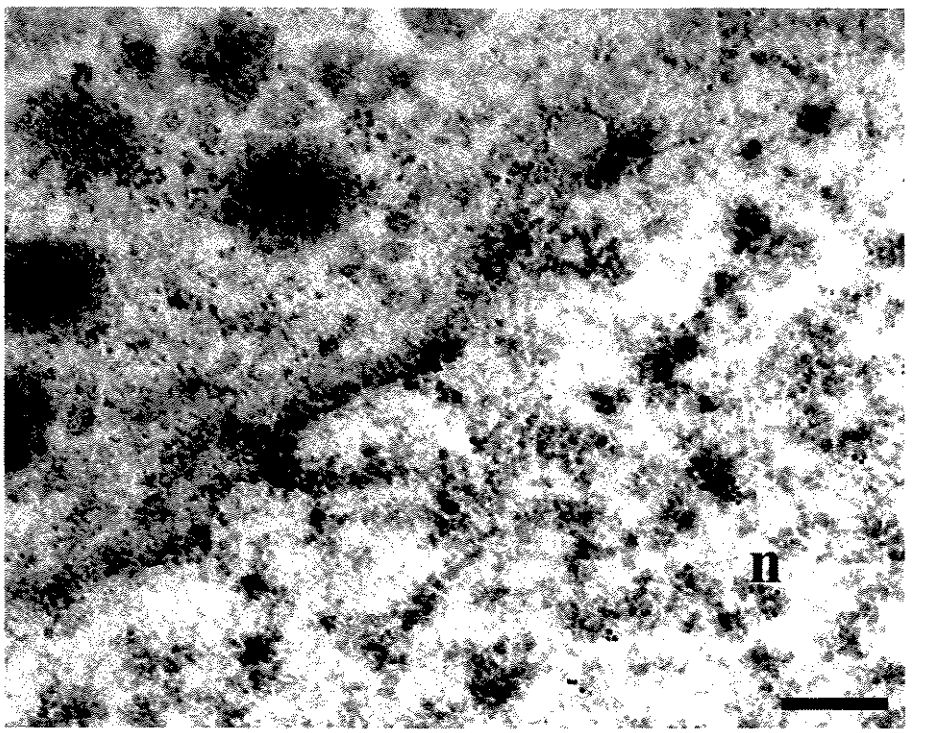

Figure 4. Magnification of an SHR DRG neuron showing CGRP located in vesicles and free in the cytosol. In the nucleus (n), this neuropeptide is associated with peripheral deposits or disperse heterochromatin. Bare 0,33 μm.

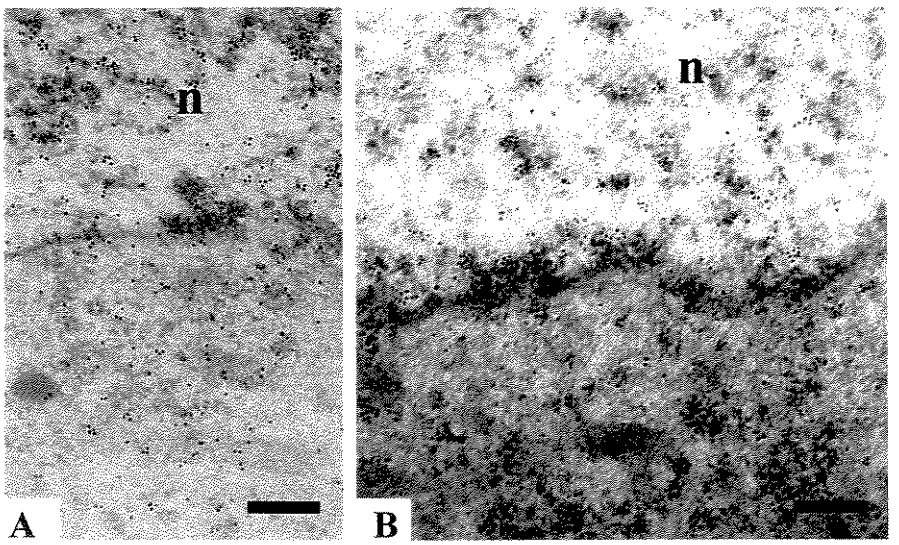

Figure 5. NK_1R in neuron nuclei (n) in WKy (A) and SHR (B) of 14-weak-old. Note the different levels of packing of the chromatin (because the cells are in different phases of protein synthesis and metabolic activity). The nuclei of SHR neurons have more NK_1R, which suggests that these receptors may be involved in the level of chromatin packing. (B) Shows the peripheral deposition of heterochromatin associated with the nuclear envelop and nuclear lamina. Bars = 0,33 μm.

