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Review

Viral vectors as tools for studies of central cardiovascular control

S. Kasparov[a,*], A.G. Teschemacher[b], D.-Y. Hwang[c], K.-S. Kim[c], T. Lonergan[a], J.F.R. Paton[a]

[a] *Department of Physiology, University of Bristol, Bristol, UK*
[b] *Department of Pharmacology, University of Bristol, Bristol, UK*
[c] *Molecular Neurobiology Laboratory, McLean Hospital, Harvard Medical School, Belmont, USA*

Abstract

During the last few years physiological genomics has been the most rapidly developing area of physiology. Given the current ease of obtaining information about nucleotide sequences found in genomes and the vast amount of readily available clones, one of the most pertinent tasks is to find out about the roles of the individual genes and their families under normal and pathological conditions. Viral gene delivery into the brain is a powerful tool, which can be used to address a wide range of questions posed by physiological genomics including central nervous mechanisms regulating the cardio-vascular system. In this paper, we will give a short overview of current data obtained in this field using viral vectors and then look critically at the technology of viral gene transfer.

Contents

*Corresponding author. Tel.: +44-117-928-7818; fax: +44-117-928-8923.
E-mail address: sergey.kasparov@bris.ac.uk (S. Kasparov).

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1. Introduction. Why viral vectors?

Increased or decreased expression of certain genes may be a cause of a pathological state or reflect second-order compensatory process. Gene arrays can be used to reveal an alteration in some genes within the brain of a model of a pathological state such as the spontaneously hypertensive rat (Busche et al., 2001). In some cases the nature of these genes may be known and there is some initial information about their physiological function (Busche et al., 2001), but this may not always be the case. Currently, perhaps the most popular approach to investigate gene function is to generate a knock-out animal in which the resulting phenotype may give clues. In fact, a vast amount of knock-out mice have already been produced some of which are commercially available. However, the knock-out technology has a number of draw-backs which, from our point of view, limit its value in physiological genomics, especially when it comes to understanding highly complicated systems such as central cardio-vascular control networks. Detailed analysis of these limitations (restricted to the mouse, dependent on the progenitor strain, in absolute majority of cases persistent throughout pre- and postnatal life and therefore evoking multiple compensatory effects at various stages of development) is outside of the scope of this paper. It suffices to draw attention to one particular problem of the knock-out strategy: the lack of the spatial/cellular specificity and temporal control.

Considering this problem, it is well-known that central cardiovascular control involves a co-ordinated action of a number of brain nuclei all of which perform different functions. For example, if a potassium channel involved in the maintenance of the resting membrane potential is knocked-out, this may increase activity of both, sympathetic and parasympathetic outflows, as well as elevate activity in other brain areas impinging upon the autonomic motor nuclei. Thus,

any conclusion as to the role of this potassium channel in controlling autonomic activity would be meaningless. Therefore, site-specific genetic perturbation is an important requirement for experiments addressing functional genomics of central blood pressure control.

But site specificity is not all that is necessary. All brain nuclei are heterogeneous in terms of their histochemistry and connectivity. Many nuclei harbour some excitatory glutamatergic and some inhibitory GABAergic neurones and changes in both of these transmitter systems have been implemented in pathological hypertension (Chalmers, 1998; de Wardener, 2001; Haywood et al., 2001; Arnolda et al., 1992; Yamada et al., 1995). Additionally, catecholaminergic cell groups (e.g. A2 in the Nucleus Tractus Solitarius or NTS, A6 in the Locus Coeruleus, C1 in the rostral ventro–lateral medulla) have been implicated in pathological hypertension (Dampney et al., 1984; Dampney, 1994; Lu et al., 1996; MacLean et al., 1990a, b). Moreover, each group of neurones is embedded in a network of glia and blood vessels. Thus, returning to the example of knocking-out a potassium channel, even if restricted to a single nucleus, such unfocused change will have unpredictable impact on blood pressure control. Therefore, a selective genetic manipulation of a certain neuronal phenotype localised to a particular brain region may be of great value. In addition, targeting of other cell types, such as glia or endothelial cells may become necessary like in our recent studies (Paton et al., 2002).

Finally, temporal control of expression of a gene is essential. For example, the spontaneously hypertensive rat only starts developing high blood pressure at an age of 4–5 weeks. It is possible that at that time certain genetic mechanisms become operational which account for the development of this pathological blood pressure. It may therefore become desirable to time any genetic manipulation exactly at this transitional stage and not before. In addition, it is optimal if the effects of genetic manipulation can be compared with control values obtained in the same animal (Kishi et al., 2002, 2003; Waki et al., 2003; Paton et al., 2001, 2003).

In conclusion, in order to be able to interpret the outcome of a genetic manipulation, at least in the context of central cardio-vascular control, it has become essential to restrict the intervention to specific nuclei or even better to a specific cell type within that nucleus and to have temporal control over expression of transgenes. One way to meet all these requirements is to use somatic gene transfer directed towards selected nuclei in order to increase or decrease expression of a particular gene. In a chronic experiment a parameter such as blood pressure can be monitored, a transgene delivered and the physiological outcome determined. However, transfer of genes into an intact brain in vivo remains a challenging task. While transfections of cell lines including those of neural origin are relatively trivial using various transfection reagents, mature neurones in the living brain are either resistant to these procedures or become damaged. This is why viral vectors, which can effectively deliver transgenes into brain cells, and even integrate them into the host genome for long term expression (many months, see references below), are so attractive.

2. The use of viral vectors for understanding central cardio-vascular control mechanisms

Viral vectors in the field of central cardio-vascular control have been used to both, enhance (via overproduction or "overexpression") and inhibit (via expression of dominant negative proteins or other transgenes) functions of certain signalling cascades. In addition, in peripheral studies, adeno-associated virus (AVV) based vectors have been extensively used for delivery of antisense

nucleotides (see references in Phillips, 2001a, b). There is no doubt that viral vectors will be used for delivery of new genomic tools such as siRNA (Xia et al., 2002; Shen et al., 2003).

