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Junctional Adhesion Molecule-1 Is Upregulated in Spontaneously Hypertensive Rats

Evidence for a Prohypertensive Role Within the Brain Stem

Hidefumi Waki, Beihui Liu, Masao Miyake, Kiyooki Katahira, David Murphy, Sergey Kasparov, Julian F.R. Paton

Abstract—Junctional adhesion molecule-1 (JAM-1) forms part of the tight junction between adjacent endothelial cells. Using microarray technology, we showed previously that JAM-1 was differentially expressed in the brain stem of spontaneously hypertensive rats compared with normotensive Wistar–Kyoto (WKY) rats. In this study, we quantified the expression of JAM-1 in the brain stem of spontaneously hypertensive rats and WKY rats and established whether any differential expression was confined to this region of the brain or was ubiquitous throughout the central nervous system and, indeed, the whole body. Because the nucleus tractus solitarius plays a pivotal role in arterial pressure regulation, we assessed whether JAM-1 in this region affects the chronic regulation of arterial pressure. Real time RT-PCR revealed that JAM-1 mRNA was upregulated in multiple regions of the brain and all of the peripheral vascular beds studied. In the nucleus tractus solitarius, the level of JAM-1 mRNA was significantly higher in both young (3-week-old, prehypertensive) and adult male spontaneously hypertensive rats (15 to 18 weeks old) than that of age-matched WKY rats (fold differences; prehypertensives: 1.01 ± 0.06 versus 1.59 ± 0.13 ; $n=10$; $P<0.01$; adult: 1.08 ± 0.14 versus 2.86 ± 0.57 ; $n=10$; $P<0.01$). After adenoviral-mediated expression of JAM-1 in the nucleus tractus solitarius of adult WKY rats (15 weeks old; $n=6$), systolic pressure was increased from 120 ± 4 to 132 ± 4 mm Hg ($P<0.01$). Our data suggest that JAM-1 expression in the spontaneously hypertensive rat is upregulated throughout the body compared with the WKY rat and that this is not secondary to the hypertension. When JAM-1 is expressed in the nucleus tractus solitarius, it raises arterial pressure, suggesting a novel prohypertensive role for this protein within the brain stem. (*Hypertension*. 2007;49:1321-1327.)

Key Words: hypertension ■ brain stem ■ inflammation ■ baroreflex control ■ adhesion molecules

Essential hypertension is a complex polygenic trait with underlying genetic components, many of which remain unknown. Unmasking these genes may provide novel information about blood regulation and could be potential future targets for therapeutic intervention. This is pertinent, because $\approx 50\%$ of patients with hypertension on antihypertensive medication continue to have elevated levels of blood pressure (www.heartstats.org).¹ Despite accumulating evidence that gene expression profiles are altered in essential hypertension,^{2,3} prohypertensive genes remain unclear. One confounding factor is that hypertension itself induces changes in gene expression as a secondary effect.

We are exploring the role of the autonomic nervous system in the etiology of hypertension. It is documented that human essential hypertension is associated with a high level of sympathetic nerve activity in humans.^{4–6} Grassi⁶ postulated that neurogenic hypertension is maintained by sympathetic

overactivity and that this may even be causative. Consistent with that viewpoint, Smith et al⁵ have shown sympathetic overactivity in white coat hypertensive subjects. Furthermore, in a rat model of hypertension (the spontaneously hypertensive rat [SHR]), sympathetic nerve activity is also substantially elevated relative to Wistar–Kyoto (WKY) rats.^{7,8} It thus appears that the sympathetic nervous system may play a key role in the manifestation of hypertension in both humans and animal models. Although numerous brain stem regions can control sympathetic nerve activity, the nucleus of the solitary tract (NTS) is one of the key central regions playing a role in the regulation of both the set point of arterial pressure,^{9–11} as well as baroreflex gain control, a feedback mechanism essential for homeostatic regulation of arterial pressure.^{10,12,13} A preliminary cDNA microarray experiment¹⁴ suggested several differentially expressed genes in the NTS between SHR and their progenitor strain, the WKY rat. One of them

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was junctional adhesion molecule (JAM)-1. This gene was upregulated in both adult and juvenile (prehypertensive) SHR and, hence, not secondary to the high blood pressure. JAM-1 is a member of the immunoglobulin superfamily of adhesion molecules that form tight junctions between adjacent endothelial cells and, therefore, form a part of the blood–brain barrier.^{15,16} JAM-1 has other functions, such as promotion of leukocyte-endothelial adhesion and subsequent inflammation.¹⁷ This might be important, because leukocyte accumulation within the systemic circulation was deemed to be a contributing factor for the hypertension in the SHR because of enhanced hemodynamic resistance.¹⁸ In addition, JAM-1 is also a risk factor for both atherosclerosis and atherothrombosis,^{17,19} both of which can be related to hypertension.²⁰

In this study, we have quantified the level of JAM-1 gene expression in the brain stem of SHR relative to WKY rats using real-time RT-PCR and assessed JAM-1 expression in other regions of the brain, as well as numerous peripheral tissues. Our findings indicate that, in the SHR brain (including NTS), expression of JAM-1 mRNA is increased and shifted toward a transcript containing an extended 3' untranslated region (3'UTR); this effectively leads to higher levels of immunoreactive JAM-1 protein. Importantly, chronic overexpression of JAM-1 in the NTS of WKY rats using viral gene transfer raised arterial pressure supporting a novel prohypertensive role for this protein.

Methods

Procedures were carried out according to the United Kingdom Home Office Guidelines on Animals (Scientific Procedures) Act of 1986. The animals were housed individually, allowed normal rat chow and drinking water ad libitum, and kept on a 12-hour light/12-hour dark cycle.

