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Targeting brain stem centers of cardiovascular control using adenoviral vectors: impact of promoters on transgene expression

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Lonergan, Tina, Anja G. Teschemacher, D. Y. Hwang, K.-S. Kim, Anthony E. Pickering, and Sergey Kasparov. Targeting brain stem centers of cardiovascular control using adenoviral vectors: impact of promoters on transgene expression. *Physiol Genomics* 20: 165–172, 2005. First published November 23, 2004; doi:10.1152/ physiolgenomics.00120.2004.—Adenoviral vectors (AVV) are widely used as tools for exploring gene function in studies of the central autonomic control, but the cellular specificity of the promoters commonly used in these vectors has not been studied. We evaluated AVV with four "wide-spectrum" promoters, human cytomegalovirus promoter (HCMV), synapsin-1 promoter (Syn1), tubulin-α1 promoter (Tα1), and neuron-specific enolase (NSE) for their ability to express enhanced green fluorescent protein (EGFP) within the dorsal vagal complex and the adjacent brain stem. They were compared with the PRSx8 promoter, specifically designed to target catecholaminergic neurons. AdHCMVEGFP, AdSyn1EGFP-WHE (woodchuck hepatitis enhancer element), AdTα1EGFP, and AdNSEEGFP were unable to drive expression of EGFP in dopamine β-hydroxylase-immunoreactive neurons of the A2 cell group, although the adjacent dorsal vagal motonucleus and especially hypoglossal motoneurons did express high levels of EGFP. AdPRSx8EGFP efficiently drove EGFP expression in the A2 cell group but also in choline acetyltransferase-positive vagal motoneurons. However, catecholaminergic neurons could be selectively and efficiently transduced via a retrograde route by injecting the vector into their target areas. Thus AVV with "wide-spectrum" promoters have strikingly different activity in the diverse cellular populations within brain stem cardiovascular control centers. The PRSx8 promoter is a valuable tool for the study of the role of catecholaminergic neurons.

adenovirus; catecholamine; dorsal vagal complex; acetylcholine

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ABNORMALITIES IN THE CARDIOVASCULAR system might result from increased or decreased expression of genes in autonomic control centers in the brain stem. One rapidly emerging technique used to study the role of particular genes in cardiovascular disease involves focal gene transfer, using adenoviral vectors (AVV), into various brain stem centers of autonomic control (11, 14–16, 25, 32, 36, 41, 43, 45). AVV allow relatively good spatial and temporal control of gene expression. However, even when the injection is spatially localized, the viral vectors are taken up by numerous cell types in the brain. Therefore, expression of a transgene in different cell types may severely complicate the interpretation of the physiological outcome. Conversely, because of the inevitable transductional tropism of the viral vectors and differences in the activity of the promoters

used to drive transgene expression, the particular cell type responsible for the physiological effect under study might not express the transgene (15), giving rise to a false-negative outcome of the experiment. To safeguard against this problem, to date, many studies have employed "constitutive" promoters such as human cytomegalovirus (HCMV), human synapsin-1 (Syn1), tubulin-α1 (Tα1), and neuron-specific enolase (NSE) for transgene expression in brain nuclei. It is known that such promoters may discriminate between neurons and glia; for example, HCMV has been reported to be more active in glia than neurons (17, 18), whereas Syn1, Tα1, and NSE are referred to as "pan-neuronal" and assumed to be active in all neurons (7, 8, 17, 18). However, the activity of these vectors in diverse neuronal populations essential for cardiovascular control, such as the dorsal vagal complex, has not been documented.

Located in the caudal brain stem, the dorsal vagal complex contains neurons of mixed phenotypes such as the A2 noradrenergic cell group, GABAergic and glutamatergic nucleus tractus solitarius (NTS) neurons, and cholinergic neurons of the dorsal motonucleus of the vagus (DMNX). Catecholaminergic neurons play a key part in the autonomic control of blood pressure, and altered catecholaminergic signaling has been implicated in rat models of hypertension (20, 29, 35, 44). It is believed that this excessive central catecholaminergic activity eventually translates to heightened sympathetic outflow leading to hypertension and end organ damage. The DMNX comprises preganglionic vagal motoneurons, which together with the motoneurons of the nucleus ambiguus are critical for the generation of parasympathetic tone of the heart, a parameter typically impaired in hypertension (1, 4–6).

Given the growing interest in long-term genetic manipulation of signaling in autonomic control centers, we evaluated a range of promoters placed in an AVV backbone, for their ability to drive expression of enhanced green fluorescent protein (EGFP) in the dorsal vagal complex. The promoters chosen were HCMV, Syn1, Tα1, and NSE. We also evaluated the artificial PRSx8 promoter, which was specifically designed to target catecholaminergic neurons (12).

Here we report that AVV incorporating the above-mentioned promoters show marked differences in their activity in different neuronal populations, with all of them, except for PRSx8 being inactive in catecholaminergic neurons. We also describe a highly efficient and selective retrograde transduction of noradrenergic neurons using injections of AVV with the PRSx8 promoter into their projection areas.

EXPERIMENTAL METHODS

Viral constructs. AdHCMVEGFP and AdSyn1EGFP-WHE were a kind gift from J. Uney, University of Bristol, UK (9). AdNSE-EGFP

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and AdTα1EGFP were generously provided by S. Kügler, University of Göttingen, Germany (17).

AdPRSx8EGFP was constructed by homologous recombination of the shuttle vector pXCX-PRSx8-EGFP and pJM17 in HEK293 cells, followed by clone isolation (2, 10). pXCX-PRSx8-EGFP was cloned by inserting EGFP from pEGFP-N1 (Clontech) into pXCXCMV (obtained from Professor J. Uney, University of Bristol) using the *Hin*dIII and *Not*I restriction sites. The CMV promoter was then removed by *Mlu*I and *Bam*HI digestion (*Mlu*I site blunted) and replaced with the *Hin*dIII-*Bam*HI fragment (*Hin*dIII site blunted) from pK18130, which contained the 240-bp PRSx8 promoter sequence (12). Except for the expression cassettes, all constructs contained essentially the same adenoviral serotype 5 backbone with deletions in the E1/2 and E3 parts of the viral genome.