Our laboratory is interested in the role of nitric oxide in baroreceptor reflex control at the level of the NTS. We used (Paton et al., 2001) a number of pharmacological tools to demonstrate that various blockers of nitric oxide production prevented a well-documented inhibitory action of angiotensin II (AngII) on this reflex in the NTS (Casto and Phillips, 1985, 1986). We chose to employ viral gene transfer of a dominant negative form of endothelial nitric oxide synthase (eNOS) for two reasons: first, we believed that it would be more selective for the ''endothelial'' NOS isoform (as opposed to the ''neuronal'' or nNOS isoform) than the available pharmacological antagonists. Secondly, we wanted to evaluate the role of NO in NTS chronically as long-term pharmacological blockade of eNOS restricted to NTS is not technically achievable. To suppress endogenous eNOS in the NTS, an AVV was used to express a truncated form of eNOS (TeNOS) under the control of the human cytomegalovirus (HCMV) promoter (Kantor et al., 1996). TeNOS lacks catalytic activity but retains the NH_2-terminal sequences required for co-translational NH_2-terminal glycine myristoylation and membrane localisation. TeNOS acts as a dominant negative inhibitor of wild-type eNOS activity through heterodimerisation with the native protein (Lee et al., 1995). We were able to demonstrate that in animals which had received 3 bilateral injections in the NTS of the TeNOS AVV 5–6 days prior to the test, AngII which normally strongly suppresses baroreceptor reflex when administered into the NTS, was ineffective Fig. 1 (see also: (Paton et al., 2001). The NO precursor L-arginine which also inhibited baroreceptor reflex when injected into the NTS of naïve rats, also failed to modulate baroreflex in

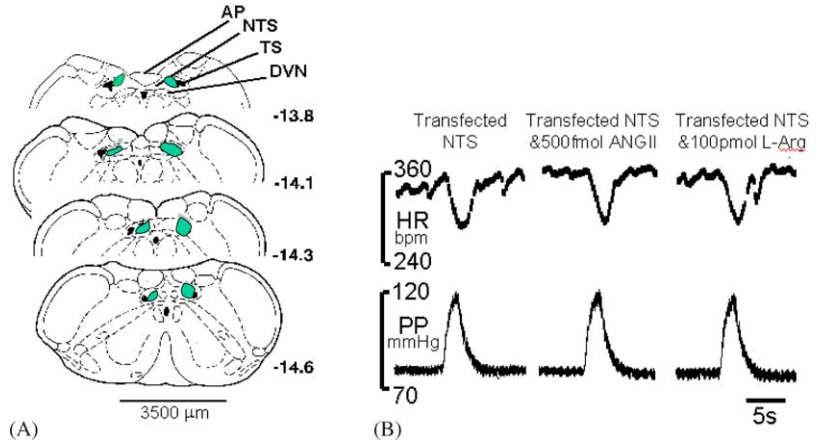


Fig. 1. Adenoviral gene transfer of a dominant negative eNOS protein (TeNOS) demonstrates the involvement of eNOS in AngII action on baroreceptor reflex in NTS. AVV HCMV-TeNOS and AVV to express EGFP were co-injected in the NTS of rats 5–6 days before they were used to prepare working heart–brainstem preparations. Baroreceptor reflex bradycardia was assessed using pressure ramps before and after NTS microinjections of AngII. AngII was unable to suppress the baroreflex in contrast to the data obtained in naïve rats in this and other studies from this laboratory. NO precursor L-arginine was also ineffective in TeNOS expressing animals, in contrast to naïve rats in which it blocks baroreflex similar to AngII. Reproduced with permission from Paton et al. (2001). (A) Sites of viral transfection identified by EGFP fluorescence documented on diagrams after Paxinos and Watson (1986). (B) Baroreflex bradicardia was evoked by perfusion pressure ramps before and after microinjection of AngII and L-arginine into the NTS.

pre-transfected animals. There appeared to be no evidence of any aberrant effects of the AVV, since (i) the baseline baroreceptor reflex sensitivity was not altered by TeNOS expression and, (ii) an EGFP-expressing AVV did not cause any detectable alterations in baroreflex function and multiple other variables measured.

More recently, the same construct was used chronically. In conscious freely moving rats instrumented with radiotransmitters to record their blood pressure and heart rate, the spontaneous baroreceptor reflex gain was determined by a time-series method. TeNOS expression evoked a gradual increase in the gain of the reflex from days 7 to 21 after gene transfer, peaking at day 21 (1.68 ± 0.20 ms/mmHg). This value was significantly higher than that before gene transfer (1.13 ± 0.09 ms/mmHg; $P < 0.001$) and in rats in which either EGFP only was expressed in the NTS or a saline-treated group. In addition, heart rate decreased from 336 ± 6 to 318 ± 8 b.p.m. ($P < 0.05$) 21 days after TeNOS gene transfer. However, in these normotensive animals gene transfer did not affect arterial pressure. These findings indicate that in the conscious rat eNOS is constitutively active within the NTS and is a factor regulating baroreceptor reflex gain and heart rate but not resting blood pressure (Waki et al., 2003). Our preliminary data indicate that when TeNOS was expressed in 10–11 week old SHR, baroreflex sensitivity increased, while systolic blood pressure decreased. In weight-matched Wistar-Kyoto control rats, similar changes in the baroreceptor reflex sensitivity were observed but arterial pressure was unaffected by TeNOS (Kasparov et al., 2002b). These results suggest that elevated activity of endogenous eNOS in the NTS is an important factor determining levels of arterial pressure in this model of pathological hypertension. For reasons which will become apparent later, it is worth mentioning that in all of these vectors transgene expression was driven by the HCMV promoter.