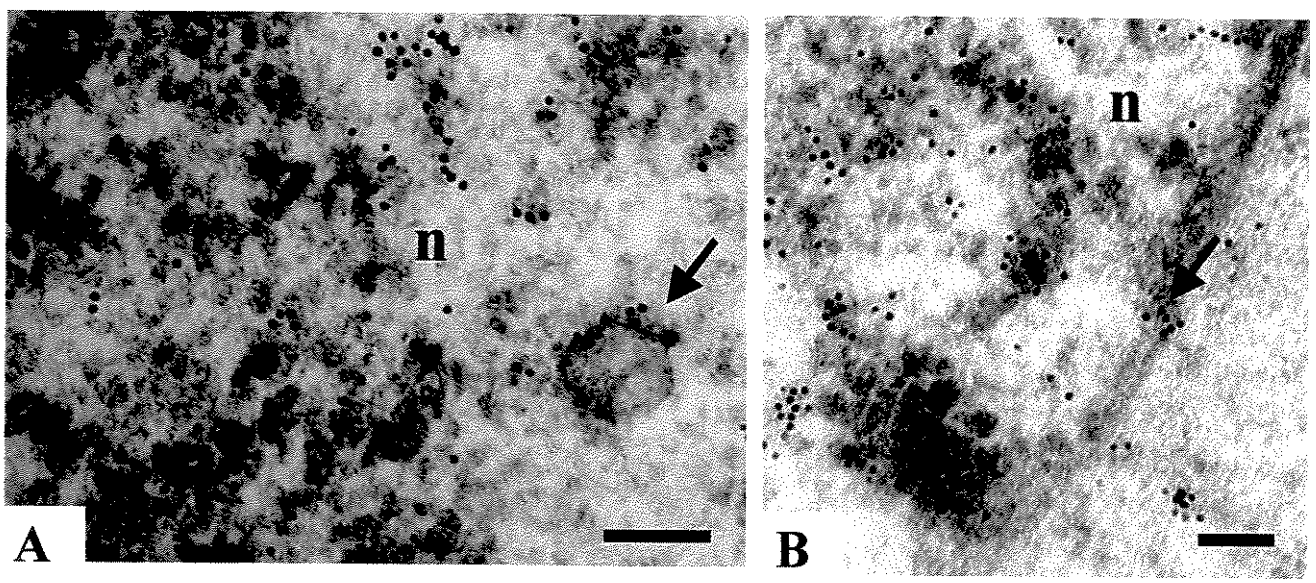

Figure 6. (A) Is a higher magnification of the nucleoplasm of an SHR neuron. The arrow indicates a vesicle with NK_1R in the membrane. (B) Another view of the nuclear-cytosolic interface. A cluster of NK_1R (arrow) can be seen between the cytosol and nucleus where there is no pore complex. This image shows the insertion of this receptor in the internal and external nuclear membrane and in the inter-membrane space of the nucle ar envelope.Bars = 0,1 μm.

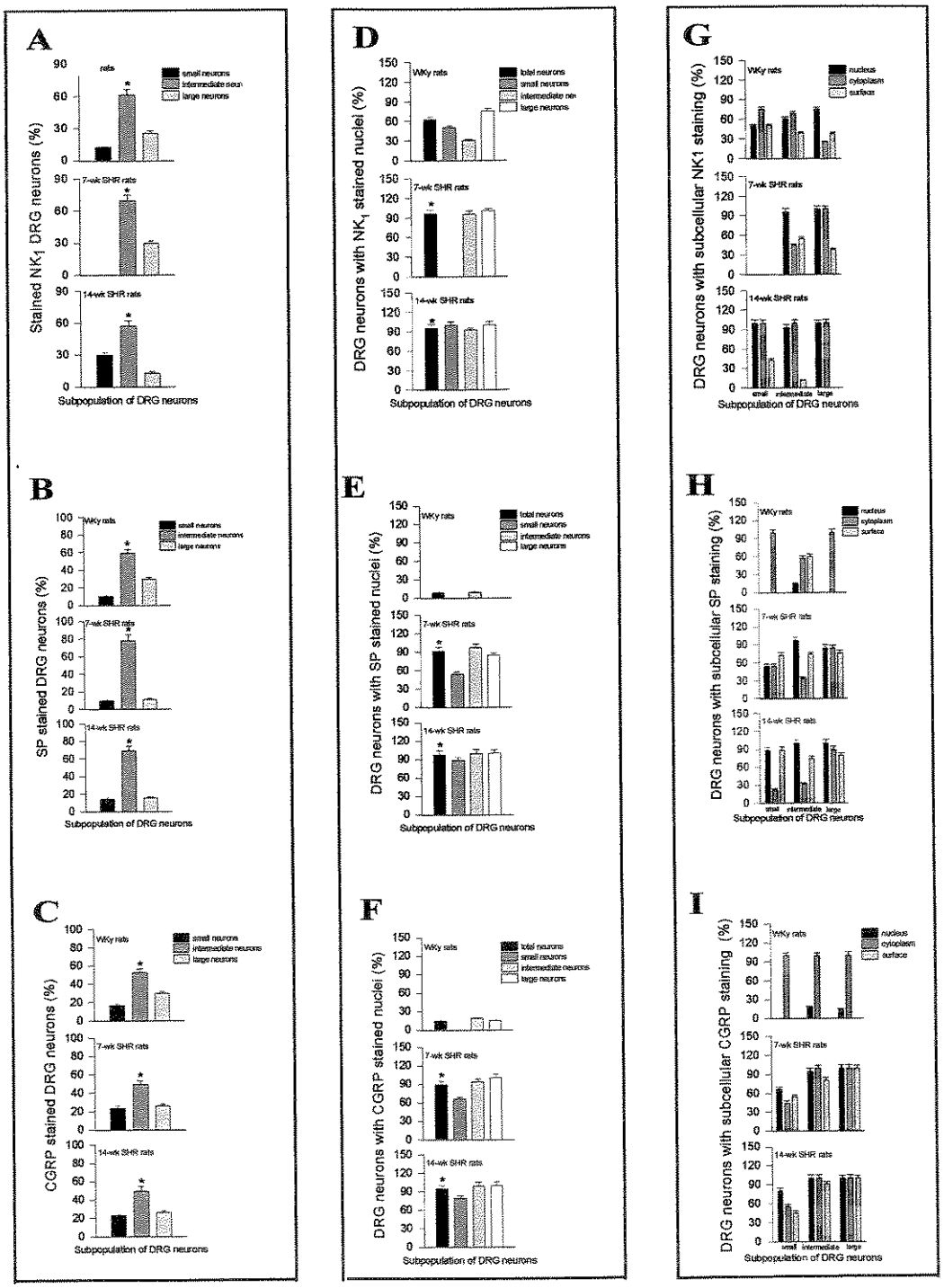

Figure 7. Distribution of NK_1R-, SP- and CGRP-positive immunoreactivity in five DRG sections cells based on optical CLSM sections. **A**, **B** and **C** represent a unimodal distribution, for size. In **D**, **E** and **F**, nuclear distribution for the cell size. The sub cellular distribution in DRG cell subpopulations are represented in **G**, **H** and **I**.

4. DISCUSSÃO GERAL

Um dos objetivos deste estudo foi avaliar a influência da atividade neural renal sobre a manipulação tubular de sódio em ratos Okamoto-Aoki jovens e adultos. Nossos resultados demonstram que SHR jovens retêm mais sódio e desenvolvem hipertensão após a sexta semana de vida, embora mantidos em dieta normossódica. Estes resultados confirmam aqueles, obtidos em pesquisas anteriores, mostrando diferenças significativas na resposta natriurética após denervação renal bilateral. Indicando efeito tubular direto, a denervação renal em SHR causou aumento da natriurese secundária à elevação significativa na rejeição tubular urinária de sódio. Estes resultados sugerem que a influência da atividade neural sobre a reabsorção de sódio pelos túbulos proximais pode ser importante na patogênese da hipertensão em SHR.