Delivery of viral vectors. Male Wistar rats (75–150 g) were anesthetized with a mixture of ketamine (60 mg/kg) and medetomidine (250 µg/kg). They were placed in a stereotaxic head holder, and the dorsal surface of the medulla was exposed through a midline incision in the neck. Bilateral microinjections (1 µl over 2 min) of AVV were made in the dorsal vagal complex, at the level of the calamus scriptorius and 300 µm rostral and caudal to it, 300–400 µm from the midline and 350–400 µm ventral to the dorsal surface of the medulla. In the first series of experiments, the following AVV were injected at the same titer of 2.2×10^9 pfu/ml: AdPRSx8EGFP ($n = 5$), AdHCMVEGFP ($n = 2$), AdSyn1EGFP-WHE (WHE = woodchuck hepatitis enhancer element, $n = 2$), AdNSEEGFP ($n = 2$), and AdTα1EGFP ($n = 2$). In a second series of experiments, to test whether this would affect their expression profile, the following AVV were injected at ~10-fold less titers: AdHCMVEGFP (2.7×10^8 pfu/ml, $n = 3$), AdSyn1EGFP-WHE (4.4×10^7 pfu/ml, $n = 2$), AdNSEEGFP (3×10^8 pfu/ml, $n = 2$), and AdTα1EGFP (3.3×10^8 pfu/ml, $n = 2$).

Finally, since in our preliminary experiments we noticed possible retrograde transduction of catecholaminergic neurons with AdPRSx8EGFP (15), in the third series of experiments AdPRSx8EGFP was injected into several regions in the spinal cord (a well-known projection area for several major catecholaminergic groups; 24). In two rats, we centered injections in the intermediolateral cell column (IML, $2 \times$ bilateral injections; 0.8 mm lateral to midline and 0.5 mm ventral, between thoracic segments 1 and 2, each injection: 1 µl over 2 min). In two other animals, injections were centered on the dorsal horn at the L3 level (500 µl bilateral injections, 0.5 mm lateral to midline, 400 µm ventral at two rostrocaudal extents separated by ~1 mm, using 5×10^9 pfu/ml). These injections were aimed at the descending catecholaminergic projections onto sympathetic preganglionic neurons and the dorsal horn, respectively.

In each case the wound was then sutured and treated with antiseptic powder, and the rat was allowed to recover for 6–7 days.

Immunohistochemistry. Rats were terminally anesthetized (pentobarbital sodium, 100 mg/kg ip) and perfused intracardially with 0.9% saline and 4% formaldehyde in 0.1 M phosphate buffer, at pH 7.4. Brain stems were removed and postfixed overnight and then placed in 30% sucrose for 24 h. Serial 60-µm or 40-µm sections were cut on a freezing microtome. In the first series of experiments, alternate sections from the brain stems of rats injected with 2.2×10^9 pfu/ml AdHC-MVEGFP, AdSyn1EGFP-WHE, AdNSEEGFP, and AdTα1EGFP were processed for dopamine β-hydroxylase (DBH), neuron-specific nuclear protein (NeuN, mouse anti-NeuN, 1:500, Chemicon), or glial fibrillary acidic protein (GFAP, mouse anti-GFAP, 1:500, Chemicon). In the second series of experiments using lower viral titers, sections were only processed for DBH. Of the five rats injected with AdPRSx8EGFP, three were processed for DBH (mouse anti-DBH, 1:1,000, Chemicon) and two for choline acetyltransferase (ChAT, rabbit anti-ChAT, 1:500, Chemicon).

Details of the immunohistochemical procedures were described previously (19). Briefly, sections were washed for 30 min with 50% ethanol, followed by three 30-min washes in Tris-phosphate buffered saline (TPBS). Sections were then incubated in primary antibody and 5% normal horse serum (NHS) in TPBS, for 72 h. This was followed by overnight incubations in either biotinylated donkey anti-mouse F(ab)$_2$ fragments or Texas Red donkey anti-rabbit F(ab)$_2$ fragments (1:500; Jackson Immunolabs, West Grove, PA) and 2% NHS in TPBS, then ExtrAvidin-Cy3 in TPBS (1:1,000, Sigma). Washes were performed between incubations (TPBS, three for 30 min), at room temperature. Sections were then mounted using Vectashield (Vector Labs).

Confocal images were obtained using a Leica SP spectral confocal microscope. Images were taken at 1- to 2-µm intervals through the thickness of the section. The two channels (EGFP, excitation 488 nm and emission 500–530 nm; Cy3, excitation 543 nm and emission 590–650 nm) were scanned separately to avoid "bleed" of fluorescence between channels and merged using Leica software.

To assess the activity of the AVV in catecholaminergic neurons, the presence of DBH immunoreactivity was determined in EGFP-expressing cells in the NTS. Cells were counted bilaterally over three comparable sections at different rostrocaudal levels within the transfected area (approximately bregma $-14.6, -14.2, -13.8$ mm; 27). To compare the expression profile of AdHCMVEGFP, AdSyn1EGFP-WHE, AdTα1EGFP, and AdNSEEGFP within the NTS and DMNX, three comparable sections at different rostrocaudal levels (as above) were chosen from each experiment. The number of EGFP-expressing neurons in the NTS and DMNX were counted bilaterally, and the totals for the three sections were summed. The numbers were then expressed as means ± SE for each AVV. In the first series of experiments, NeuN was used as a marker of neuronal phenotype. In the second series, only morphological criteria were used to confirm the neuronal nature of EGFP-fluorescent cells. Cells defined as neurons had the following features: soma > 10 µm, detectable nucleus, few well defined asymmetric processes with limited branching proximal to cell body. As all of these constructs were highly active in the hypoglossal (XII) motonucleus, which lies directly ventral to the DMNX, neurons in the XII were also counted for comparison purposes.

RESULTS

Patterns of transduction following AdHCMVEGFP, AdSyn1EGFP-WHE, AdTα1EGFP, and AdNSEEGFP injection in the dorsal vagal complex. When AdHCMVEGFP, AdSyn1EGFP-WHE, AdTα1EGFP, and AdNSEEGFP were injected into the dorsal vagal complex, we regularly observed transduced cells in the NTS, DMNX, and the underlying XII motonucleus, due to the spread of the injection. Neurons of the XII could be easily identified by their location and larger cell bodies whose axons traversed toward and exited the ventral edge of the medulla (Fig. 1*A*). DMNX neurons were found dorsal to the XII and ventral to DBH-immunoreactive A2 neurons (Fig. 1*B*).

AdHCMVEGFP, AdSyn1EGFP-WHE, AdTα1EGFP, and AdNSEEGFP were inactive in DBH-immunoreactive A2 neurons (Fig. 1, *C–E*). For each vector, over 200 EGFP-expressing cells were counted, and none was found to be DBH immunoreactive in rats injected with 2.2×10^9 pfu/ml. Despite their inactivity in noradrenergic cells, AdSyn1EGFP-WHE, AdTα1EGFP, and AdNSEEGFP were highly active in other NTS neurons. The total number of EGFP-expressing NeuN-immunoreactive neurons in three different rostrocaudal sections in the NTS (see EXPERIMENTAL METHODS) were AdSyn1EGFP-WHE (60 ± 10), AdTα1EGFP (74 ± 8), and AdNSEEGFP (61 ± 25). EGFP expression in the NTS following AdSyn1EGFP-WHE and AdTα1EGFP, was restricted to neurons as was confirmed by colocalization with NeuN (Fig. 1*G*) and lack of colocalization with GFAP (Fig.