To dissect out the intracellular signalling pathway utilised by AngII to suppress baroreceptor reflex within NTS in acute experiments we used a range of adenoviral vectors (Wong et al., 2002). As shown in Fig. 2, HCMV–EGFP-injected animals were no different to naïve rats in terms of AngII action on the baroreceptor reflex in NTS. However, expression of a dominant negative Gq protein driven by the human elongation factor (EF1α) promoter (a kind gift of Professor D. Cook, University of Sydney, Australia) in NTS, strongly antagonised the depressant action of AngII. This was an important finding because Gq is an established link between G-protein coupled receptors (such as the AngII type 1 receptor) and intracellular Ca^{2+} stores. These data were ratified using conventional pharmacological approaches (Fig. 2; Wong et al., 2002). In the same study two HCMV-based AVV were used. The first one overexpressed human catalase (a kind gift of Dr. X. Fang and Dr. B. Davidson, University of Iowa) in order to reveal a potential role for reactive oxygen species. Incidentally, after NTS transfection we histochemically demonstrated high catalase activity at the site of injection. The second vector carried a dominant negative form of Akt (protein kinase B, kind gift of Dr. K. Walsh, Boston). However neither of these vectors inhibited the effect of AngII on the baroreceptor reflex, which confirmed the outcome of pharmacological experiments, Fig. 2 (Wong et al., 2001). In summary, our laboratories have used AVV to investigate a link between AngII and eNOS, a role for eNOS in the long-term control of baroreceptor reflex sensitivity in normotensive and hypertensive animals and the intracellular signalling pathway of AngII at the level of NTS.

Viral vectors have been used to investigate mechanisms of central cardiovascular control by several other groups. Recently, a number of articles employing AVV have come from the laboratory of Y. Hirooka. This group used AVV to overexpress certain proteins as opposed to

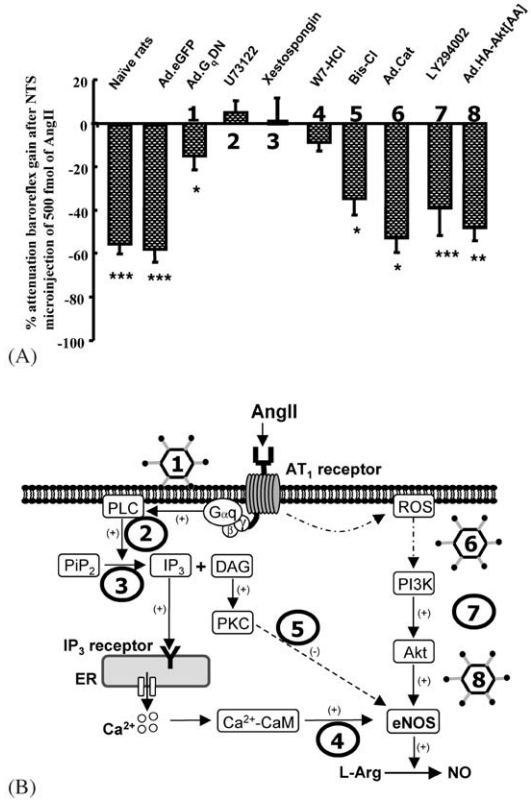


Fig. 2. Signalling pathway of Ang II in NTS analysed using AVV gene delivery and pharmacological drugs. (A) Bar chart to illustrate the effect of gene transfer and pharmacological tools on Ang II—induced depression of baroreceptor reflex gain (sensitivity). Note that the degree of the baroreceptor reflex inhibition was similar in naïve rats and rat pretransfected with AVV HCMV-EGFP. This illustrates the lack of non-specific effects of viral gene transfer on NTS function. Ad.GqDN expressed dominant negative protein to inhibit function of Gq protein which strongly antagonised the depressant action of AngII. For further detail see text and Wong et al. (2002). Upper panel reproduced with permission from Wong et al. (2002). (B) Plausible signalling pathways of AngII in NTS. Numbers at particular signalling links refer to the viral or pharmacological tools used to analyse them (as shown in A).

blocking their actions with dominant negatives. They were able to demonstrate that 5–10 days after injection in the NTS of an AVV that induced overexpression of eNOS, blood pressure and heart rate significantly decreased, as measured using radiotelemetry (Sakai et al., 2000). These results were interpreted as an indication of a potentiating effect of NO on transmitter (presumably glutamate) release in the NTS. We have argued that the apparent inconsistency of these results and our data may be due to the lack of spatial specificity and excessive concentration of NO in overexpression experiments. Indeed, copious amounts of NO produced as a result of HCMV-driven eNOS expression might cause different effects to presumably small concentrations released by endogenous eNOS (for more detailed discussion see Paton et al., 2002). Overexpression of eNOS in the rostral ventro-lateral medulla (RVLM) also lead to a decrease in the mean arterial blood pressure and heart rate. These effects were greater in stroke-prone SHR than in WKY.

Based on the differences in the sensitivity of the pressor responses to the RVLM injections of bicuculline in SHR and WKY the authors argue that apparently NO overproduction resulted in an apparent increase in local GABAergic input to RVLM neurons (Kishi et al., 2002). More recently that laboratory has also found that NO overproduction in the RVLM improves the impaired baroreflex control of heart rate in the SHR (Kishi et al., 2003). For more detailed account of the work coming from Y. Hirooka group see their contribution in this issue.

R. Davisson, B. Davidson and their colleagues and have been working to develop viral means of gene delivery to the supraoptic nucleus and subfornical organ in mice. Both of these structures are of key importance in cardiovascular control and express a range of genes of the renin–angiotensin cascade. Both, AVV and lentiviral vectors (LVV) derived from the feline immunodeficiency virus were used to achieve selective targeting of these cardiovascular-relevant brain structures. Interestingly, although the expression was controlled by HCMV in both types of vectors, the vesicular stomatitis (VS)-coated LVV proved to be more neurone-selective than AVV, which transduced both glia and neurones (Sinnayah et al., 2002). AVV were also used by the same group to demonstrate an involvement of reactive oxygen species in the pressor and dipsogenic responses induced by centrally administered AngII in mice (Zimmerman et al., 2002). In that study superoxide dismutase isoforms were overexpressed in mouse brain using intracerebroventricular injection of AVV with the HCMV promoter. Overexpression of both, mitochondrial and cytoplasmatic isoforms strongly antagonised responses to icv injections of AngII indicating a role for reactive oxygen species in AngII signalling pathways. For further details see their contribution in this issue.