Differential Gene Expression in the SHR and WKY Rats

Extraction of RNA

Both 3- (SHR, n=10; WKY, n=10) and 15- to 18-week-old (SHR, n=10; WKY, n=10) male rats were humanely killed by cervical dislocation. Regions of the brain (cerebellum, cerebral cortex, NTS, paraventricular nuclei, rostral ventrolateral medulla, and supraoptic nuclei) and internal organs (eg, liver, lung, kidney, spleen, skeletal muscle, and heart) were dissected (or punched if brain) rapidly from each animal and homogenized in 400 μ L of TRIzol reagent (Invitrogen). To avoid contamination with genomic DNA, the RNA samples were treated with RNase-free DNase I (Roche Diagnostics GmbH). RNA purity was verified by performing PCR on samples not treated with reverse transcriptase.

Quantitative RT-PCR

β -Actin, JAM-1, JAM-3, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 genes were tested in this study; primer details are available in an online data supplement at <http://hyper.ahajournals.org>. For JAM-1, 2 different sets of primers were used (for the coding part of the transcript, nucleotides 430 to 652 or nucleotides 1145 to 1385 for the 3'UTR, the numbers refer to the GeneBank entry BC065309) to determine potential variation in splicing between SHR and WKY. Real-time RT-PCRs were carried out using a DNA Engine Opticon 2 system (MJ Research) and the QuantiTect SYBR Green RT-PCR kit (Qiagen), as described recently.¹¹ Expression of target genes was assessed in relation to a housekeeping gene (β -actin) using the comparative ($2^{-\Delta\Delta C_T}$) method²¹ in each sample. Fold differences against average values of WKY were calculated.

Immunohistochemistry for JAM-1 in the Brain Stem

Methods for JAM-1 immunohistochemistry are detailed in the data supplement.

Telemetric Recording of Arterial Pressure

Male rats (WKY, 15 weeks old; n=12) were anesthetized with ketamine (60 mg/kg) and medetomidine (250 μ g/kg) intramuscularly. A radio transmitter (Data Sciences International; TA11PA-C40) was implanted to record arterial pressure from the abdominal aorta as described previously.²² Anesthesia was reversed with atipamezole (1 mg/kg). Transmitter implantation occurred 7 to 10 days before the first control arterial pressure readings were obtained.

In Vivo Gene Transfer into NTS

Ad-CMV-JAM-1 (3.24×10^{10} pfu/mL), a replication-deficient recombinant adenoviral vector, driving the expression of JAM-1 under control of the human cytomegalovirus (CMV) promoter was constructed using standard methods of homologous recombination from IMAGE5599898 clone. As a control, Ad-CMV-eGFP (3.20×10^{10} pfu/mL) expressing enhanced green fluorescent protein (eGFP) was used. After control readings, animals were reanesthetized. Four 100-nL bilateral microinjections of viral suspension (either Ad-CMV-JAM-1 or Ad-CMV-eGFP) were made into the NTS at separate sites spanning ± 500 μ m rostral/caudal to the calamus scriptorius, 350 to 600 μ m from midline, and 500 to 600 μ m below the dorsal surface of the medulla. Each injection was made over 1 minute. To avoid postsurgical cardiovascular effects, animals were allowed to recover for 5 days before blood pressure data were sampled. Data analysis was performed using the Hey-Presto system²³ on the day before NTS microinjection of virus and on days 5 to 7 and 14 after microinjections. On these days, arterial pressure was measured continuously for 5 minutes every hour from 9:00 AM until 8:05 AM on the following day. Heart rate (HR) was derived from the interpulse interval. Averaged systolic blood pressure (SBP) and HR were calculated. The spontaneous cardiac baroreflex gain (sBRG) was also determined from spontaneous changes in SBP and HR as described previously.^{22,23}

Data Analysis

Group data were expressed as mean \pm SEM. To evaluate time-dependent effects of JAM-1 expression, we used 2-way repeated-measures ANOVA and the Scheffe test for multiple comparisons of cardiovascular variables across time and between different groups. A paired/unpaired *t* test was also used for comparisons between 2 groups. Differences were considered significant if $P < 0.05$.

Results

Quantitative Comparison of JAM-1 Gene Expression in WKY Rats and SHRs

In WKY rats using 50 cycles of PCR, we failed to amplify the JAM-1 mRNA transcript containing 3'UTR (nucleotides 1145 to 1385 in BC065309; JAM-1/1145 to 1385) in the rostral ventrolateral medulla, supraoptic nuclei, cerebellum, liver, and heart. In contrast, expression was evident in the same tissues of the SHR (Figure 1 and 2). However, the JAM-1/1145 to 1385 transcript was detectable in the paraventricular nuclei, cerebral cortex, lung, kidney, spleen, and skeletal muscle of WKY rats but was clearly less abundant than that present in the SHR (Figures 1 and 2). Using real-time RT-PCR, we confirmed that the threshold cycle of β -actin of these tissues was the same between SHRs and WKY rats, indicating that the total RNA concentration in each sample was identical (data not shown). Because of its role in central control of arterial

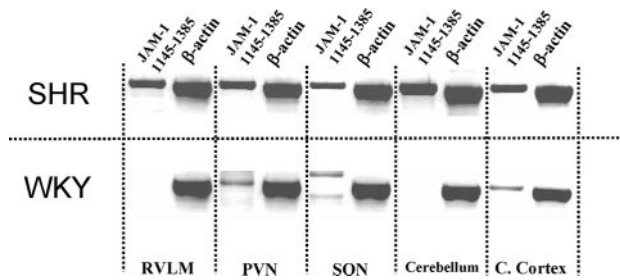


Figure 1. Endogenous JAM-1 gene expression in various brain regions of SHR and WKY rats. JAM-1 3'UTR-containing transcript (nucleotides 1145 to 1385 in GeneBank entry BC065309) was highly expressed in the supraoptic nuclei (SON), paraventricular nuclei (PVN), rostral ventrolateral medulla (RVLN), cerebellum, and cerebral cortex of SHR. In contrast, JAM-1/1145 to 1385 expression was observed only in the PVN and cerebral cortex of WKY, but the level of expression was lower than that in SHR. Note nonspecific bands in samples from SON of WKY rats.

pressure and baroreceptor reflex function, all of the remaining studies focused on the NTS.