1*H*). AdNSEEGFP largely transduced neurons, but cells of glial morphology were occasionally observed (2–3 cells per experiment). Similarly, in the second series of rats injected with lower titers of the above-mentioned vectors, none was able to drive EGFP expression in DBH-immunoreactive A2 neurons, although EGFP was expressed in non-DBH-immunoreactive neurons in the NTS following injection of AdSyn1EGFP-WHE (22 ± 7), AdTα1EGFP (21 ± 11), and AdNSEEGFP (19 ± 7).

Interestingly, nearly all cells transduced by AdHCMVEGFP in the NTS were glial, as confirmed by GFAP immunohistochemistry (Fig. 1*I*), although, very rarely, EGFP was found within NeuN-immunoreactive cells (3 ± 1.5 per experiment). In two additional animals, we explored the possibility that AdHCMVEGFP may transiently express EGFP in A2 catecholaminergic neurons. Forty-eight hours following the injection of AdHCMVEGFP, robust EGFP expression was observed in the NTS, DMNX, and XII, but none of more than 300 EGFP-fluorescent cells was DBH immunoreactive.

All four vectors were approximately equally effective in expressing EGFP in neurons of the DMNX. In the first series of experiments using 2.2×10^9 pfu/ml, the density of EGFP (NeuN-immunoreactive) neurons was as follows: AdSyn1EGFP-WHE, 21 ± 3; AdTα1EGFP, 24 ± 6; AdNSEEGFP, 19 ± 8; and AdHCMVEGFP, 11 ± 1. In the second series of experiments using lower vector titers, slightly lower numbers of neurons were observed in the DMNX without striking differences between the vectors: AdSyn1EGFP-WHE, 8 ± 7; AdTα1EGFP, 17 ± 14; AdNSEEGFP, 7 ± 4; and AdHCMVEGFP, 8 ± 4.

Finally, all four AVV resulted in strong EGFP expression in NeuN-immunoreactive XII neurons, and again, no radical difference was observed in the number of neurons expressing EGFP for all four vectors in both the first series (Syn1, 89 ± 14; Tα1, 84 ± 2; NSE, 121 ± 19; HCMV, 80 ± 16) and second series of experiments using lower AVV titers (AdSyn1EGFP-WHE, 107 ± 30; AdTα1EGFP, 107 ± 30; AdNSEEGFP, 72 ± 34; AdHCMVEGFP, 118 ± 57). Noticeably, the level of EGFP expression in XII neurons targeted with AdHCMVEGFP was very high (Fig. 1*A*).

AdHCMVEGFP also produced robust EGFP expression in glial (GFAP-immunoreactive) cells in the DMNX and XII motonucleus.

The tropism of each vector for neurons (based on cell counts from the first series of experiments) is summarized in Table 1.

Expression profile of AdPRSx8EGFP. Within the dorsal vagal complex, AdPRSx8EGFP was the only vector efficient in driving EGFP expression in the noradrenergic neurons of the A2 cell group (Fig. 2*A*). However, AdPRSx8EGFP also triggered EGFP expression in some DBH-negative neurons ventral to the NTS. We confirmed that these neurons were cholinergic using immunohistochemistry for ChAT in two animals (Fig. 2*B*). No EGFP-expressing cells were observed in the XII motonucleus. In two additional experiments, decreasing the titer of AdPRSx8EGFP 10-fold to 2.2×10^8, did not eliminate the EGFP expression in the DMNX and also decreased the number of catecholaminergic neurons labeled (data not shown).

Previously we noticed that some EGFP-expressing noradrenergic neurons could be found in the brain stem after AdPRSx8EGFP injections into the hypothalamus (15). To test whether retrograde transduction can be used for efficient and selective targeting of noradrenergic neurons from their projec-

tion areas, we injected AdPRSx8EGFP into the IML (a known target area for C1 adrenergic neurons in the rostral ventrolateral medulla, RVLM; 24, 30, 31, 39). This resulted in numerous EGFP-expressing neurons in the RVLM (Fig. 2*C*), all of which were DBH immunoreactive. For example, we found 156 retrogradely labeled neurons in the RVLM of one animal (counted in every second section to avoid double counting between 0 to 800 μm caudal from the facial nucleus). Injections in the dorsal horn retrogradely labeled dense clusters of noradrenergic neurons in the locus coeruleus (LC), where numerous fluorescent cells were present in each section (Fig. 2*D*). Unlike traditional retrograde tracers which usually only label only the soma and proximal dendrites, AdPRSx8EGFP produced extensive labeling of the dendritic tree of most cells and even labeled axonal varicosities (Fig. 2*E*).

DISCUSSION

We have demonstrated that commonly used promoters such as HCMV, Syn1, NSE, and Tα1 are unable to drive transgene expression in catecholaminergic neurons, when incorporated into an adenoviral backbone. However, these vectors were highly effective in the two adjacent groups of cholinergic motor neurons. Moreover, HCMV was apparently unable to provide sustainable expression in other neuronal phenotypes present within the NTS. Furthermore, we have shown that while the artificial PRSx8 promoter efficiently drives transgene expression in catecholaminergic neurons, it also allows expression of transgene in vagal preganglionic cholinergic motor neurons. Finally, we found that catecholaminergic neurons can be selectively and efficiently transduced, following injection of AdPRSx8EGFP into their target locations.

Transgene expression driven by AdHCMVEGFP, AdSyn1EGFP-WHE, AdTα1EGFP, and AdNSE-EGFP in the dorsal vagal complex is targeted. It is clear that the promoters used in the above-mentioned vectors have very different expression profiles in the dorsal vagal complex. All of these constructs are highly active in the XII nucleus and reasonably active in the adjacent DMNX. However, none of these promoters could drive EGFP expression in the noradrenergic A2 cell group. This phenomenon does not appear to be limited to the A2 cell group, as AdHCMVEGFP also did not transduce any DBH-immunoreactive neurons in the C1 adrenergic cell group following injection into the RVLM (data not shown). Furthermore, when AdHCMVDsRed (expressing red coral fluorescent protein DsRed) was coinjected with AdPRSx8EGFP in the A6 cell group, no colocalization of EGFP and DsRed was observed (15). Finally, a completely different construct incorporating both HCMV (driving EGFP) and PRSx8 (driving β-galactosidase) as a single AVV (12) shows the same pattern of EGFP expression in the dorsal vagal complex (data not shown) as AdHCMVEGFP used in this study.