3. Viral vectors as experimental tools

The success of viral gene delivery experiments depends on the ability of the transgene to appear in the relevant cell type, at a concentration that gives rise to a physiological response. This will depend primarily on two factors: (i) the ability of the viral vector to deliver its genome into the cell and, (ii) the activity of the promoter used for transgene expression.

Several types of viral vectors are currently in use but the most popular types in studies of central mechanisms of cardio-vascular control are AVV and retroviral vectors including LVV (Katovich et al., 2001; Wang et al., 1999; Sinnayah et al., 2002; Stec et al., 1999; Kishi et al., 2003; Waki et al., 2003; Wong et al., 2002; Paton et al., 2001; Irnaten et al., 2002; Hirooka et al., 2001). It is generally accepted that these viruses can infect various cell types present in the brain (i.e. neurones, glia, vascular cells). The AVV has also been successfully used by a number of groups mainly for gene delivery to peripheral targets (Cucchiarini et al., 2003; Phillips, 2001a, b; Peel and Klein, 2000; Davidson and Chiorini, 2003). Even the most advanced versions of these vectors cannot accommodate inserts of more than $\sim$4 kb which may limit their use in some cases (Kugler et al., 2003). We will not discuss these vectors any further as our laboratory has no first-hand experience with them.

AVV have been increasingly used for gene transfer in physiological research since pioneering studies in 1993 (Akli et al., 1993; Davidson et al., 1993). However, despite extensive application of AVV for gene delivery in numerous cellular systems (Griesbeck et al., 1997), our knowledge regarding some essential properties of adenoviral-mediated gene expression (such as preferential

transfection of some cell types) is mainly based on these early reports. The information about the expression profile of the LVV in the brain is even more limited (but see: Bienemann et al., 2003; Mazarakis et al., 2001; Mitrophanous et al., 1999; Coleman et al., 2003).

4. AVV and LVV as delivery vehicles

AVV and LVV are different in many respects. From the practical point of view, the capacity of the vector is one of its most important characteristics. The majority of laboratories use AVV with deletions in E1 region (with additional deletions in E2 or E3) which can accommodate approximately 7 kb of a transgene. This leaves enough space for a sufficiently long promoter and 1–2 genes. HIV-derived LVV which we are currently using, has nearly all of the viral genome deleted and in principle can accommodate ~11–12 kb. These theoretical limits may be very hard to reach in practice. Nevertheless, these size limitations are important when cell specificity is desired, because in most cases, researchers attempt to achieve such specificity by using long sequences found upstream of a gene, as promoters (Jin et al., 2001). Another practically important difference is the ability of LVV to stably insert the transgene into the host genome, while the transgenes delivered by AVV remain episomal, which leads to a fairly rapid elimination of the transgene, especially in dividing cells.

AVV particles are very different to LVV in that they are non-enveloped and their ability to invade cells is dependent entirely on the proteins present in their fibres and capsid. It has been demonstrated that the serotype affects the neuronal tropism of the AVV and apparently vectors with the Ad17 fibre may be best for targeting neurones (Chillon et al., 1999). Nevertheless, most of the AVV vectors currently used have been derived from the serotype 5. Adenovirus fibre protein interacts with a range of protein receptors present on the membranes of many cells, such as the Major Histocompatibility Complex (MHC) class I molecule and the ''coxsackievirus–adenovirus'' receptor (CAR). Modifications of the AVV fibre protein may change its tropism and confer useful properties to these vectors in terms of their targeting for certain cell types (for review see, Wickham, 2000). Recently, using this approach, Omori et al. (2002) have managed to re-direct AVV to microglia, which lack CAR by incorporating the Arg-Gly Asp motif containing peptide into the HI loop of the fibre knob. This motif enables interaction with αV integrins, ubiquitously expressed on the surface of mammalian cells. Similarly, Baker and his colleagues were able to modify the HI loop to de-target AVV from CAR and direct it instead to endothelial cells (Nicklin et al., 2001; White et al., 2001). Thus modifications of adenoviral capsid can lead to the development of vectors with an even better ability to preferentially target different cell types present in brain, such as glia or the endothelium of blood vessels.

It is clear that the properties of the viral particle may affect the ability of the vector to invade remote neurones retrogradely. There has been some controversy regarding the ability of the conventional AVV to transduce retrogradely. In our earlier studies, we unilaterally microinjected a mixture of AVV driving expression of EGFP under the control of HCMV promoter, and Fluo-Ruby (a red-shifted conventional retrograde tracer) into the right hippocampus of rats. Seven days later, numerous cells retrogradely labelled with Fluo-Ruby were found both ipsi- and contralaterally in many brain areas, but none of them expressed EGFP (Kasparov and Paton, 2000). However a clear retrograde transduction using AVV was obtained after microinjections