Quantitative Comparison of the Levels of JAM-1 Gene Expression in the NTS of WKY Rats and SHR

In the NTS, the level of JAM-1 mRNA transcript representing the protein-coding sequences (JAM-1/430 to 652) was significantly higher in both young and adult SHR compared with age-matched WKY rats (adult WKY rats and SHR: 1.08 ± 0.14 versus 2.86 ± 0.57 , respectively, $n=10$ for each strain, $P<0.01$; young WKY rats and SHR, 1.01 ± 0.06 versus 1.59 ± 0.13 respectively, $n=10$ for each strain, $P<0.01$; Figure 3). With regard to JAM-1/1145 to 1385, we failed to amplify it in the NTS of both young and adult WKY rats. In contrast, it was present in the NTS of SHR in both young and adult animals (Figure 3). It was notable that the threshold cycle of β -actin was the same between the 2 strains of rats (Figure 3), indicating that the total RNA concentration in each sample was identical.

Immunocytochemical Evidence for JAM-1 Presence in the NTS of SHR and WKY Rats

Although in the commissural NTS some JAM-1 immunoreactivity was detectable in WKY rats, it was clearly lower compared with the SHR in all 6 of the rats studied (compare Figure 4A with 4B). Moreover, in the SHR but not WKY rats, JAM-1 was extensively colocalized with RECA-1, a marker for endothelial cells consistent with JAM-1 expression in endothelium (Figure 4C).

Comparison of Levels of JAM-3, ICAM-1, and Vascular Cell Adhesion Molecule-1 Gene Expression in the NTS of WKY Rats and SHR

With the heightened mRNA of JAM-1 in the NTS of the SHR, we ascertained the specificity of the differential expression by comparing it with other vascular adhesion molecules. However, in both young and adult WKY rats and SHR, there were no differences in the level of JAM-3, ICAM-1, and vascular cell adhesion molecule-1 gene expression in the NTS (see the data supplement), suggesting that the upregulation of JAM-1 in the SHR was specific.

Adenoviral Expression of JAM-1 in the NTS of the WKY Rat

We next tested for any functional impact of JAM-1 expression on arterial pressure regulation. With JAM-1 being upregulated in the NTS of the SHR (compared with WKY rats), we decided to overexpress it in the NTS of WKY rats. Before viral injection, baseline levels of SBP, HR, and sBRG in the Ade-CMV-JAM-1 group ($n=6$) were 120 ± 4 mm Hg, 360 ± 8 bpm, and -2.31 ± 0.20 bpm/mm Hg, respectively. These values were not different from those of the Ade-CMV-eGFP group ($n=6$; SBP, 116 ± 3 mm Hg; HR, 360 ± 7 bpm; sBRG, -1.99 ± 0.13 bpm/mm Hg; Figure 5). Five to 7 days after injection of the Ade-CMV-JAM-1, SBP significantly increased (control data above; 7 days after viral transduction: 132 ± 4 mm Hg; $P<0.01$), whereas no changes were found in the SBP of the Ade-CMV-eGFP group (control data above; 7 days after viral transduction: 121 ± 3 mm Hg). The average maximum increment of SBP occurred between 5 and 7 days and was 16.2 ± 2.8 mm Hg; this value was significantly higher than that in the Ade-CMV-eGFP group (4.7 ± 2.9 mm Hg; $P<0.05$). In contrast, HR was significantly decreased, whereas sBRG did not change 5 to 7 days after viral transduction in the Ade-CMV-JAM-1 group (7 days after viral transduction: HR, 311 ± 8 bpm, $P<0.01$; sBRG, -2.34 ± 0.27 bpm/mm Hg, P value not significant; Figure 5). Fourteen days after Ade-CMV-JAM-1 transduction, the SBP remained elevated compared with control levels (ie, 130 ± 5 mm Hg; $P<0.05$), whereas HR changes returned to pretransfection levels (361 ± 10 bpm; Figure 5). In the Ade-CMV-eGFP group, all of these parameters were unaffected by viral transduction except for HR, which showed a transient bradycardia on day 5 after viral injection (eg, from 360 ± 7 to 304 ± 13 bpm; $P<0.01$; Figure 5).

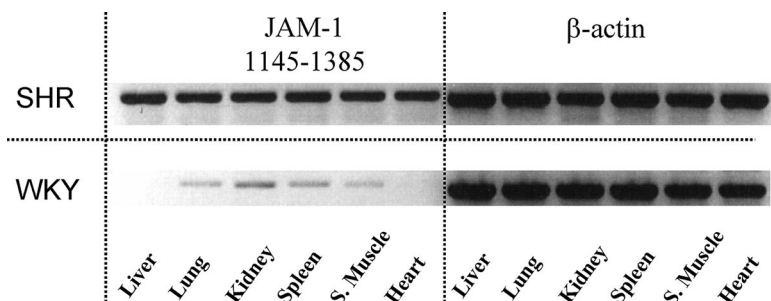


Figure 2. Comparison of endogenous JAM-1 gene expression in different organs of SHR and WKY rats. JAM-1 3'UTR-containing transcript (nucleotides 1145 to 1385) was highly expressed in the liver, lung, kidney, spleen, skeletal muscle (s. muscle), and heart of SHR, whereas in WKY rats the level of expression was lower and could be clearly detected in only lung, kidney, spleen, and skeletal muscle.