Concerning the HCMV promoter, it is not surprising that it may be silenced in some neuronal populations. For example, in the hippocampus, an AVV with the HCMV promoter only led to a transient expression of EGFP, whereas the synapsin promoter in the same AVV backbone still produced strong EGFP expression even after 9 mo (9). We have tested the possibility that HCMV-driven expression in noradrenergic neurons is extremely short-lived by looking at the expression
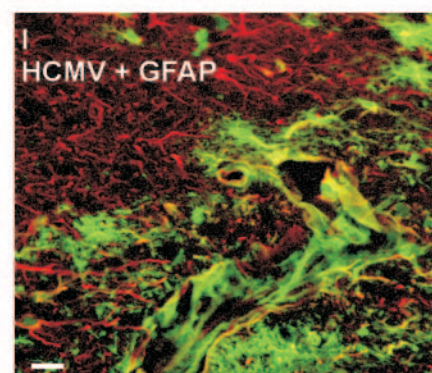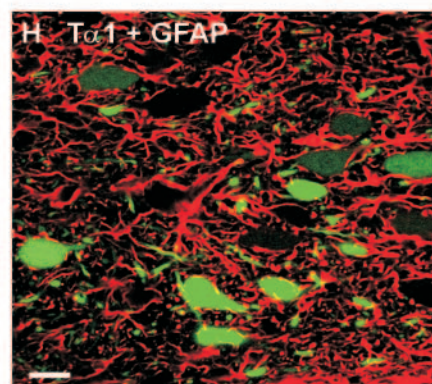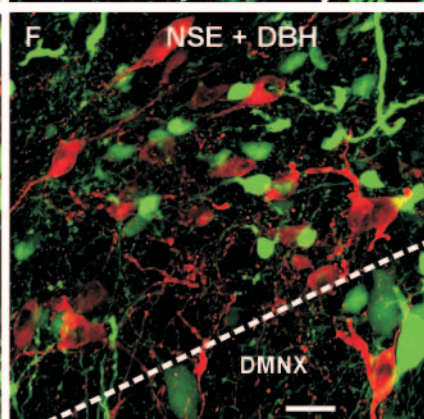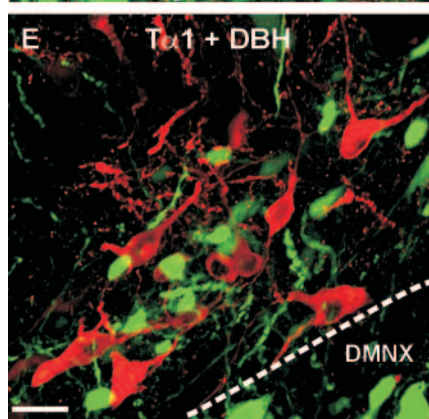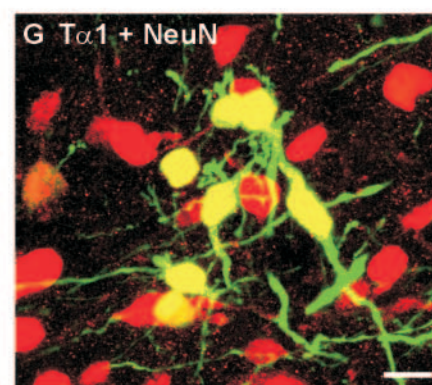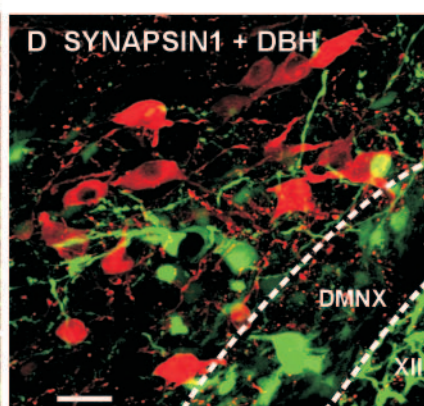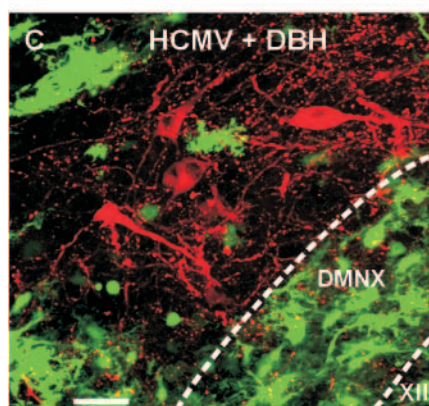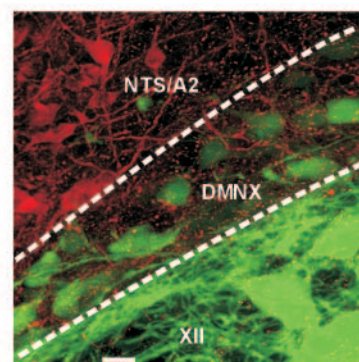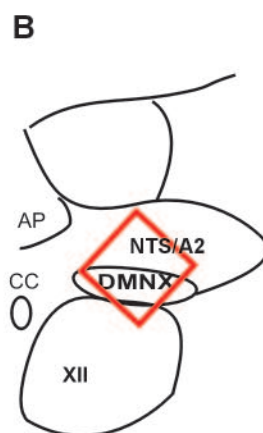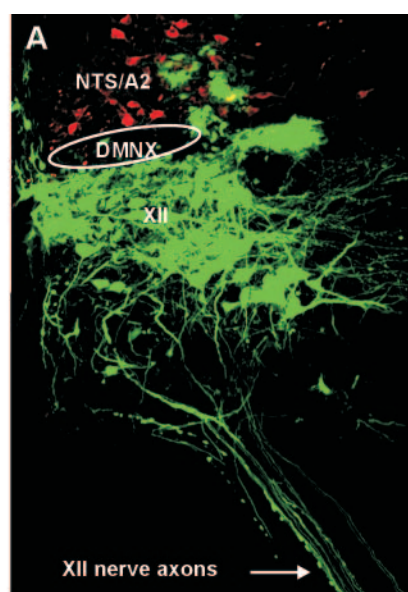
pattern after 48 h. Despite the very high overall level of expression, no DBH-positive neurons could be found, indicating that the mechanisms which suppress HCMV activity in these cells are already active at that stage. Moreover, not only do the catecholaminergic neurons appear to be resistant to HCMV-driven expression, but also so do other neuronal phenotypes abundant in NTS, such as glutamatergic and GABAergic interneurons. Indeed, only a few EGFP-fluorescent NeuN-positive cells (e.g., neurons) were found in NTS of AdHCM-VEGFP-injected rats. Some authors have concluded that HCMV preferentially expresses transgene in glia over neurons (9, 17, 18), but our study demonstrates that this is not necessarily the case. In fact HCMV was very active in XII motoneurons, whereas in the NTS, most of the transduced cells were of glial morphology. Moreover, it appears that in several previously published studies where HCMV was successfully used to express transgenes in neurons, the transduced areas contained cholinergic neurons (13, 22). The reasons for this selective expression in glia and certain neural phenotypes are not known but might be related to the activity of the CREB pathway in these phenotypes (for further discussion see Ref 15). Kügler and colleagues (18) have also suggested that AVV-transduced glia may actively suppress the expression of transgene in certain neurons by secreting cytokines. In a more recent study, it was demonstrated that a very rapid shutdown of HCMV-driven transgene expression in skeletal muscle was due to the extensive methylation of this promoter (3), another possibility that has to be investigated.