Além disso, entre a 3ª e 7ª semanas, os SHR apresentaram aumento na retenção de sódio e excreção de sódio urinário significativamente diferente, indicando anormalidades em mecanismos renais. Com 12 semanas a excreção de sódio, nestes animais, foi normalizada.

Similarmente, BIANCHI et al. (1975), demonstraram que em MHS jovens existe um período de elevação no balanço positivo de sódio durante o desenvolvimento da hipertensão e, após oito semanas, tais diferenças são abolidas. Previamente, HERLITZ et al. (1979), reportaram que SHR de sete semanas excretam menos sódio urinário que os WKy. Com 12 semanas a excreção de sódio é igual (ROMAN e COWLEY, 1985; HARRAP, 1986; HALL et al., 1996), entretanto, quando a pressão de perfusão em SHR é reduzida a níveis observados em WKy, SHR excreta menos sódio urinário (ROMAN e COWLEY, 1985). Nossos e outros resultados indicam que os rins de SHR precisam de maior pressão arterial para excretar a mesma quantidade de sal que WKy.

A retenção de sódio poderia contribuir para o desenvolvimento da hipertensão por interagir com uma variedade de mecanismos como a vasoconstriçao (SOFOLA et al., 2002), a expansão do volume extracelular efetivo (SCHAFER, 2002) ou a hiperatividade do sistema

nervoso simpático (STRAZZULLO et al., 2001). Entretanto, o mecanismo exato pelo qual ocorre retenção de sódio em SHR jovens permanece desconhecido. Alguns estudos evidenciam alterações funcionais na vasculatura glomerular associada a redução do taxa de filtração glomerular e do fluxo sanguíneo renal bem como no metabolismo de potássio (DILLEY et al., 1984; ZHOU e FROHLICH, 2001). Entretanto, nós não observamos alterações significativas no CCr, nem tampouco nas concentrações de potássio plasmática e urinária, sugerindo que a elevação pressórica teria efeito direto antinatriurético nos segmentos tubulares proximais e não nas porções onde se localizam os trocadores sódio-potássio.

Estudos genéticos têm auxiliado no esclarecimento da gênese de possíveis alterações na manipulação de sódio renal que causariam elevação na pressão sanguínea. Dentre os genes cuja mutação pode exercer efeitos sobre a pressão arterial, a grande maioria codifica proteínas envolvidas diretamente no transporte de sódio tubular ou na sua regulação endócrina e parácrina (ZHANG et al., 1998; WILSON et al., 2001). Entretanto, tais mutações acometem menos de 1% dos pacientes hipertensos. A grande maioria dos casos de hipertensão arterial resulta da interação de variáveis múltiplas como estilo de vida, fatores metabólicos e genéticos e não em conseqüência da alteração em um único gene.

A influência do sistema nervoso simpático (SNS) sobre a função renal durante o desenvolvimento pressórico é pouco explorada. Vários investigadores sugerem o envolvimento do SNS na patogênese da hipertensão arterial devido a sua influência no controle da função renal. Assim, a elevação no tônus adrenérgico renal eferente, poderia promover retenção urinaria de sal devido à constrição arteriolar e/ou ao aumento da reabsorção tubular. O estimulo elétrico de nervos renais, em experimentos agudos, produz aumento na reabsorção de sódio, particularmente pelo segmento contorcido do túbulo proximal (DIBONA, 2002). O tráfego neural eferente renal é elevado em SHR de oito semanas (JUDY e FARREL, 1980). Além disso, tanto o estímulo

elétrico dos nervos renais, em baixa freqüência, quanto à infusão intra-renal de norepinefrina podem produzir hipertensão (COLLIS et al., 1980; PATEL et al., 1981). Isso ocorre devido ao aumento na reabsorção de sódio pelo túbulo proximal e alça de Henle, sem que ocorram alterações na hemodinâmica renal (DIBONA e KOPP, 1997; DIBONA, 2000). Em contrapartida, estes e outros autores (RUDD et al., 1986; OPARIL, 1987; KLINE, 1987) têm demonstrado que a denervação renal bilateral, em SHR de três a oito semanas, abole o desenvolvimento de hipertensão durante três semanas, paralelamente à redução na reabsorção de sódio tanto pelo túbulo proximal quanto pela alça de Henle e túbulo distal. Nossos resultados demonstram aumento na rejeição tubular proximal de fluido e baixa atividade reabsortiva compensatória distal. O aumento na excreção fracional de sódio, observado três semanas após denervaçao, evidencia a relação funcional entre a atividade neural renal e a atenuação na excreção de sódio, em SHR, que leva ao desenvolvimento de hipertensão nesta linhagem. Já a elevação pressórica, observada oito semanas após a denervação, esteve associada ao aumento no conteúdo de norepinefrina, um indicador de reinervação renal (OPARIL, 1987; KLINE, 1987).

Nossos resultados sugerem que a atividade neural renal, associada ao aumento na reabsorção de sódio proximal, medeia, pelo menos em parte, a elevação pressórica em linhagens geneticamente hipertensas. Porém, não podemos descartar a possível ação de uma série de fatores humorais que poderiam estar envolvidos na natriurese aqui observada. Entretanto, tais resultados permitem que especulemos sobre a influência neural na habilidade de manipulação tubular renal e no equilíbrio hidro-eletrolítico.

Sabemos ainda que, embora o efeito da denervação possa refletir inibição na atividade simpática, tal procedimento priva os rins também da inervação sensora. A inervação aferente renal gera efeitos envolvidos no reflexo renorenal simpático e na excreção urinaria de sódio

(DIBONA e KOPP, 1997; GONTIJO et al., 1999). Nossos experimentos não nos permitem distinguir entre os efeitos da denervaçao eferente e aferente.

No que se refere à inervação aferente renal sabemos que o estimulo de MRs e CRs, presentes nas terminações neurais pélvicas, é transmitido a neurônios presentes nos gânglios da raiz dorsal. Posteriormente, tal estimulo é transmitido ao corno dorsal da medula espinhal, causando liberação de neuropeptídeos como SP e CGRP, que estão estocados, freqüentemente, nas mesmas vesículas (NIIJIMA, 1975; DONOVAN et al., 1983; KNUEPFER e SCHRAMM, 1987; KUMMER et al., 1989; KNIGHT et al., 1991; TAMAKI et al., 1992).