into the caudate putamen (Bilang-Bleuel et al., 1997). Transfected cells (predominantly dopaminergic) were found up to 2 mm from the site of injection. Interestingly, in this case the Rous sarcoma virus long terminal repeat was used as a promoter, rather than HCMV. The authors observed numerous retrogradely transfected cells in substantia nigra compacta. Initially we thought that this could mean that dopaminergic (or all catecholaminergic) neurones are selectively sensitive to retrograde transduction (Kasparov and Paton, 2000). However, Nishimura et al. (1998) were able to achieve retrograde transfection using intra-hippocampal AVV injections (especially when the virus was suspended in a hypertonic 1 M mannitol solution). The expression in these AVV was controlled by the modified chicken β-actin promoter (Niwa et al., 1991), rather than HCMV and the neurones retrogradely transduced in these experiments in the cortex were likely to be glutamatergic, not catecholaminergic. On the other hand, Irnaten et al. (2002) used an AVV with the HCMV-driven construct to retrogradely transduce cholinergic neurones of the nucleus ambiguus from the pericardial sac and Sinnayah et al. (2002) found retrogradely transfected neurones in subfornical organ after injection of AVV with HCMV promoter into the supraoptical nucleus (the exact transmitter phenotype of these cells is not known). Taken together, these data indicate that AVV *are* able to retrogradely invade cells other than catecholaminergic neurones. In light of our recent observations about the lack of HCMV activity in noradrenergic neurones and its high activity in cholinergic neurones (see below), we hypothesised that the lack of retrograde transduction in our earlier experiments with HCMV-driven AVV construct could be largely due to HCMV, rather than the AVV themselves. We tested this hypothesis by injecting AVV with the PRSx8 promoter, which is highly active in noradrenergic and adrenergic cells, into the paraventricular area of hypothalamus in the rat. As illustrated in Figs. 3 and 8 noradrenergic neurones expressing EGFP were found in the NTS

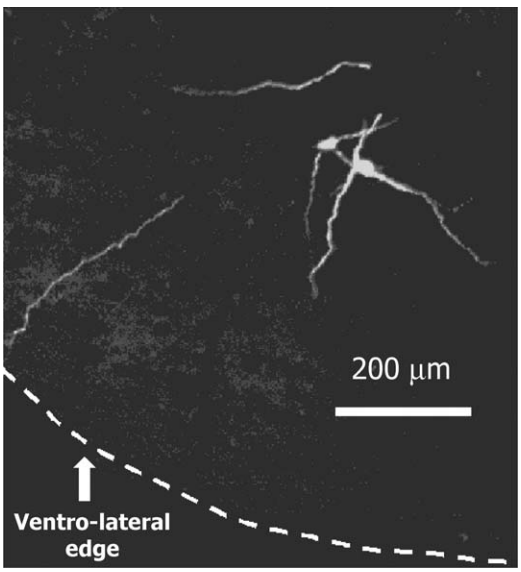


Fig. 3. Noradrenergic neurones in the brainstem retrogradely transduced using PRSx8-EGFP AVV. A photograph of the C1 area of the brainstem from a rat which had received a bilateral injection of AVV PRS8-EGFP in the periventricular area of hypothalamus, 5 days before the sections were prepared.

and in the ventrolateral medulla 5 days after the injection. These cells could have *only* contracted the transgene via retrograde invasion from the axonal arborisations within the hypothalamus, because these areas are separated by many *millimetres* of brain tissue, ruling out diffusion. Therefore, we believe that the expression in remote sites after AVV microinjections may occur albeit at a fairly low level, provided that the promoter used is active in these projection neurones. Clearly, AVV are much less efficient in retrograde transduction than, for example, the rabies or pseudorabies virus.

In contrast to AVV, the LVV capsid is wrapped into an additional "coat". The coat is associated with the capsid but it is a separate protein encoded by separate genes. Because the coat is involved in the binding of the viral particle to the cellular membrane, modifications of the coat can alter the tropism of the vector. Most vectors used currently, irrespectively of the virus they have been derived from (i.e. HIV or feline immunodeficiency virus) are pseudotyped, i.e. employ a foreign protein coat such as the VS glycoprotein G (Iwakuma et al., 1999; Sinnayah et al., 2002; Coleman et al., 2003). The VS coat enables LVV to deliver transgenes to a wide range of cells, including neurones and glia (Coleman et al., 2003). Pseudotyping with other coats confers new properties. VS-pseudotyped vectors seem to have negligible ability for retrograde transfection. For example, VS-pseudotyped feline immunodeficiency virus did not retrogradely transduce neurones in subfornical organ when injected into the supraoptical nucleus in contrast to an AVV (Sinnayah et al., 2002). This feature of VS-pseudotyped LVV may be important for functional studies where the impact of a gene manipulation needs to be restricted to a single injected brain area. When an EF1α-EGFP LVV with the VS coat was injected into the hypothalamus of the rat, no GFP-expressing neurones in remote areas such as brain stem could be found (Kasparov, Paton, Huentelman and Raizada—unpublished observation). Other envelopes, however alter this property of LVV. For example, rabies-G envelope conferred the ability for the retrograde axonal transfection to the equine infectious anaemia virus bearing HCMV-βGal expression cassette (Mazarakis et al., 2001). When pseudotyped using the VS coat, these vectors were completely unable to transduce spinal motor neurones after an intramuscular injection, while rabies-pseudotyped vectors induced β-Gal in 27% of the retrogradely labelled (cholinergic) motor neurones. Moreover, after injection into the spinal cord, rabies-G pseudotyped vectors induced expression in cortico-spinal neurones. Interestingly, pseudotyping also affected the degree of immune response to the viral vectors and was minimal when VS coat was used (Mazarakis et al., 2001).

5. Promoters and their impact on the properties of viral vectors

5.1. HCMV

Since the pioneering studies of Wilkinson and Akrigg (1992) the HCMV immediate-early promoter has been extensively used to drive expression of viral transgenes. This promoter is often referred to as "constitutively active", a reflection of the plethora of response elements and enhancer sequences, including several calcium/cAMP response elements (CRE) and NF-χB binding sites, an AP-1 site, a serum-response element (SRE), etc. Although this promoter is expected to be active in most cell types in the brain, it remains unclear to what extent its function

is affected by the regulatory pathways of the host cell, in particular the activity of the CRE binding protein (CREB). In cell lines HCMV-driven expression was clearly sensitive to forskolin and dibutyryl-cAMP suggesting that the CREB pathway is essential for HCMV promoter function (Wilkinson and Akrigg, 1992). More recently this has been confirmed in neurones directly. Using AVV (Wheeler and Cooper, 2001) have found that HCMV–EGFP transfected cultured sympathetic ganglion neurones and hippocampal neurones did not express EGFP. However, prolonged exposure to 40 mM K^+ (to evoke depolarisation) triggered EGFP expression (Wheeler and Cooper, 2001). The authors argue that depolarisation opens voltage (and nifedipine)-sensitive Ca^{2+} channels and this leads to activation of the CREB pathway. Mutation of all five CRE abolished this induction. We have addressed this issue using acute rat brainstem slices transfected using AVV with the HCMV–EGFP expression cassette (Stokes et al., 2003a). In these experiments expression of EGFP could be significantly attenuated using Rp-cAMPs which blocks a number of cAMP-dependent cascades, in particular PKA (Fig. 4). Hence, it is possible that the transgene expression may be discriminative in favour of cell type (neuronal, glial and vascular) where this cascade is highly active for some reason. Moreover, it is possible that this expression profile may change subject to external influences.