Consistent with previously published data, Syn1- and Tα1-driven EGFP expression appeared to be restricted to cells of neuronal morphology (7, 18, 33, 42), whereas the NSE promoter also allowed expression in cells of glial morphology (17, 18). It was more surprising to discover that all three neuronal-specific promoters were unable to drive EGFP expression in catecholaminergic neurons, at least not to levels detectable by spectral confocal microscopy (on the basis of our previous studies, we estimate the threshold of detection in these studies to be 100–300 nm of EGFP) (37). It is known, for example, that synapsin 1 is not expressed in all neuronal types in the retina (21) and in the thalamus (40). Thus it is possible that this gene is inactive in catecholaminergic neurons, and therefore the same pattern of transcriptional control could apply to the exogenously introduced proximal promoter sequence used in the AVV. It is more difficult to explain why the Tα1 and NSE promoters are inactive in catecholaminergic neurons. Both proteins are believed to ubiquitously expressed in neurons (15). However, no study has specifically addressed whether indeed

Table 1. *Activity of AVV containing the promoters HCMV, Syn1, Tα1, and NSE, in neurons (NeuN-immunoreactive) of the NTS, DMNX, and XII motonucleus*

	HCMV	Syn1	Tα1	NSE
NTS (excluding A2 neurons)	+	++++	++++	++++
DMNX	++	++	++	++
XII	+++++	+++++	+++++	+++++

The cell counts are based on data obtained in animals injected with 2.2×10^9 pfu/ml. Plus symbol (+) indicates that the promoter was able to induce EGFP expression in NeuN-immunoreactive cells: + = 0–10 cells, ++ = 10–30 cells, +++ = 30–50 cells, ++++ = 50–80 cells, and +++++ = >80 cells per animal. AAV, adenoviral vectors; HCMV, human cytomegalovirus; Syn1, synapsin-1; Tα1, tubulin α1; NSE, neuron-specific enolase; EGFP, enhanced green fluorescent protein; NTS, nucleus tractus solitarius; DMNX, dorsal motonucleus of the vagus; XII, hypoglossal.

all neuronal phenotypes express these proteins nor whether they are colocalized with markers of catecholaminergic neurons. In addition, if the lack of expression in some phenotypes is due to the active suppression of viral transgenes, as mentioned above in connection with HCMV, then it could be that Syn1-, Tα1-, NSE-containing expression cassettes can also be silenced in catecholaminergic neurons. Finally, it is also possible that the sequences used as promoters in these vectors are insufficient to drive expression in some neuronal populations such as catecholaminergic neurons. Whether the same is true for other neuronal phenotypes such as GABAergic, serotonergic, dopaminergic neurons etc. remains to be established.

Targeting of catecholaminergic neurons using AdPRSx8EGFP. The PRSx8 promoter is a synthetic construct based on the binding motif for the transcription factors Phox2a/2b, found in the DBH promoter (12). These transcription factors are critical for the expression of the catecholaminergic phenotype (23, 26, 34). The earlier published data consistently demonstrated that the PRSx8 promoter was able to efficiently drive expression in noradrenergic neurons in the LC (12), where essentially all EGFP-expressing neurons were DBH immunoreactive (15). However, when the same construct was injected into the dorsal vagal complex where catecholaminergic neurons are adjacent to cholinergic motoneurons, the latter also expressed EGFP. This is not entirely surprising: while these neurons release a different neurotransmitter in the postnatal brain, they are believed to be ontogenetically related to catecholaminergic neurons and are also known to express Phox2a/2b (38). We attempted to avoid EGFP expression in cholinergic neurons by altering the AVV titer, but this ap-