Os efeitos atribuídos à SP têm sido relacionados à sua interação com o receptor NK_1R localizado na membrana plasmática (PENNEFATHER, 2004) porém, sua ocorrência e distribuição sub-celular em GRD ainda não foi demonstrada. Desta forma, este estudo apresenta novas evidências de localização nuclear deste receptor em células neurais do GRD. A localização nuclear pode ser atribuída tanto à sua translocação da membrana para o núcleo, quanto à síntese *de novo* deste receptor. Como estas células estão expostas à SP circulante a internalização, mediada pela associação ligante-receptor e translocação nuclear, pode ser uma possibilidade. NK_1R, em sistema nervoso central de ratos *in vivo*, localiza-se primariamente na membrana plasmática e após fosforilação do receptor ocorre internalização do complexo ligante-receptor, sendo a SP degradada em lisossomos e NK_1R defosforilado, e reciclado para a membrana celular (MANTYH et al., 1995). Estes autores verificaram também que a internalização endossomal do complexo ocorre em toda superfície neuronal incluindo os dendritos expostos ao agonista. Os neurônios pseudounipolares do GRD não apresentam dendritos e nossos resultados indicam a presença do receptor nas projeções de membrana dos corpos celulares de neurônios e células satélite. Tais achados são importantes se considerarmos que, após a ligação do agonista, ocorre

internalização e reorganização estrutural da membrana o que, conseqüentemente, provoca alterações na inter-relação destas células e nas propriedades eletrofisiológicas do pericário.

Estão surgindo evidências que demonstram a localização de receptores de fatores de crescimento ligados à proteína G, nas membranas nucleares e regiões perinucleares. Estudos têm ainda, demonstrado a localização nuclear dos receptores de angiotensina II, AT1 (RE et al., 1984; BOOZ et al., 1992), do fator de crescimento epidermal (CARPENTIER et al., 1986), de insulina (VIGNERI et al., 1978; HORVAT, 1978), de interferon β (KUSHNARYOV et al., 1985), muscarínicos colinérgicos (LIND e CAVANAGH, 1993), do fator de crescimento neural (YANKNER e SHOOTER, 1979), de prostaglandina E_2 (PGE_2) (BHATTACHARYA et al., 1998; 1999), e opioide (BELCHEVA et al., 1993). Como já citado estes receptores podem ser internalizados após ligação com o agonista ou sintetizados e enviados ao núcleo. Os receptores AT1 e EGF-R são translocados para o núcleo após a ligação com o agonista (LU et al., 1998; MARTI et al., 2001). Por outro lado PGE_2 é captada pela via transportadora de prostaglandina e irá agir em receptores nucleares (GOBEIL et al, 2002). Para que os NK_1Rs intracelulares tenham relevância funcional, eles devem ter uma fonte de ligantes intracelulares e, estudos em seres humanos têm demonstrado a ocorrência de duas isoformas de NK_1R indicando ativação diferencial de efetores intracelulares que poderiam gerar efeitos biológicos diferentes para SP. Além disso, receptores NK_1 apresentam seletividade distinta ao agonista em diferentes tecidos (IVERSEN et al., 1987), sugerindo a possibilidade de sítios de ligação e/ou vias de segundo mensageiro distintos para este receptor. Hoje sabemos que as vias de sinalização nucleares influem na regulação do transporte nuclear, na expressão gênica e na formação do envoltório nuclear.

Componentes chave de várias vias de sinalização estão presentes no envoltório nuclear, no núcleo e no recentemente descrito retículo nucleoplasmático, o que fundamenta a presença de cascatas de sinalização nucleares. Evidencias sugerem a presença de proteína-G no núcleo (WILLARD e CROUCH, 2000). Estudos biofísicos e microscópicos suportam a presença de efetores como adenilato ciclase (YAMAMOTO et al., 1998), fosfodiesterase (LUGNIER et al., 1999), diacilglicerol quinase (TOPHAM et al., 1998), fosfolipase A_2 (FATIMA et al., 2003), fosfolipase C (FAENZA et al., 2000), fosfolipase D (BALDASSARE et al., 1997), e fosfatidilinositol 3-quinase (DIDICHENKO e THELEN, 2001).

As membranas nucleares contêm Ca^{2+}-ATPase sarco/endoplasmática (SERCA)-bombas (ABRENICA e GILCHRIST, 2000) bem como canais de Ca^{2+}-rianodine (ABRENICA e GILCHRIST, 2000) e canais de Ca^{2+} IP_3-sensíveis (GUIHARD et al., 1997). Processos de sinalização, associados ao envoltório nuclear, envolvem produtos do metabolismo lipídico como 1,2-diacilglicerol (DG) e 1,4,5- trifosfato de inositol (IP3) (IRVINE, 2002). DG recruta ou ativa proteína quinase C, que fosforila proteínas nucleares gerando liberação de cálcio, via IP3, das cisternas nucleares aumentando a concentração nucleoplasmático de cálcio. Além disso, o estímulo de receptores de PGE2 (BHATTACARYA et al., 1998) provoca influxo de cálcio nuclear.

Os sinais nucleares de Ca^{2+}, que são independentes dos citosólicos, regulam a transcrição gênica, a apoptose, o reparo gênico, atividade das polimerases e ativação de tropoisomerases. Além disso, os efeitos regulatórios mediados pela liberação de Ca^{2+} no nucleoplasma, alteram o estado conformacional dos complexos de poro nucleares, inibindo a difusão de moléculas entre núcleo e citosol (STEHNO-BITTEL et al., 1995). Moléculas menores que 9 nm ou 60 kDa difundem-se passivamente pelos complexos de poro nucleares. NK_1R, SP e CGRP têm 46, 1.5 e

3.8 KDa, respectivamente, e quando sintetizados pelos neurônios podem ser translocados para o núcleo através de processos mediados pelo Ca^{2+}.

A localização sub-celular de NK_1R pode refletir sua importância nas respostas iniciais à SP, quando localizado na membrana plasmática, estando sua presença nuclear relacionada a repostas somente significativas em longo prazo. A resposta biológica a insulina também é caracterizada por efeitos primários na superfície celular seguidos por alterações, em longo prazo, no metabolismo celular (LOSTROH e KRAHL, 1974).