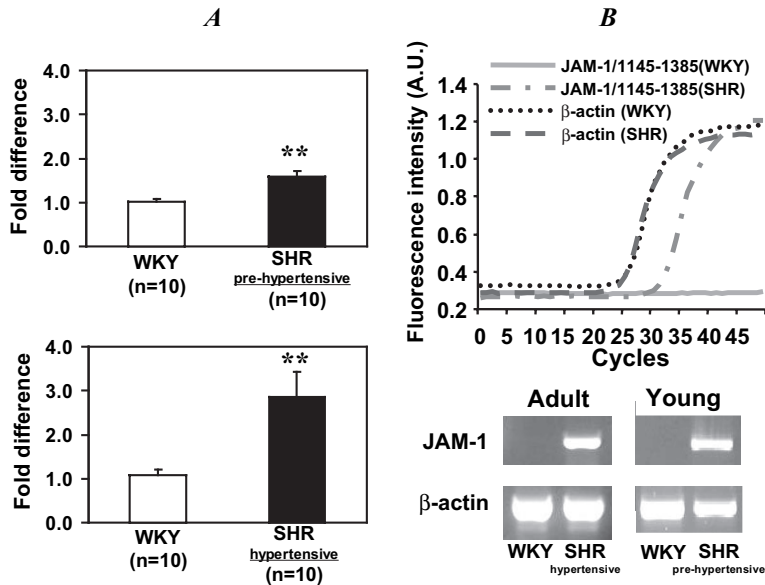


Figure 3. Endogenous JAM-1 gene expression in the NTS of SHR and WKY rats. A, Results of real-time RT-PCR for the protein-coding part of JAM-1 transcript (nucleotides 430 to 652 in GeneBank entry BC065309) in the NTS of SHR and WKY rats. JAM-1 gene expression was higher in both young (prehypertensive, top) and adult (bottom) SHRs compared with age-matched WKY rats. ** $P < 0.01$. B, A typical plot of a real-time RT-PCR experiment (top, adult rats) and a photograph of a gel run with the final product (bottom, adult and immature rats) for JAM-1/1145 to 1385 in the NTS of WKY rats and SHRs. Note that the young SHR was prehypertensive, suggesting that JAM-1 expression was not secondary to the high blood pressure. In WKY, JAM-1/1145 to 1385 could not be amplified within 50 cycles of PCR.

Location of NTS Sites Transduced With Ad-CMV-JAM-1 and AdV-CMV-eGFP

JAM-1 immunoreactivity was elevated after Ad-CMV-JAM-1 transduction compared with endogenous JAM-1 immunoreactivity (Figure 4). Numerous NTS cells including glia and blood vessels were immunopositive for JAM-1 on day 7 of posttransduction (Figure 4). In contrast, JAM-1 immunoreactivity in Ad-CMV-eGFP rats (data not shown) was equivalent to control (naïve rats) as depicted in Figure 4A. Moreover, based on the localization of JAM-1 immunoreactivity

and eGFP expression, highly comparable NTS regions were transduced with both viruses. These regions spanned -13.8 to -14.6 mm relative to Bregma (ie, 400 μm rostral and caudal to calamus scriptorius), 300 to 800 μm from midline and 400 to 800 μm below the dorsal surface of the medulla.

Discussion

We have demonstrated that expression of JAM-1 mRNA is elevated in multiple organs in the SHR compared with WKY rat. In the brain stem (as exemplified in the NTS) JAM-1 protein is

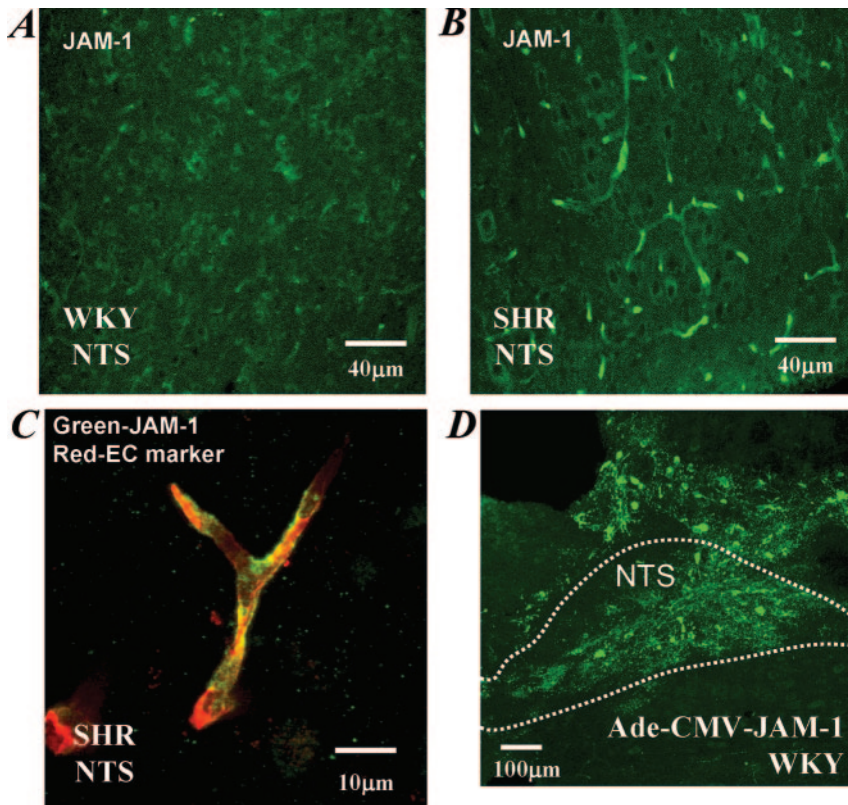


Figure 4. Cellular localization of JAM-1 in the NTS. JAM-1 immunoreactivity was higher in SHR-NTS (B) compared with WKY-NTS (A). JAM-1 staining was colocalized with the endothelium as revealed using RECA-1, an endothelial cell (EC) marker (C). D, after injection of Ad-CMV-JAM-1 in the NTS of the normotensive WKY rat, JAM-1 was extensively expressed in NTS, including glia and blood vessels.