Fig. 1. Expression profile of AdHCMVEGFP, AdSyn1EGFP-WHE, AdTα1EGFP, and AdNSEEGFP, following injection into the dorsal vagal complex. *A*: low-power view of the dorsal vagal complex area injected with AdHCMVEGFP. Although no dopamine β-hydroxylase (DBH)-positive (red) A2 neurons expressed enhanced green fluorescent protein (EGFP) and no EGFP-expressing neurons of dorsal motonucleus of the vagus (DMNX) are visible at this magnification, there is robust EGFP expression in the hypoglossal nucleus (XII). Axons of the XII nucleus can be seen traversing toward the ventral surface of the medulla. AdSyn1EGFP-WHE, AdTα1EGFP, and AdNSEEGFP were equally efficient in transducing XII motor neurons. *B*: EGFP expression in the dorsal motonucleus of the vagus (DMNX) was evident following injection of AdHCMVEGFP, AdSyn1EGFP-WHE, AdTα1EGFP, and AdNSEEGFP into the dorsal vagal complex. In this example, AdNSEEGFP was used. EGFP-expressing neurons (green) of the DMNX can be clearly seen, dorsal to the XII nucleus (also expressing EGFP) and ventral to the DBH-immunoreactive (red) A2 neurons. The red box on the cartoon in *B* (*left*) indicates the area and orientation of the image in the *right* and in subsequent images. *C–H*: following injection into the dorsal vagal complex, AdHCMVEGFP (*C*), AdSyn1EGFP-WHE (*D*), AdTα1EGFP (*E*), and AdNSEEGFP (*F*) failed to induce EGFP expression (green) in DBH-immunoreactive (red) A2 neurons, as indicated by the lack of colocalization of EGFP and DBH. Despite the lack of expression of EGFP in catecholaminergic neurons, the neuronal promoters did express EGFP in other neuronal types as indicated by the colocalization (yellow) of NeuN (red) and EGFP (green, *G*; AdTα1EGFP in this example). Adenoviral vectors (AVV) with neuron-specific promoters did not express EGFP in glial cells, as indicated by the lack of colocalization of EGFP and glial fibrillary acidic protein (GFAP; *H*). AdHCMVEGFP largely produced EGFP expression in glia in the nucleus tractus solitarius (NTS), as indicated by the colocalization (yellow) of EGFP (green) with GFAP (red, *I*). Images are merged confocal projection stacks (40–50 μm) or single confocal planes (*G* and *H*). Scale bars are 20 μm.
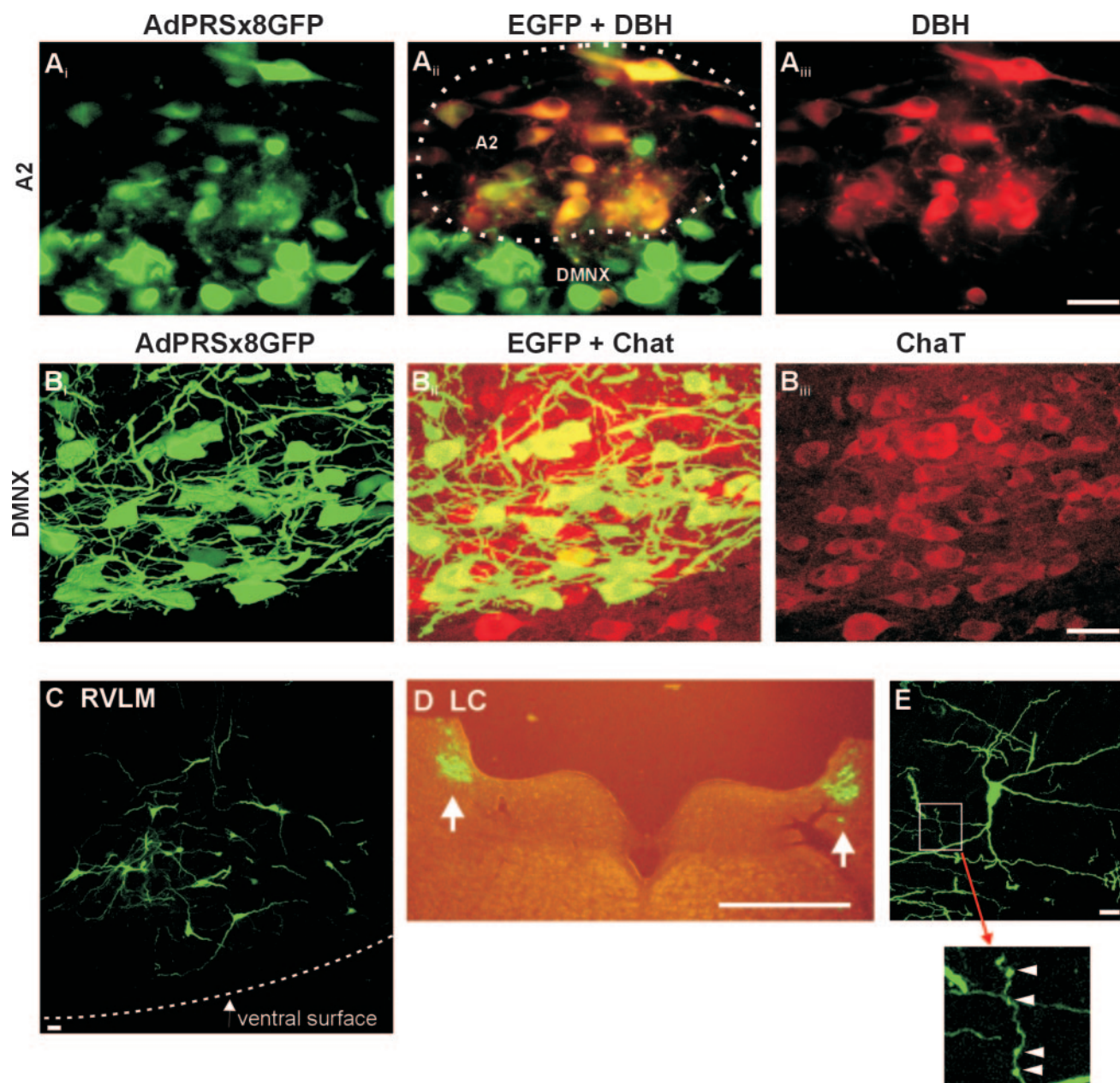
Fig. 2. Expression profile of AdPRSx8EGFP, in the dorsal vagal complex. *A*: following injection of AdPRSx8EGFP into the dorsal vagal complex, EGFP-expressing neurons (green, *A_i*) were partially colocalized (*A_ii*) with DBH-immunoreactive A2 neurons (red, *A_iii*). The remaining EGFP-expressing neurons appeared to belong to the cholinergic population of the DMNX. *B*: the cholinergic phenotype of these cells was confirmed in separate studies where EGFP expression (green, *B_i*) was colocalized (*B_ii*) with choline acetyltransferase (ChAT) immunoreactivity (red, *B_iii*). *C–E*: selective retrograde transduction of catecholaminergic neurons. Following injection of AdPRSx8EGFP into the intermediolateral cell column (IML) or locus coeruleus (LC), many EGFP-expressing adrenergic C1 neurons (green) are observed throughout the extent of the rostral ventrolateral medulla (RVLM, *C*) and LC (*D*, arrows), respectively. Many of the retrogradely labeled neurons were intensely fluorescent, and fine structural details became apparent including extensive dendritic arbor and axonal varicosities (*E*, also see *inset*, arrowheads indicate varicosities). *A* and *B*: merged confocal projection stacks (40–50 µm) or a single confocal plane (*C*) or a superimposition of transmitted light and low-power confocal image (*D*). Scale bars are 20 µm (in *D* = 1 mm).

proach did not provide the desired degree of specificity, suggesting a fairly high level of Phox2 expression in the DMNX. This unsolicited expression in cholinergic neurons that can be found adjacent to most of the catecholaminergic cell groups may complicate experimentation with PRSx8-based constructs in vivo.