Estudos recentes têm evidenciado a ocorrência de elevações na concentração endógena de SP e CGRP e aumento na expressão de NK_1R, na medula espinal após ativação periférica de nociceptores (SEYBOLD et al., 2003). Assim, a expressão gênica pode ser mediada por receptores sensoriais, controlando o conteúdo neuronal de neuropeptideos (MENARD et al., 1996; POWELL et al, 2000). Além disso, poderiam ainda haver outras moléculas sinalizadoras envolvidas no controle da expressão gênica de NK_1R.

SP e CGRP estão colocalizados em neurônios primários aferentes (HELKE et al., 1980; DONOVAN et al., 1983; KUMMER et al., 1989; KNIGHT et al., 1991; TAMAKI et al., 1992). CGRP produz elevação na biodisponibilidade de SP (GONTIJO et al., 1999) e pode aumentar a expressão de receptores ativados por SP (SEYBOLD et al., 2003). Tais interações podem ainda variar nas três sub populações de neurônios que expressam NK_1R, SP e CGRP no GRD.

Até o momento, os efeitos induzidos por SP eram atribuídos à sua associação com NK_1Rs presentes na membrana plasmática. Nossos resultados evidenciam a presença destes receptores associados à heterocromatina de cerca de 30 % das três populações de neurônios presentes nos GRD (pequenos médios e grandes). Além disso, a presença nuclear deste receptor foi também evidenciada nas células de Schwann e satélite. Os mecanismos de captação nuclear e associação a sítios aceptores presentes na cromatina, bem como seu papel junto à heterocromatina, ainda

deverão ser investigados. De qualquer forma, para que os NK_1Rs nucleares tenham relevância funcional é necessária uma fonte de ligante que pode ser proveniente da captação de SP extracelular e/ou produção deste neuropeptídeo pelas células neurais que deveriam, neste caso, agir intracelularmente como mediadores "intácrinos".

Como citado anteriormente, a colocalização nuclear de NK_1R, SP e CGRP em neurônios do GRD pode refletir interações funcionais entre estes neuropeptideos em animais normotensos. Já em SHR de sete semanas, nós verificamos decréscimo significativo na expressão de NK_1R na membrana celular. Tal decréscimo progrediu tornando difícil a imuno detecção deste receptor na membrana dos neurônios de animais com 14 semanas. Estudos anteriores (KOPP et al., 1984; 1996a; 1996b; KOPP e SMITH, 1993) têm demonstrado que o aumento na pressão pélvica renal leva à elevação ipsilateral da ARNA, liberação pélvica ipsilateral de SP e excreção contralateral de sódio urinário em WKy. Entretanto, um aumento, da mesma magnitude, no estimulo neural sensor em SHR não produz elevação na ARNA ipsilateral nem tampouco a excreção de sódio urinário contralateral. Tal perda da atividade reflexa renorenal, em SHR, está associada ao decréscimo na liberação pélvica de SP (KOPP et al, 1987; 1998). Porém, a administração pélvica de SP não produz aumento na ARNA de SHR em concentrações que produzem elevação significativa deste parâmetro em WKy (KOPP et al., 1998). Estudos prévios (MOSS, 1984; KOPP et al., 1987; GONTIJO e KOPP, 1994; GONTIJO et al., 1999) em ratos normotensos, demonstram que SP e CGRP produzem resposta similar no reflexo renorenal. Além disso, o tratamento com agonistas dos receptores de SP e CGRP ou com capsaicina, que depleta a SP dos neurônios sensores, bloqueou a resposta da ARNA ao aumento da pressão pélvica renal (KOPP e SMITH, 1991b; 1993; GONTIJO e KOPP, 1994; GONTIJO et al., 1999). Tais resultados sugerem que a perda da sensibilidade dos receptores sensoriais pélvicos em SHR, não está relacionada somente ao decréscimo na liberação pélvica de SP, mas pode estar associado à

redução na quantidade de NK_1R, como revelado em nosso trabalho pelas técnicas de imunofluorescência e imunobloting, em SHR de sete e 14 semanas. Estas observações são relevantes, podendo gerar uma nova linha de investigação sobre a etiologia da perda sensorial na pelve renal associada ao estabelecimento de hipertensão em SHR.

Os mecanismos, possivelmente envolvidos na ativação de receptores sensores renais, incluem ativação do cálcio intracelular em resposta a liberação de SP e CGRP nas terminações neurais. Recentes estudos *in vitro*, em cultura de neurônios do GRD, têm demonstrado que PDBu aumenta a liberação de SP, mediada por cálcio, nestes neurônios (BARBER e VASKO, 1996). As sinalizações produzidas pela via da fosfoinositidase C-PKC são alteradas em SHR (TAKATA e KATO, 1996), de modo que o tratamento com o ativador de PKC (PDBu) não produz aumento na ARNA em SHR (KOPP e SMITH, 1996; KOPP et al., 1998). Estes resultados estão de acordo com aqueles obtidos em estudos prévios, demonstrando a incapacidade de SP em produzir resposta sensorial nestes animais (MORI et al., 1993).

O stress mental pode resultar em redução na concentração de SP na membrana celular de neurônios em modelos hipertensos (FAULHABER et al., 1987), e vários estudos demonstram, tanto em seres humanos quanto em ratos hipertensos, aumento na sensibilidade à dor (ZAMIR e SHUBER, 1980; VIRUS et al., 1981; SITSEN e De JONG, 1983; GUASTI et al., 1995; SCHOBEL et al., 1996; IRVINE e WHITE, 1997). Estes estudos sugerem que a perda da resposta sensorial renal em SHR está, parcialmente, relacionada a alterações tanto na síntese quanto na secreção de SP, por estes neurônios sensores. Entretanto, contrariamente a esta sugestão, nossos resultados demonstram significativo aumento no conteúdo de SP em neurônios dos GRD de SHR. Nossos resultados sugerem que a perda da capacidade de elevação da ARNA, em resposta a ativação química ou mecânica em SHR, está relacionada ao decréscimo na expressão e/ou na localização de NK_1R nas membranas, tanto no pericário quanto no axônio, de

neurônios sensores pélvicos. Observações em tecido vascular também têm concluído que ocorre diminuição na expressão de receptores para SP, na membrana axonal, em modelos de hipertensão (VIRUS et al., 1981; POMPEI et al., 1993; PANZA et al., 1994; EGASHIRA et al., 1995; HUANG e KOLLER, 1996; QUYYUMI et al., 1997).