Recently we have made some observations that imply HCMV-driven expression is not as ubiquitous in the brain as sometimes thought. A striking result was obtained in experiments in organotypic brainstem slice cultures transfected with two AVV at the same time: the first AVV contained an HCMV-DsRed (a far-red shifted fluorescent protein from coral) expression cassette, while in the second AVV expression of EGFP was controlled by the PRSx8 artificial promoter

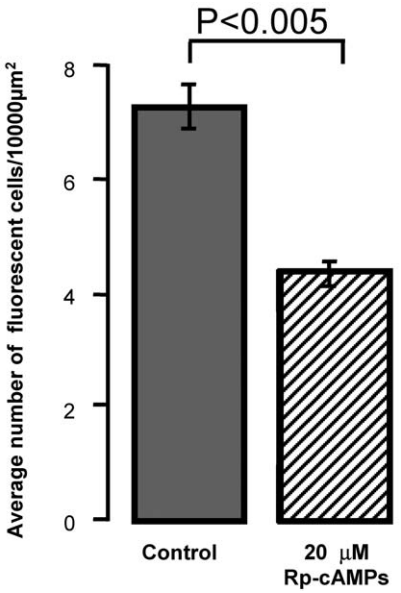
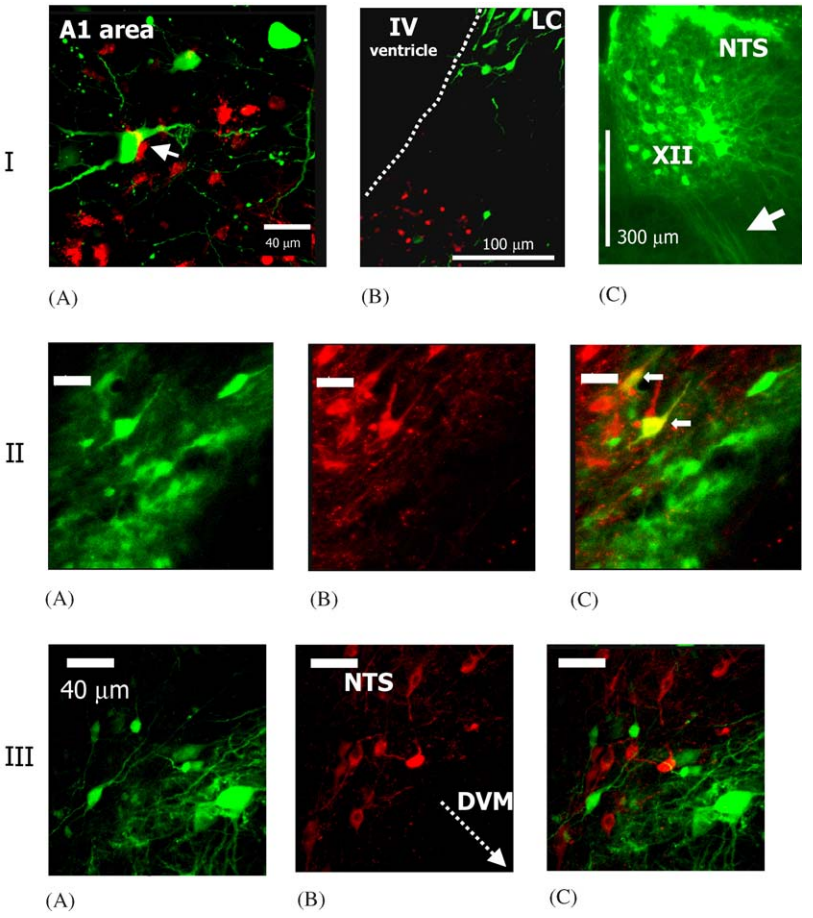


Fig. 4. Blockade of cAMP signalling inhibits HCMV-driven expression in acute brainstem slices. Acute rat brainstem slices were prepared and transfected as described in Stokes et al. (2003a) with AVV HCMV-EGFP. Some slices were incubated with 20 μm of the cAMP antagonist Rp-cAMPs which strongly inhibited EGFP accumulation and therefore reduced the number of fluorescent cells visible after 10 h of incubation.

(see below), which is highly active in noradrenergic and adrenergic neurons. As shown in Fig. 5 in the A1 noradrenergic group no co-localisation of DsRed and EGFP could be detected in numerous (> 50) cells using spectral confocal microscopy (for details see, Kasparov et al., 2002a). In contrast, when HCMV–DsRed AVV was co-applied with HCMV–EGFP AVV > 90% of cells (many of which looked glial) expressed both proteins. These results were consistent with the outcome of in vivo experiments where noradrenergic neurones in the locus coeruleus did not express DsRed after injection of a mixture of PRSx8-EGFP and HCMV–DsRed AVV, although red fluorescent cells were visible in the adjacent areas (Fig. 5). Interestingly, HCMV–EGFP AVV caused robust expression in motor cholinergic nuclei, such as the motor nucleus of the hypoglossal nerve and dorsal vagal motor nucleus (Fig. 5). The ability of AVV with HCMV promoter to efficiently target glia has also been documented in vivo (Kugler et al., 2003) and in acute brain slices (Stokes et al., 2003a). Moreover, AVV with HCMV–EGFP can transduce local cerebral vascular endothelium after brain microinjections (Kasparov and Paton, 2000). It follows, that HCMV-controlled vectors may discriminate against some neuronal phenotypes (at least in the brainstem). It has been proposed that some unidentified inhibitory factors released by HCMV-transduced glia may explain HCMV inactivity in some neuronal types (Kugler et al., 2001). The



same factors might explain the dramatic fall in HCMV-driven expression reported recently by Glover et al. (2003).