Previously we noticed that when AdPRSx8EGFP was injected in the hypothalamus, some retrogradely transduced neurons were found in the brain stem (15), suggesting that noradrenergic neurons may be transduced via retrograde route, from their projection areas. The present study proves the efficacy of this approach: indeed, numerous catecholaminergic cells were found at the expected locations. The number of cells observed in this study following injection of AdPRSx8EGFP into the IML is comparable to that observed following retrograde tracing using cholera toxin β-chain (e.g., see Ref. 28). Moreover, the present experiments show that retrograde targeting may be used to alter the function of these cells as it allows high

levels of transgene expression, as judged from the brightness of EGFP fluorescence (Figs 2, *C–E*). Obviously, this approach will work best after injections in the areas where there is a high concentration of catecholaminergic terminals. For example, injection of AdPRSx8EGFP into the IML allowed selective targeting of C1 adrenergic neurons in the RVLM projecting to that area. Injections of the same construct into the dorsal horn of the lumbar spinal cord selectively transduced another important noradrenergic projection, which originates from the LC. Interestingly, when the PRSx8 promoter was incorporated into a lentiviral backbone, no retrograde labeling of catecholaminergic neurons was observed (T. Lonergan, A. G. Teschemacher, and S. Kasparov, unpublished observation). We believe the retrograde approach can be used to study various aspects of function of different populations of central catecholaminergic neurons and their contribution to disorders such as pathological hypertension.

In conclusion, this study demonstrates the need for careful choice of promoter for gene manipulation in brain stem autonomic nuclei, and probably other brain regions. It illustrates the fact that the "constitutive" or "pan-neuronal" promoters may in fact have a specific pattern of expression and have strikingly different activity profiles in different subsets of neurons even within the same nucleus. Importantly, within the brain region tested, the most commonly used promoter, HCMV, was only active in cholinergic neurons. In the context of genetic manipulation of catecholaminergic neurons, to date PRSx8 turns out to be the only promoter active in these neurons, at least within an AVV backbone. Selective up- or downregulation of genes in these neurons via a retrograde route opens a whole new avenue for studies of their role in central autonomic control and in hypertension.

GRANTS

REFERENCES

1. **Amerena J and Julius S.** The role of the autonomic nervous system in hypertension. *Hypertens Res* 18: 99–110, 1995.
2. **Bett AJ, Haddara W, Prevec L, and Graham FL.** An efficient and flexible system for construction of adenovirus vectors with insertions or deletions in early regions 1 and 3. *Proc Natl Acad Sci USA* 91: 8802–8806, 1994.
3. **Brooks AR, Harkins RN, Wang P, Qian HS, Liu P, and Rubanyi GM.** Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. *J Gene Med* 6: 395–404, 2004.
4. **Davrath LR, Goren Y, Pinhas I, Toledo E, and Akselrod S.** Early autonomic malfunction in normotensive individuals with a genetic predisposition to essential hypertension. *Am J Physiol Heart Circ Physiol* 285: H1697–H1704, 2003. First published June 12, 2003; doi:10.1152/ajpheart.00208.2003.
5. **Ferrari AU, Daffonchio A, Franzelli C, and Mancia G.** Cardiac parasympathetic hyperresponsiveness in spontaneously hypertensive rats. *Hypertension* 19: 653–657, 1992.
6. **Friberg P, Karlsson B, and Nordlander M.** Sympathetic and parasympathetic influence on blood pressure and heart rate variability in Wistar-Kyoto and spontaneously hypertensive rats. *J Hypertens Suppl* 6: S58–S60, 1988.
7. **Gloster A, Wu W, Speelman A, Weiss S, Causing C, Pozniak C, Reynolds B, Chang E, Toma JG, and Miller FD.** The Tα1 α-tubulin promoter specifies gene expression as a function of neuronal growth and regeneration in transgenic mice. *J Neurosci* 14: 7319–7330, 1994.
8. **Glover CP, Bienemann AS, Heywood DJ, Cosgrave AS, and Uney JB.** Adenoviral-mediated, high-level, cell-specific transgene expression: a SYN1-WPRE cassette mediates increased transgene expression with no loss of neuron specificity. *Mol Ther* 5: 509–516, 2002.
9. **Glover CP, Bienemann AS, Hopton M, Harding TC, Kew JN, and Uney JB.** Long-term transgene expression can be mediated in the brain by adenoviral vectors when powerful neuron-specific promoters are used. *J Gene Med* 5: 554–559, 2003.
10. **Graham FL and Prevec L.** Methods for construction of adenovirus vectors. *Mol Biotechnol* 3: 207–220, 1995.
11. **Hirooka Y, Sakai K, Kishi T, Ito K, Shimokawa H, and Takeshita A.** Enhanced depressor response to endothelial nitric oxide synthase gene transfer into the nucleus tractus solitarii of spontaneously hypertensive rats. *Hypertens Res Clin Exp* 26: 325–331, 2003.
12. **Hwang DY, Carlezon WA Jr, Isacson O and Kim KS.** A high-efficiency synthetic promoter that drives transgene expression selectively in noradrenergic neurons. *Hum Gene Ther* 12: 1731–1740, 2001.
13. **Irnaten M, Walwyn WM, Wang J, Venkatesan P, Evans C, Chang KS, Andresen MC, Hales TG, and Mendelowitz D.** Pentobarbital enhances GABAergic neurotransmission to cardiac parasympathetic neurons, which is prevented by expression of GABA$_A$ ϵ subunit. *Anesthesiology* 97: 717–724, 2002.
14. **Ito K, Hirooka Y, Kishi T, Kimura Y, Kaibuchi K, Shimokawa H, and Takeshita A.** Rho/Rho-kinase pathway in the brainstem contributes to hypertension caused by chronic nitric oxide synthase inhibition. *Hypertension* 43: 156–162, 2004.
15. **Kasparov S, Teschemacher AG, Hwang DY, Kim KS, Lonergan T, and Paton JFR.** Viral Vectors as Tools for Studies of Central Cardiovascular Control. *Prog Biophys Mol Biol* 84: 251–277, 2004.
16. **Kishi T, Hirooka Y, Kimura Y, Sakai K, Ito K, Shimokawa H, and Takeshita A.** Overexpression of eNOS in RVLM improves impaired baroreflex control of heart rate in SHRSP. Rostral ventrolateral medulla Stroke-prone spontaneously hypertensive rats. *Hypertension* 41: 255–260, 2003.
17. **Kügler S, Kilic E, and Bahr M.** Human synapsin 1 gene promoter confers highly neuron-specific long-term transgene expression from an adenoviral vector in the adult rat brain depending on the transduced area. *Gene Ther* 10: 337–347, 2003.
18. **Kügler S, Meyn L, Holzmuller H, Gerhardt E, Isenmann S, Schulz JB, and Bahr M.** Neuron-specific expression of therapeutic proteins: evaluation of different cellular promoters in recombinant adenoviral vectors. *Mol Cell Neurosci* 17: 78–96, 2001.
19. **Lonergan T, Goodchild AK, Christie MJ, and Pilowsky PM.** Presynaptic delta opioid receptors differentially modulate rhythm and pattern generation in the ventral respiratory group of the rat. *Neuroscience* 121: 959–973, 2003.
20. **Lu D, Yu K, Paddy MR, Rowland NE, and Raizada MK.** Regulation of norepinephrine transport system by angiotensin II in neuronal cultures of normotensive and spontaneously hypertensive rat brains. *Endocrinology* 137: 763–772, 1996.
21. **Mandell JW, Czernik AJ, De Camilli P, Greengard P, and Townes-Anderson E.** Differential expression of synapsins I and II among rat retinal synapses. *J Neurosci* 12: 1736–1749, 1992.
22. **Mohan RM, Heaton DA, Danson EJ, Krishnan SP, Cai S, Channon KM, and Paterson DJ.** Neuronal nitric oxide synthase gene transfer promotes cardiac vagal gain of function. *Circ Res* 91: 1089–1091, 2002.
23. **Morin X, Cremer H, Hirsch MR, Kapur RP, Goridis C, and Brunet JF.** Defects in sensory and autonomic ganglia and absence of locus coeruleus in mice deficient for the homeobox gene Phox2a. *Neuron* 18: 411–423, 1997.
24. **Palkovits M and Brownstein MJ.** Catecholamines in the dentral nervous system. In: *Handbook of Experimental Pharmacology*, edited by Trendelenburg U and Weiner N. New York: Springer-Verlag, 1989, p. 1–26.
25. **Paton JFR, Deuchars J, Ahmad Z, Wong LF, Murphy D, and Kasparov S.** Adenoviral vector demonstrates that angiotensin II-induced depression of the cardiac baroreflex is mediated by endothelial nitric oxide synthase in the nucleus tractus solitarii of the rat. *J Physiol* 531: 445–458, 2001.
26. **Pattyn A, Goridis C, and Brunet JF.** Specification of the central noradrenergic phenotype by the homeobox gene Phox2b. *Mol Cell Neurosci* 15: 235–243, 2000.