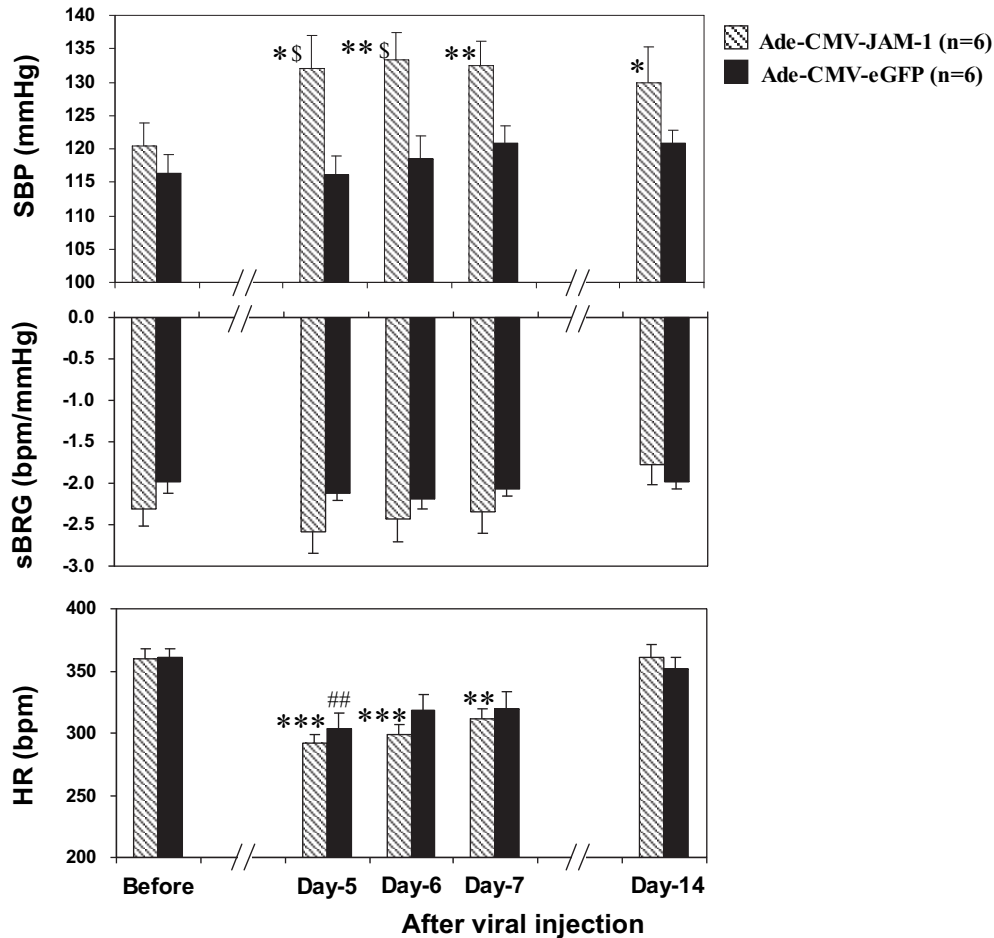


Figure 5. JAM-1 overexpression in the NTS of WKY rats raises arterial pressure. Five days after NTS injection of Ad-CMV-JAM-1, SBP was elevated significantly in WKY rats. This remained above control levels for the 14-day observation period. HR was transiently decreased, whereas sBRG was unaffected. In the Ade-CMV-eGFP group, all of these parameters were unaffected by viral transduction except for HR on day 5. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared before and after Ad-CMV-JAM-1 transduction. ## $P < 0.01$ compared before and after Ad-CMV-eGFP transduction. \$ $P < 0.05$ compared with Ad-CMV-eGFP transduction.

localized in endothelial cells, and the level of expression is higher quantitatively in the SHR compared with WKY rats. To assess the functional consequence of heightened JAM-1 expression in the NTS, we used an adenovirus to chronically overexpress JAM-1 in this brain stem structure of normotensive WKY rats and found that this produces hypertension, consistent with a prohypertensive role of JAM-1.

JAM-1 Expression: SHRs Versus WKY Rats

With regard to the JAM-1/1145 to 1385 transcript, we found that the level of this transcript in WKY varied between organs, whereas it was present in all of the organs analyzed in the SHR. Regarding the NTS, JAM-1/1145 to 1385 transcript was not detectable in WKY rats, whereas it was clearly present in the SHR. This indicates that, in the SHR, JAM-1 mRNA is spliced differently to WKY rats. In the NTS (and other brain areas studied), it appears to have an extended 3'UTR, a known feature present on many translationally efficient mRNAs. Alternatively, it is possible that the WKY transcripts lack 5'UTR because of differences in the transcription termination mechanism. When primers for the protein-coding part of JAM-1 mRNA were used (JAM-1/430 to 652), the transcript could be clearly identified in the NTS of WKY rats, although the level of expression was much lower than that of SHRs (see Figure

3). These results suggest that in normotensive WKY rats, a significant fraction of JAM-1 mRNA lacks 3'UTR sequences, annotated in GeneBank BC065309 entry, making it unstable and effectively reducing the amount of JAM-1 mRNA available for protein expression. Furthermore, processing of JAM-1 mRNA is shifted toward the 3'UTR-containing transcript in many brain regions and bodily organs in the SHR compared with the WKY rat. JAM-1 is one of the proteins that forms the tight junction in both endothelial cells¹⁶ and some types of epithelial cells.¹⁵ Our immunocytochemistry data demonstrated that JAM-1 protein in the brain stem (as exemplified in NTS) is abundantly expressed in endothelial cells where the level of expression is higher in SHRs compared with WKY rats. Importantly, JAM-1 expression in the SHR is not secondary to hypertension, because prehypertensive SHRs also showed a high level of JAM-1 gene expression, at least in NTS.

Under certain conditions endothelial JAM-1 shows a transmembrane luminal orientation thereby protruding into the bloodstream. With its leukocyte binding site (via a leukocyte function-associated antigen-1), JAM-1 is known to cause leukocyte adhesion.^{15,19,24–26} In addition, JAM-1 can activate platelet aggregation.^{15,26} In this context, it is curious that leukocyte adhesion²⁷ and platelet aggregability²⁸ are enhanced in both the SHRs and humans with essential hypertension. All of these

abnormalities can cause accumulation of these cells leading to release of inflammatory mediators, atherosclerosis, and atherothrombosis.^{17,19,29} One outcome of the latter is an increased total peripheral resistance that contributes to the hypertension.^{18,20,30} In addition, any inflammatory response may, in part, be because of altered gene/protein expression in endothelial cells affecting, for example, JAM-1 expression, thereby increasing the adhesion of blood cell types to the vessel wall.^{17,25} This is entirely consistent with leukocyte adhesion after JAM-1 expression in the NTS of the WKY rat, which is normally devoid of such pathology (see the data supplement).