These observations shed new light on our own data obtained using several AVV with HCMV promoter. For example, as-mentioned above, TeNOS (the dominant negative for eNOS) expression in the NTS using AVV with the HCMV promoter antagonised the effect of AngII. Because TeNOS expression was not limited to either neurones or blood vessels we were unable to pinpoint the exact site of AngII action in that study (Paton et al., 2001). Given, that the noradrenergic neurones are the likeliest neuronal phenotype targeted by AngII (Lu et al., 1996; Sumners et al., 1987; Jenkins et al., 1995) the lack of HCMV activity in these neurones further strengthens our argument that within the NTS AngII acts on the endothelium to release NO which affects the activity of the adjacent neuronal networks (Paton et al., 2002). This argument awaits confirmation using other vectors with cell-specific promoters.

5.2. *EF1α promoter*

Elongation factor 1α (EF1α) is a ubiquitous nucleotide-binding protein involved in the elongation of polypeptide chains during synthesis of proteins on the ribosome (Riis et al., 1990). Elongation factors are highly conserved throughout evolution and are involved in functions other

Fig. 5. Expression driven by HCMV, EF1α and synapsin promoters in brainstem areas involved in cardio-vascular control. I. AVV HCMV-driven expression. Panel A—AVV PRSx8 (see below) was used to express EGFP in noradrenergic neurones in the A1 area in cultured brainstem slices which were also transfected with AVV HCMV-DsRed. None of the EGFP expressing noragrenergic cells also expressed DsRed. Rather, HCMV-driven DsRed appeared in small glial cells some of which were tightly attached to the noradrenergic neurones (arrow). Thus, HCMV promoter is inactive in noradrenergic neurones in A1 area. Living cells were imaged using $\times 63$ water immersion objective using Leica spectral confocal microscope. The EGFP channel (500–530 nm band) and DsRed channel (600–650 nm band) were overlaid by Leica confocal software. Panel B—AVV PRSx8-EGFP was mixed with AVV HCMV-DsRed and injected into several spots in the area of locus coeruleus. The locus coeruleus cells in the upper left part of the image all express EGFP exclusively while injection in a more ventral site resulted in mainly DsRed expression in some other cells. Two EGFP-positive cells in that area also do not co-express DsRed. Maximum projection of a 20 μm stack. Other parameters as described above. Panel C—AVV HCMV-EGFP cause robust expression in motor cholinergic nuclei such as dorsal vagal nucleus and hypoglossal nucleus as evidenced by multiple axons (arrow) traversing towards the ventral surface of the brainstem. However in the NTS area individual neurones are not distinguishable because the expression in that area is mainly non-neuronal. This is a wide field image taken with a CCD camera using $\times 10$ objective. II. LVV EF1α-EGFP driven expression in NTS/DVM area. Panel A—EGFP, panel B—DBH staining (using a CY3-coupled secondary antibody), panel C—overlay of A and B. Note that EGFP co-localises with DBH staining in some cells (arrows) indicative of successful targeting of noradrenergic neurones using EF1α promoter. Large green DBH-negative neurones in the ventral area of the image are likely to be vagal motor neurones of the DVM. In addition, some small cells with astrocyte-like phenotype are visible. Thus, EF1α appears not to discriminate for/against these multiple cell types. These images are the maximum intensity *Z*-projections of 10 μm stacks taken using a Leica SP spectral confocal microscope. The two channels were separately scanned (EGFP: excitation 488, emission 500–530 nm; CY3: excitation 543 nm, emission 590–650 nm) and overlaid by Leica software. III. AVV synapsin-EGFP-WHE driven expression in NTS (A2) /DVM area. Panel A—EGFP, panel B—DBH staining, panel C—overlay of A and B. Note that the DBH-positive neurones (red) did not express EGFP, although the virus clearly has spread over the area of DBH-positive cells. These data suggest that the synapsin promoter may be inactive in noradrenergic neurones. Most of the EGFP expressing neurones tend to occupy more ventral area partially overlapping with DVM. Parameters of imaging—as above.

than protein synthesis, such as organisation of the mitotic apparatus, signal transduction, developmental regulation and ageing. EF1α is expressed in cells at very high levels, the four EF1 subunits together may constitute up to 5% of the total protein in actively proliferating cells (Sanders et al., 1996). This implies a high rate of transcription of this gene and high activity of its promoter. Along with HCMV, EF1α promoter-driven expression is regarded as "constitutive".

EF1α has been used by a number of authors including ourselves (Poronnik et al., 1998; Coleman et al., 2003; Wong et al., 2002). The levels of EGFP expression in transfected brain cells (many of which looked neuronal) are fairly high although they seemed to be somewhat lower than those achievable with HCMV. Transgene (EGFP) was stable for at least 90 days (Coleman et al., 2003) when EF1α-EGFP LVV was injected into the hypothalamus.

At this time the cellular expression profile of this promoter in the brain is unknown. Therefore, we have tested whether EF1α also discriminates against noradrenergic cells, similar to HCMV. As shown in Fig. 5 EF1α-EGFP LVV (generated by Dr. Huentelman, Professor Raizada's laboratory, University of Florida) caused EGFP expression in both, DBH-positive and DBH-negative neurones and some apparently glial cells. Thus, EF1α is likely to have a wider spectrum of neuronal targets than HCMV but more information is required to accurately assess the properties of this promoter.