27. **Paxinos G and Watson C.** *The Rat Brain in Stereotaxic Coordinates*. London: Academic, 1986.

28. **Phillips JK, Goodchild AK, Dubey R, Sesiashvili E, Takeda M, Chalmers J, Pilowsky PM, and Lipski J.** Differential expression of catecholamine biosynthetic enzymes in the rat ventrolateral medulla. *J Comp Neurol* 432: 20–34, 2001.

29. **Reja V, Goodchild AK, and Pilowsky PM.** Catecholamine-related gene expression correlates with blood pressures in SHR. *Hypertension* 40: 342–347, 2002.

30. **Ross CA, Armstrong DM, Ruggiero DA, Pickel VM, Joh TH, and Reis DJ.** Adrenaline neurons in the rostral ventrolateral medulla innervate thoracic spinal cord: a combined immunocytochemical and retrograde transport demonstration. *Neurosci Lett* 25: 257–262, 1981.

31. **Ruggiero DA, Cravo SL, Golanov E, Gomez R, Anwar M, and Reis DJ.** Adrenergic and non-adrenergic spinal projections of a cardiovascular-active pressor area of medulla oblongata: quantitative topographic analysis. *Brain Res* 663: 107–120, 1994.

32. **Sakai K, Hirooka Y, Matsuo I, Eshima K, Shigematsu H, Shimokawa H, and Takeshita A.** Overexpression of eNOS in NTS causes hypotension and bradycardia in vivo. *Hypertension* 36: 1023–1028, 2000.

33. **Schoch S, Cibelli G, and Thiel G.** Neuron-specific gene expression of synapsin I. Major role of a negative regulatory mechanism. *J Biol Chem* 271: 3317–3323, 1996.

34. **Seo H, Hong SJ, Guo S, Kim HS, Kim CH, Hwang DY, Isacson O, Rosenthal A, and Kim KS.** A direct role of the homeodomain proteins Phox2a/2b in noradrenaline neurotransmitter identity determination. *J Neurochem* 80: 905–916, 2002.

35. **Seyedabadi M, Goodchild AK, and Pilowsky PM.** Differential role of kinases in brain stem of hypertensive and normotensive rats. *Hypertension* 38: 1087–1092, 2001.

36. **Sinnayah P, Lindley TE, Staber PD, Davidson BL, Cassell MD, and Davisson RL.** Targeted viral delivery of Cre recombinase induces conditional gene deletion in cardiovascular circuits of the mouse brain. *Physiol Genomics* 18: 25–32, 2004. First published April 6, 2004; doi:10.1152/physiolgenomics.00048.2004.

37. **Stokes CEL, Murphy D, Paton JFR, and Kasparov S.** Dynamics of a transgene expression in acute rat brain slices transfected with adenoviral vectors. *Exp Physiol* 88: 459–466, 2003.

38. **Tiveron MC, Hirsch MR, and Brunet JF.** The expression pattern of the transcription factor Phox2 delineates synaptic pathways of the autonomic nervous system. *J Neurosci* 16: 7649–7660, 1996.

39. **Tucker DC, Saper CB, Ruggiero DA, and Reis DJ.** Organization of central adrenergic pathways: I. Relationships of ventrolateral medullary projections to the hypothalamus and spinal cord. *J Comp Neurol* 259: 591–603, 1987.

40. **Ullrich B and Sudhof TC.** Differential distributions of novel synaptotagmins: Comparison to synapsins. *Neuropharmacology* 34: 1371–1377, 1995.

41. **Waki H, Kasparov S, Wong LF, Murphy D, Shimizu T, and Paton JFR.** Chronic inhibition of eNOS activity in NTS enhances baroreceptor reflex in conscious rats. *J Physiol* 546: 233–242, 2003.

42. **Wang S, Wu H, Jiang J, Delohery TM, Isdell F, and Goldman SA.** Isolation of neuronal precursors by sorting embryonic forebrain transfected with GFP regulated by the T alpha 1 tubulin promoter. *Nat Biotechnol* 16: 196–201, 1998.

43. **Wong LF, Polson JW, Murphy D, Paton JF, and Kasparov S.** Genetic and pharmacological dissection of pathways involved in the angiotensin II-mediated depression of baroreflex function. *FASEB J* 16: 1595–1601, 2002.

44. **Yang H and Raizada MK.** Role of phosphatidylinositol 3-kinase in angiotensin II regulation of norepinephrine neuromodulation in brain neurons of the spontaneously hypertensive rat. *J Neurosci* 19: 2413–2423, 1999.

45. **Zimmerman MC, Lazartigues E, Lang JA, Sinnayah P, Ahmad IM, Spitz DR, and Davisson RL.** Superoxide mediates the actions of angiotensin II in the central nervous system. *Circ Res* 91: 1038–1045, 2002.

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