NK_1R tem vasta distribuição em vários tecidos e órgãos. A alta concentração deste receptor no sistema nervoso sensor demonstra que NK_1R tem papel importante no controle da atividade neural. Como já citado, a ativação de NK_1R medeia a resposta sensora da pelve renal em ratos normotensos (KOPP e SMITH, 1991a; 1991b: 1993; KOPPet al., 1997). Nossos resultados, demonstrando redução na imunolocalizaçao e na expressão de NK_1R na membrana de neurônios dos GRD, podem explicar a perda da atividade receptora sensorial em SHR.

Em complementação aos resultados obtidos por imunofluorescência, o estudo de imunolocalizaçao com ouro coloidal por microscopia eletrônica de transmissão, nos demonstrou, em SHR, a ocorrência de grande quantidade de SP e NK_1R em vesículas citosólicos do pericário. Além disso, nesses animais verificamos aumento na quantidade deste neuropeptídeo e de seu receptor em associação a heterocromatina paralelamente a redução da localização destes na membrana. Estes resultados indicam que a perda da resposta sensorial renal em SHR (KOPP et al., 1987; 1998), poderia resultar de alterações no transporte de NK_1R para a membrana celular e/ou defeito na via de sinalização que resulta da interação NK_1R-SP.

SP e CGRP são vasodilatadores potentes e a síntese destes neuropeptídeos ocorre nos DRGs. Em modelos de hipertensão (mineralocorticoid-salt hypertension) tem sido verificado aumento nos níveis de RNAm e na imunoreatividade para CGRP, tanto em GRDs quanto na medula espinhal (SUPOWIT et al., 1994, 1995, 1997). Após a síntese, CGRP é transportado em vesículas, tanto em axônios que inervam os vasos sanguíneos quanto naqueles que se dirigem a áreas responsáveis pelo controle da pressão sanguínea na medula espinhal. O aumento na síntese

deste potente vasodilatador representa uma resposta compensatória no sentido de atenuar o aumento na pressão sanguínea. Entretanto, não sabemos se sua expressão é aumentada apenas como resultado da elevação pressórica ou se existe a interferência de outros parâmetros. O significativo aumento no conteúdo de SP e CGRP em DGRs antes da ocorrência de elevação pressórica, observado em SHR de sete semanas, sugere-nos que não é somente a hipertensão que provoca tal aumento no conteúdo destes neupeptídeos neuronais. Desta forma, sugerimos a ocorrência de alterações específicas em SHR, por fatores não identificados, que modulariam a síntese e secreção de SP e CGRP. Os resultados da literatura, nos quais foi feita detecção de imunoreatividade para CGRP em seres humanos hipertensos, variam consideravelmente sendo que alguns investigadores demonstram aumento (MASUDA et al., 1992), outros não detectaram alterações (SCHIFTER et al., 1991), e outros detectaram decréscimo na expressão deste neuropeptídeo (EDVINSSON et al., 1989). Tal variabilidade tem sido atribuída à natureza heterogênea da hipertensão ou aos diferentes procedimentos utilizados. Também em modelos de roedores hipertensos, têm sido relatados resultados contraditórios quanto ao conteúdo de CGRP (SUPOWIT et al., 1995; SUPOWIT et al., 2001).

Uma possível explicação, para a elevação no conteúdo de neuropeptideos no pericário, seria a ocorrência de alterações no transporte axonal de SP e CGRP em SHR. Outra possibilidade de interferência na resposta sensorial seria a ocorrência de transporte axonal assimétrico entre as terminações centrais e periféricas (TOMLINSON et al., 1988) como observado em modelos experimentais de diabetes.

Embora não tenhamos identificado os fatores que medeiam o aumento na expressão neuronal de CGRP em SHR, podemos sugerir como candidatos neutotrofinas, bradicinina, prostaglandinas e o sistema nervoso simpático. A expressão de neurotrofina é significativamente maior em SHR durante as quatro primeiras semanas de vida e, da 2ª a 10ª semana, este aumento

foi evidenciado no tecido cardíaco e na artéria mesentérica. Tal elevação, em gânglios simpáticos e em órgãos vastamente inervada de SHR, indica que a neurotrofina pode ter papel preponderante no desenvolvimento de uma rede neural, possivelmente por atuar tanto na manutenção quanto na ramificação de neurônios simpáticos (ZHANG e RUSH, 2001). O fator de crescimento neural (NGF) atua via receptores tirosino-quinase (trkA) para controlar a síntese de vários neuropeptideos e regular a excitabilidade da membrana neuronal (EHLERS et al., 1995). Usando cultura primária de neurônios do GRD de ratos adultos, LINDSAY (1988) demonstrou que NGF pode aumentar diretamente tanto a síntese quanto a secreção de CGRP. O início da sinalização por NGF ocorre via associação com um receptor pelo qual tem alta afinidade (trkA) em terminações neurais nociceptoras (CHAO e HEMPSTEAD, 1995; KAPLAN e MILLER, 1997). O complexo ligante-receptor é então internalizado e transportado retrogradamente do axônio para o corpo celular (EHLERS et al., 1995). As neurotrofinas poderiam produzir rápida elevação na fosforilação de resíduos tirosina de receptores trk que se propagaria retrogradamente ao longo do axônio. Desta forma estes eventos podem ser mediados pelo transporte do complexo ligante-receptor e podem também, envolver processos indefinidos de transporte axonal devido a grande velocidade da resposta (EHLERS et al., 1995; BHATTACHARYA et al., 1997; SENGER e CAMPENOT, 1997). Sabe-se que a ativação da via de segundos mensageiros do receptor trk, como proteínas G, quinases reguladas por sinais extracelulares (ERKs), e fosfolipase C-γ (PLC-γ) participam do rápido transporte retrógrado axonal, porém o mecanismo exato é desconhecido (JOHANSSON et al., 1995). Estudos têm demonstrado que os neurônios têm capacidade de transportar fatores de transcrição via axônios (SCHMIED et al., 1993; CURTIS e DI STEFANO, 1994; O'NEILL e KALTSCHMIDT, 1997). Estudos em *Aplysia* têm detectado sinal de localização nuclear (NLS) em proteínas transportadas retrogradamente e posterior transporte destas para o núcleo (SCHMIED et al., 1993). Assim, NGF pode manter o fenótipo de uma

subpopulação de neurônios nociceptores em vertebrados adultos, parcialmente, via regulação da expressão de SP e CGRP.