Another highly expressed molecule in endothelial cells is ICAM-1, which is upregulated in carotid arteries in the SHR.²⁷ However, we failed to detect a high level of ICAM-1 gene expression in the NTS of SHRs, compared with WKY rats, indicating that the level of expression of ICAM-1 in the SHR could be vascular bed specific (see the data supplement). Similarly, the levels of expression of JAM-3 and vascular cell adhesion molecule-1 in the NTS were not different between the SHR and WKY rat. These results suggest that the high level of JAM-1 mRNA in the NTS of the SHR is specific to this type of endothelial adhesion molecule and not related to a general inflammatory reaction.

JAM-1 Expression in the NTS and Hypertension

A functional role for JAM-1 for regulating arterial pressure was tested by overexpressing it in the NTS of adult normotensive WKY rats using adenoviral gene transfer. This resulted in abundant JAM-1 expression in the NTS, including glia and endothelial cells (Figure 4). Five days after viral injection, SBP was significantly higher than control rats and remained elevated into day 14 after viral injection (Figure 5). In contrast to the SBP, sBRG did not change over this period, suggesting that JAM-1 in the NTS was affecting neural mechanisms controlling the set point of arterial pressure independent of alterations in baroreflex sensitivity. However, whether the gain of the baroreceptor reflex sympathetic vasoconstrictor component was modified remains unknown, because we did not test this. In addition, HR was significantly lower between 5 and 7 days after viral transduction with Ad-CMV-JAM-1 but, unlike SBP, returned to control 2 weeks later. The transient fall in HR may be mediated by the cardiac baroreflex in response to the rising level of arterial pressure. Fourteen days after viral transduction, however, the operating point of the reflex could reset to a higher level of arterial pressure and, hence, the level of HR returns to normal. It should be noted that, at least for the first 5 days after Ad-CMV-JAM-1 transduction, the fall in HR may be a consequence of surgery and viral induced transduction in the NTS, as we have reported previously.¹¹

The increase in SBP in the WKY rats after Ad-CMV-JAM-1 overexpression was associated with an increase in both the low frequency:high frequency of HR variability and low frequency+very low frequency power of SBP variability, suggesting activation of cardiac and vasomotor sympathetic activity, respectively (see the data supplement). It should be noted that the low frequency:high frequency of HR variability increased, whereas HR was decreased 7 days after Ad-CMV-JAM-1 transduction. The fall in HR may be because of an enhanced vagal tone, because the high-frequency power of HR variability increased after Ad-CMV-JAM-1 transduction (see the data supplement).

Possible Mechanisms by Which JAM-1 in NTS Elevates Arterial Pressure

Our findings show that high level of JAM-1 expression at the level of NTS can cause hypertension in WKY rats. The question of how this arises comes about. We have recently described that adenoviruses with the human CMV promoter drive extremely low levels of gene expression in most NTS neurons.³¹ On the other hand, these vectors clearly transduce local vasculature.¹¹ Based on evidence that JAM-1 can cause leukocyte and platelet adhesion in the peripheral circulation,^{24–26} it was necessary to assess whether this could occur within the brain after inducing JAM-1 overexpression. The data that we present suggest that, at least in NTS, JAM-1 can induce leukocyte binding (see the data supplement). It remains unclear what, if any, effect this would have on NTS neurons impinging on downstream neuronal networks controlling sympathetic activity. Nevertheless, these results raise the intriguing and novel possibility that there is a link between high levels of leukocyte/platelet adhesion in the brain stem and excessive levels of sympathetic nerve activity. Thus, once adhered to the endothelium, leukocytes (and platelets) release both cytokines and reactive oxygen species, such as superoxide. These are established products from such cells^{32,33} and are known to activate brain stem cardiovascular neurons.^{34,35} Because some types of cytokines can cross the blood–brain barrier,³⁶ it now becomes pertinent to understand their actions on brain stem circuitry regulating the set point control of arterial pressure. In addition, leukocyte accumulation in capillaries may provide a significant obstacle to blood flow, resulting in localized ischaemia.³⁷ The latter could further enhance cytokine and reactive oxygen species production from affected endothelial cells.^{38,39} The functional significance of cytokines, chemokines, and reactive oxygen species activation within the central nervous system has been studied^{40–43} and includes modulation of central neurotransmitters (eg, noradrenaline,⁴⁴ 5-hydroxytryptamine,⁴⁵ glutamate,⁴⁶ and γ -aminobutyric acid^{46,47}), effects on neuronal differentiation/growth,⁴⁸ and synaptic plasticity.⁴⁹ Such actions might affect brain stem circuitry regulating sympathetic activity that may lead to significant alterations in blood pressure control.

Perspectives

We have revealed that there is widespread JAM-1 expression in the SHR compared with the WKY rat. Increased JAM-1 protein expression in brain stem structure(s) regulating arterial pressure, such as the NTS, could contribute to hypertension. It remains to be established whether a similar scenario applies to other brain regions of the SHR, such as the circumventricular organs, hypothalamus, and rostral ventrolateral medulla, and also peripheral tissues, such as the kidney and heart, and how this may contribute to hypertension.

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Disclosures

None.