Embora tenhamos demonstrado diminuição na expressão de NK_1R nos DGR de SHR, o conhecimento sobre as vias de sinalização envolvidas no controle da expressão deste receptor é extremamente pobre. Como a região promotora do receptor NK_1 contém o elemento de resposta ao AMPc (CRE), nós supomos que os níveis elevados de CGRP, nos núcleos de neurônios do DGR, poderiam regular a expressão de receptores NK_1, via proteína ligante ao elemento de resposta ao AMPc (CREB) (SEYBOLD et al., 2003). Adicionalmente, nossos resultados definem a possibilidade de um papel para CGRP e SP como moléculas sinalizadoras, podendo regular a expressão de receptores NK_1 em neurônios sensoriais de DGR. A presença de NK_1R no núcleo pode ainda, estar associada ao controle da expressão gênica no CNS, além de nos dar uma nova dimensão para o entendimento da co-transmissão de sinais via SP e CGRP em neurônios aferentes primários.

Enfim, nossos resultados indicam que alterações na expressão e localização de neuropeptídeos e NK_1R, podem estar relacionados à atenuada ARNA, com conseqüente elevação da ERSNA, retenção renal de sódio e hipertensão arterial em SHR.

5. CONCLUSÃO GERAL

- Em WKy, CGRP, SP e seu receptor NK_1 podem estar localizados na membrana, no citosol e no núcleo, associados a heterocromatina, nas três populações de neurônios do GRD. As células satélite e de Schwann do GRD apresentam a mesma distribuição de NK_1R.

- Da 4ª a 12ª semana de vida, SHR apresenta elevação na pressão arterial cerca de 6 vezes maior que a observada em WKy no mesmo período. Em SHR, tal elevação pressórica ocorre paralelamente ao aumento na reabsorção tubular, proximal e pós-proximal, de sódio e redução da natriurese.

- Em SHR, a denervaçao renal bilateral, na 3ª semana de vida, abole o aumento pressórico durante as 5 semanas seguintes. Paralelamente, estes animais apresentam diminuição na excreção urinaria fracional de sódio em virtude do aumento na rejeição tubular proximal de sódio e baixa atividade reabsortiva compensatória pós-proximal.

- A expressão nuclear de CGRP, SP e NK_1R é significativamente maior em neurônios do GRD de SHR de 7 semanas, quando comparada a de WKy. Paralelamente, nestes SHR ocorre redução progressiva do receptor NK_1 na membrana celular destes neurônios até a 14ª semana de vida.

- A pelve renal de SHR, com 7 e 14 semanas, apresenta quantidade significativamente menor de receptores NK_1, quando comparada a WKy.

- Alterações tanto na expressão quanto na localização de CGRP, SP e NK_1R, podem estar relacionados à atenuada ARNA com conseqüente elevação da ERSNA, retenção renal de sódio e hipertensão arterial em SHR.

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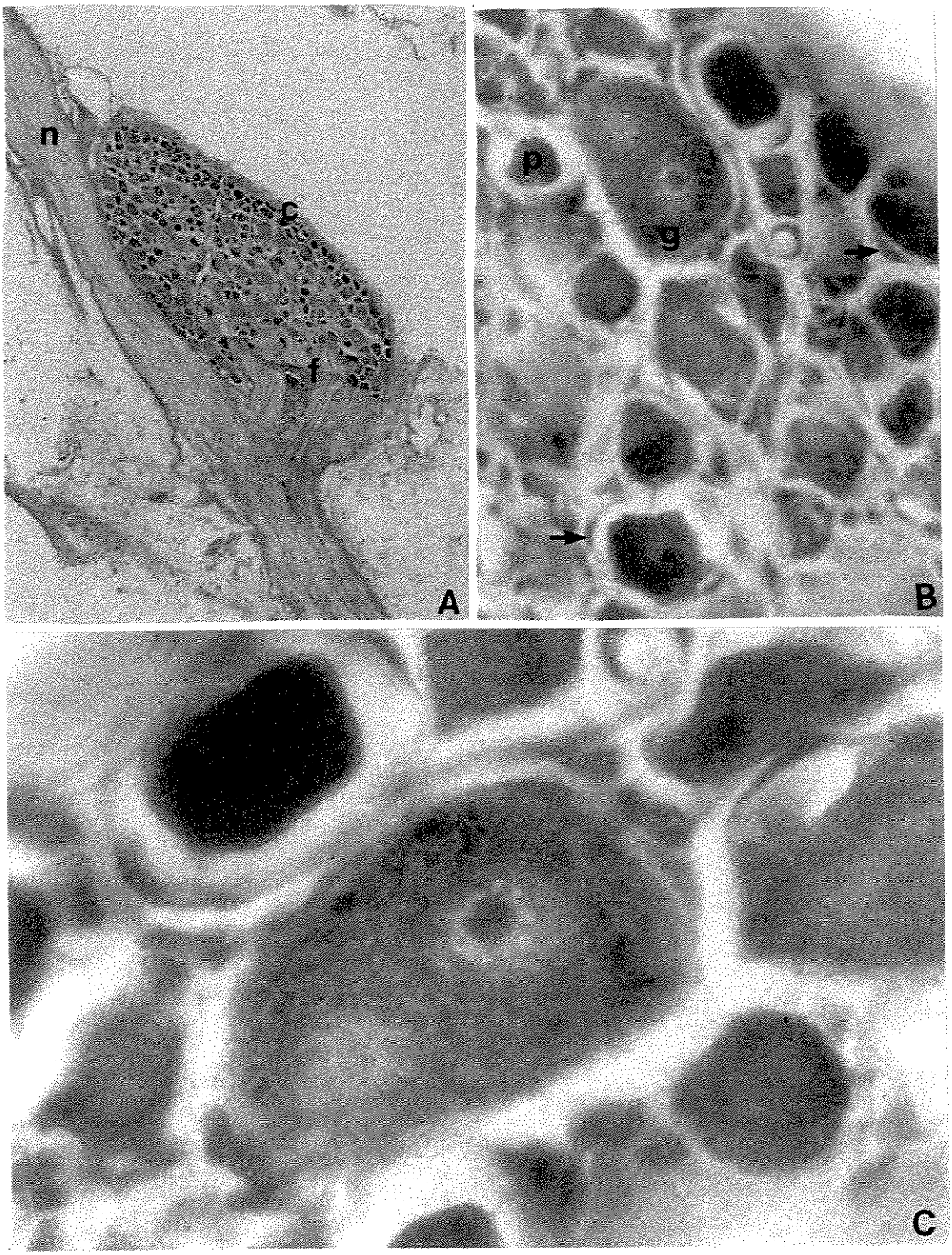
7. ANEXOS

IMAGENS DE GRDs T_{13} DE ANIMAIS DE 7 SEMANAS, OBTIDAS EM MICROSCOPIO DE FLUORESCENCIA, APÓS SEREM SUBMETIDOS A IMUNOISTOQUIMICA PARA SP:

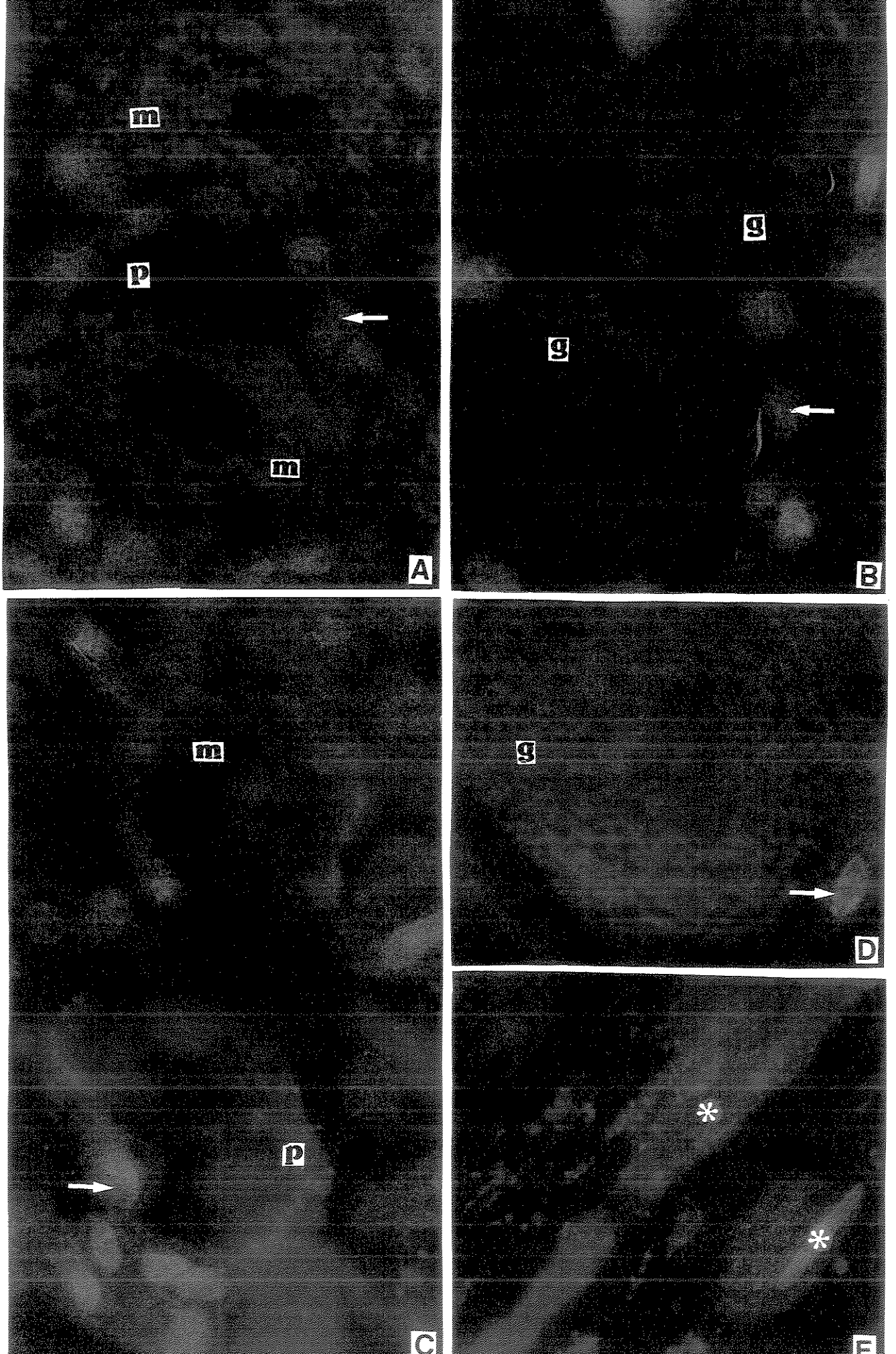
Em vermelho, podem ser observados padrões de distribuição deste neuropeptídeo em precários pequenos (**p**), médios (**m**) e grandes (**g**). Os núcleos das células satélite apresentam-se corados em azul (**setas**). Esta fluorescência foi obtida com DAPI, sendo que neste caso, o fluorocromo se liga especificamente ao DNA. **A** e **B** representam a imunolocalização obtida em animais WKy e **C** e **D** representam a marcação em SHR. Em **E**, podemos observar SP em feixes de axônios e os núcleos das células de Schwann (**asteriscos**), responsáveis pela mielinização dos axônios no sistema nervoso periférico (1.500x).

Estas foram as primeiras imagens que obtivemos e, embora já nos dessem indícios de maior concentração nuclear de SP em SHR, tal aspecto poderia ser proveniente de localização no Complexo de Golgi, que se localiza ao redor do núcleo. Isto devido ao fato desta microscopia refletir toda a fluorescência existente na espessura do corte histológico. Somente a analise em CLSM, com obtenção de secções ópticas e significativa melhora na relação sinal-ruído, nos permitiu afirmar tal localização.



GRD T_{13} DE ANIMAL WKy, (7 SEMANAS) CORADO COM HE:

A- Vista panorâmica de um corte longitudinal do gânglio, onde podem ser observadas as seguintes estruturas: a cápsula (**c**) de tecido conjuntivo denso, o nervo da raiz dorsal (**n**), feixes de fibras nervosas (**f**) e corpos celulares de neurônios de vários tamanhos (60x). **B**- Podemos observar as três populações de neurônios, que se distinguem por apresentarem pericários pequeno (**p**), médio (**m**) e grande (**g**). Observe as células satélite ao redor dos pericários (setas) (600x). **C**- Em maior aumento, neurônios, dos três tamanhos encontrados nos GRDs, podem ser observados (1.500x).


m
p
m
A
g
g
B
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C
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