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Methods

Method for Immunohistochemistry

Animals (SHR, n=6; WKY, n=18; both strains were 15 to 18 weeks old) were humanely killed by cervical dislocation. The brainstem was removed, fixed with 4% paraformaldehyde for at least 24 h, and transferred to phosphate buffered saline (PBS) containing 30% sucrose. Serial sections (40 µm) through NTS were obtained using a freezing microtome. The sections were rinsed in PBS, put in 10% serum with 0.3% Triton X-100 for 15 minutes at room temperature, rinsed again and incubated with an JAM-1 antibody, sc-17427, (Santa Cruz Biotechnology, dilution: 1:200 in PBS with 1% serum and 0.3% Triton X-100). After overnight incubation at 4 °C, the sections were rinsed in PBS and incubated with biotinylated horse anti-goat IgG (1:500 dilution, Vector Laboratories, UK) for 1 hour. The sections were rinsed and then incubated in streptavidin conjugated Alexa-Fluor 488 (1:500 dilution, Molecular Probes, USA) for 1 hour. Finally, sections received a PBS wash before mounting in Vectashield (Vector Laboratories). Sections were photographed using a Leica TCS-NT scanning laser confocal microscope. In order to determine the cell types expressing JAM-1, we used double labelling fluorescence immunohistochemistry with an endothelial cell marker (RecA1, Abcam plc, UK). A leukocyte marker - W3/25 anti-rat CD4 monoclonal antibody (kindly received from Dr S.M. Nicholls, Ophthalmology, Bristol Eye

Hospital, Bristol UK, dilution: 1:200 in PBS with 1% serum and 0.3% Triton X-100) was also used to identify leukocyte adhesion within the NTS of WKY and SHR (See Supplemental Data IV).

Method for power spectral analysis of HR and SBP variability

Power spectral analysis of SBP and HR variability was performed using a fast Fourier transform (FFT) algorithm as described by us recently¹. The magnitude of power was integrated in the very low-frequency (VLF) band (0-0.27 Hz), the low-frequency (LF) band (0.27-0.75 Hz) and the high-frequency (HF) band (0.75–3.3 Hz)¹. The HF power of HR variability, the ratio of LF to HF (LF:HF) of HR variability and the LF + VLF power of SBP variability were analyzed on the day before NTS microinjection of virus and on the 7th day after microinjections. We assumed that the HF power of HR variability is mediated by cardiac parasympathetic tone whereas the LF:HF of HR variability is an index of cardiac sympathetic tone². Similarly, the LF + VLF power of SBP variability is, in part, mediated by vasomotor sympathetic tone³.

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Legends

Figure S1. Leukocyte marker (W3/25) shows leukocyte accumulation in the NTS capillaries of hypertensive rats demonstrating a high immune environment.

Immunohistochemistry indicated endogenous leukocyte accumulation in the capillaries of the medulla including the NTS of SHR but not WKY rats (A). Following JAM-1 expression in WKY rats (which was normally devoid of leukocytes) leukocyte adhesion was evoked. Importantly, immunoreactivity for leukocytes was not found in control WKY in which adenovirus expressing eGFP had been injected previously (B). This suggested that the leukocyte adhesion was due to JAM-1 expression and not related to the presence of the adenovirus and is consistent with the leukocyte adhesion property of JAM-1.

Figure S2. Expression of JAM-1 at the level of the NTS alters autonomic cardiovascular activity.

Seven days post viral transduction with Ade-CMV-JAM-1 the LF + VLF power of SBP variability was significantly increased (from 7.32 ± 0.22 to 8.01 ± 0.29 mmHg², $p < 0.05$) indicative of raised sympathetic activity. In contrast, no changes were found in this variable in WKY rats transduced with Ade-CMV-eGFP. Seven days post viral transduction with Ade-CMV-JAM-1 both the LF:HF and HF power of HR variability were significantly

increased (LF:HF of HR variability: from 0.25 ± 0.01 to 0.29 ± 0.02 , $p < 0.05$; HF power of HR variability: from 14.2 ± 0.9 to 19.6 ± 1.5 bpm², $p < 0.05$).

* $p < 0.05$ compared before and after Ad-CMV-JAM-1 transduction. # $p < 0.05$ compared before and after Ad-CMV-eGFP transduction.

Table S1. Primer details used for quantitative RT-PCR

Gene name	Accretion No.	Primers/product details	Amplification size
β -actin	NM_031144	forward: CGTTGACATCCGTAAAGACCTC reverse: GTACTCCTGCTTGCTGATCCAC	228bp
JAM-1 (430-652)	BC065309	forward: ACTGTGCTTGTGCCTCCATC reverse: CAAAGACCAAATCCCCTGAC	223bp
JAM-1 (1145-1385)	BC065309	forward: CCTTTCCTTACCACCATTGC reverse: TCAGCCCATCTTCCTCCAGT	241bp
JAM-3	BC079429	forward: GGGAGAGCACATACAAGCAC reverse: CCAATCAACAGAGGCACCAT	410bp
β -actin*	NM_031144	QT00193473, Qiagen	145bp
VCAM-1	NM_012889	QT00178500, Qiagen	118bp
ICAM-1	NM_012967	QT00174447, Qiagen	134bp

*This set of primers was used with primers for VCAM-1 and ICAM-1

Table S2. JAM-3, ICAM-1 and VCAM-1 gene expression (fold differences) in the NTS of WKY and SHR

Gene name	WKY	SHR
ADULT		
JAM-3	1.00±0.08(n=10)	1.20±0.08(n=10)
ICAM-1	1.01±0.05 (n=10)	0.82±0.10(n=10)
VCAM-1	1.04±0.10(n=10)	1.19±0.09(n=10)
YOUNG		
JAM-3	1.02±0.10(n=6)	1.06±0.06(n=5)
ICAM-1	1.02±0.10(n=6)	1.20±0.15(n=5)
VCAM-1	1.02±0.09(n=6)	0.89±0.07(n=5)

Expression of target genes relative to β -actin gene in each sample were derived using the comparative ($2^{-\Delta\Delta CT}$) method⁴. Fold differences were calculated against the average of age-matched WKY. For JAM-3, ICAM-1 and VCAM-1 gene expression, no significant differences were found between WKY and SHR.

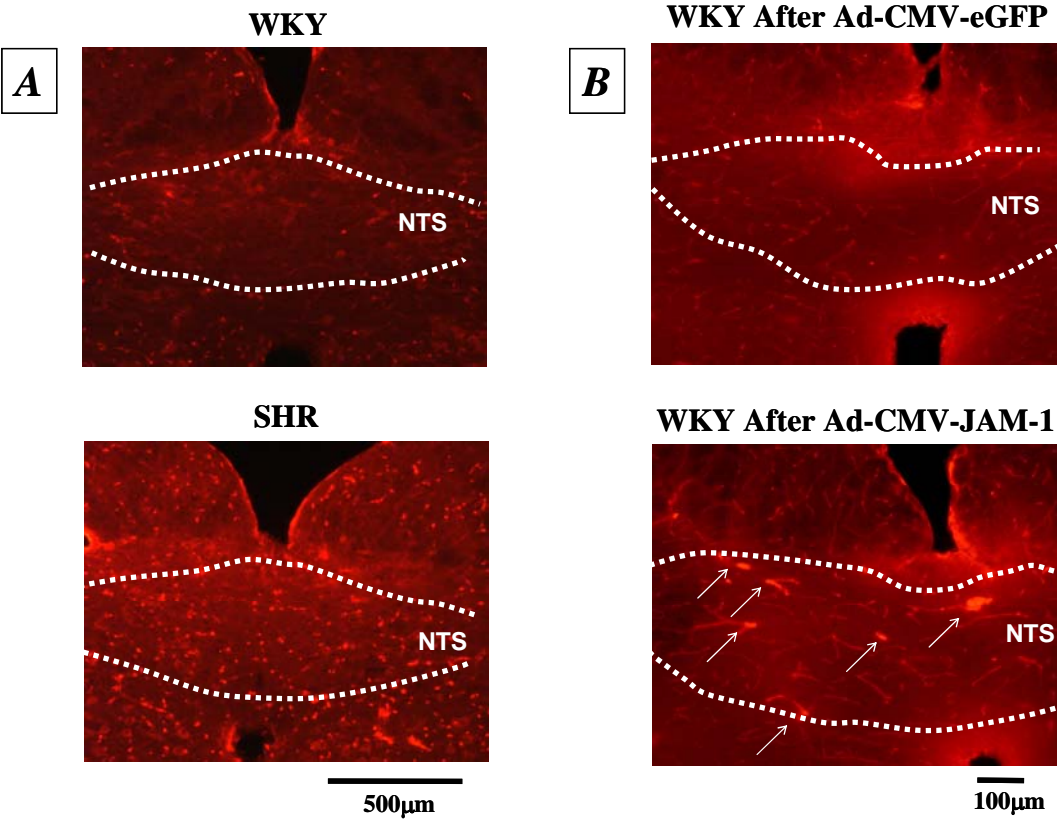


Figure S1

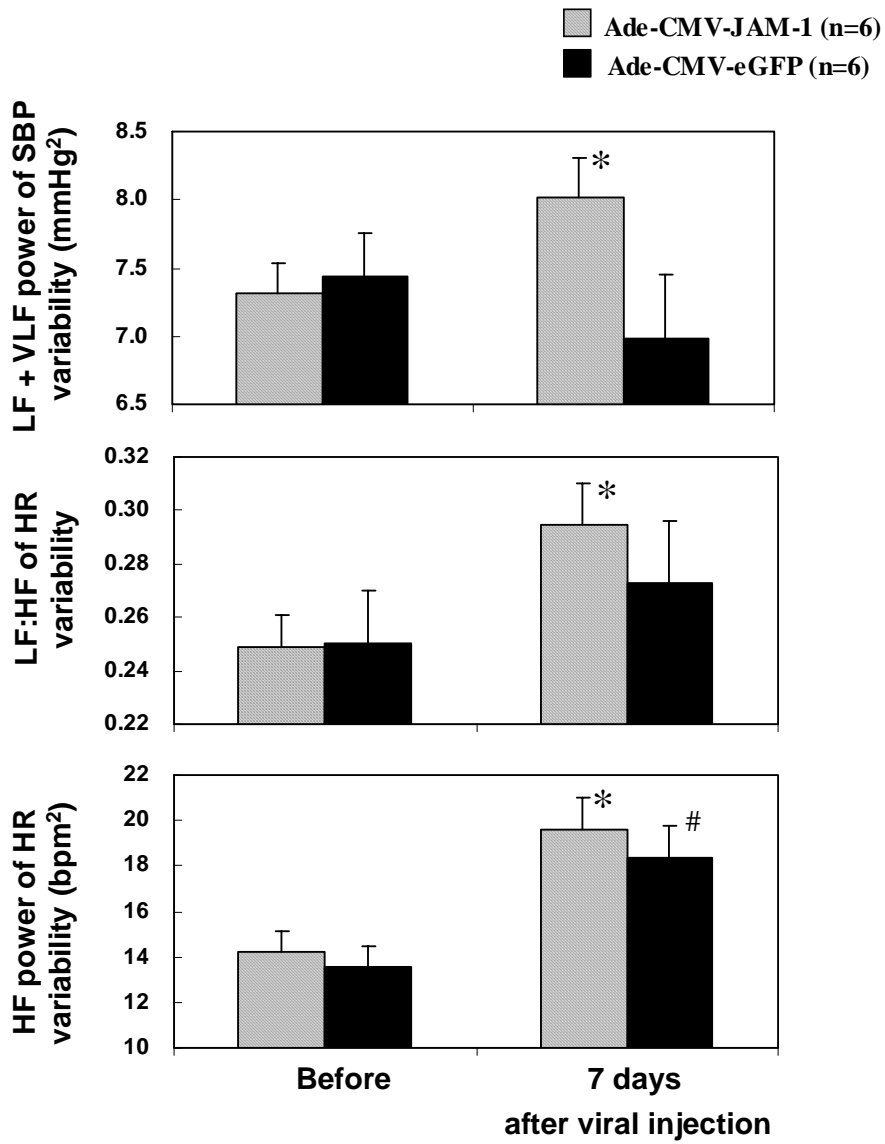


Figure S2