5.3. β-actin promoter

β-actin is one of the most abundant proteins (if not *the* most abundant) in many cell types. It is involved in a vast number of cellular functions, from cell division to synaptic release (reviewed in Qualmann and Kessels, 2002; Cousin, 2000). The activity of the human version of this promoter within the retroviral backbone has been documented for Schwann cells (Owens and Bunge, 1991; Owens and Boyd, 1991), as well as for astroglial and unidentified neurones in primary cultures (Lu et al., 1995).

6. "Cell-type" specific promoters

6.1. NSE promoter

Efficient viral expression of a transgene in "any neurone" (in contrast to any other cell type present in the brain) requires a reliable and active promoter which would only operate in neurones. Neurone specific enolase (NSE) has been used for many years as one of the reliable markers of the neuronal phenotype. NSE is a glycolytic enzyme that is localised primarily to the neuronal cytoplasm. Because NSE is a metabolic enzyme, its concentration in neuronal cells is relatively high. On the other hand, there are reasons to believe that NSE expression will not be subject to short-term oscillations such as those typical for the genes controlled by the fast genomic switches like cyclic AMP response element (CRE) or SRE. Hence, NSE might be one of the best currently available promoters for "pan-neuronal" targeting (i.e. expression in all kind of neurones but not other cells). In primary cultures transfected with plasmid DNA using lipofection, the NSE promoter resulted in GFP expression primarily in neurones (Tsuchiya et al., 2002). NSE-βgal AVV preferentially transfected neurones in hippocampus, cerebellum and striatum and the

expression lasted for 3–6 months (Navarro et al., 1999). Recently, Kugler et al. (2003) have evaluated the features of this promoter using an NSE-EGFP AVV. In their hands, the 1.8 kb NSE promoter provided preferential expression in neurones although ~30% of the EGFP-positive cells did not stain for the chosen neuronal marker "neurone-specific nuclear protein" (NeuN) suggestive of a "leak" into glial cells in the striatum. The NSE promoter was equally effective as the synapsin promoter in cortical neurones. Apparently, in the AVV backbone, the NSE promoter gives very long lasting and highly neurone-specific expression due to the neuronal tropism of that vector (Klein et al., 2002). Possibly a similar improvement in NSE selectivity could be achieved using VS-preudotyped LVV.

6.2. *Synapsin promoter*

Synapsin is a protein involved in the control of synaptic vesicle release. There are several members of the synapsin family which are differentially expressed in brain, with the highest levels of expression in the cortex and hippocampus and a relatively low level of expression in brainstem nuclei (Ullrich and Sudhof, 1995). There are several reports of synapsin promoter use for gene expression. Only ~500 bp are sufficient for highly neurone-selective targeting of neurones using AVV in the hippocampus (Ralph et al., 2000; Kugler et al., 2001) and striatum (Kugler et al., 2003). Apparently, the activity of the synapsin promoter is fairly low, but expression could be enhanced using the woodchuck hepatitis virus posttranscriptional regulatory element (Glover et al., 2002). More recently, it has been reported that this construct remains transcriptionally active in hippocampal neurones for as long as 9 months (Glover et al., 2003). The activity of this promoter in areas more closely relevant to cardiovascular homeostasis has not been documented. From the expression profile of the endogenous gene it might be expected to be low (Ullrich and Sudhof, 1995). Therefore, we have characterised expression of the AVV Syn-EGFP-WHE construct generated by Glover et al. (2002) in the brainstem at the level of the NTS (Fig. 5). It appears that although the cells expressing EGFP all looked neuronal, no expression could be detected in DBH-positive noradrenergic cells. Therefore, this promoter might be selective for certain types of neurones, rather than being "pan-neuronal" and its expression profile requires further clarification.

6.3. *GFAP promoter*

Recently, glia has attracted more and more attention as an active player in various aspects of brain function and in particular as a mediator of neurone-to-vessel signalling (Zonta et al., 2003). Glial fibrillary acidic protein (GFAP) immunostaining has been used for many years to identify certain types of cells of glial lineage, such as astrocytes. GFAP is an intermediate filament protein abundant within the cytoplasm of mature astrocytes. The GFAP promoter has been used to generate transgenic mice which express EGFP in astroglia (Nolte et al., 2001). Regulation of the GFAP promoter is not fully understood. It has been shown, however that activation of astrocytes (e.g. by brain injury or disease) leads to rapid increases of GFAP mRNA and protein (Junier et al., 1994). The same effect can be evoked by a prion but not by rabies virus infection (Titeux et al., 2002). Efficient targeting of astroglia has been achieved using an AVV system based on 2.2 kb of GFAP promoter to drive tet-OFF expression (Ralph et al., 2000).

6.4. Activity-dependent and inducible systems

One can imagine a situation when there is a need to adjust transgene expression to the physiological state of the cell (for example, an activated cell produces more transgene). The *cFOS promoter* is an example of an activity—dependent promoter. Similar to HCMV it also contains CRE but also a SRE and other consensus sequences. We have recently shown that it is sufficiently powerful to drive expression of EGFP to high levels ($>1\,\mu M$; Stokes and Kasparov, unpublished observation). This promoter is responsive to natural stimuli in vivo (for example, dehydration activates it in the paraventricular nucleus of the hypothalamus) whereas cellular depolarisation in cultured slices in vitro also activates this promoter to drive EGFP expression (Fig. 6; Stokes et al., 2003b).

A possible use of highly state/activity-dependent promoters can be illustrated by the recent work by Phillips et al. (2002) who have generated a "vigilant" vector. In that vector the expression of transgene is both, site-specific (targeted to the heart using myosin light chain MLC2v promoter) and hypoxia-responsive (conferred using the hypoxia-regulatory element from human enolase). The idea behind this approach is to enable the production of anti-hypoxic and anti-apoptotic proteins when the heart becomes ischaemic. Therefore incorporation of specific response elements in the promoters of viral vectors might be used to generate cell and state-dependent vectors for CNS studies.

6.5. Tetracycline-response element (Tre)-based systems

Tre has been derived from bacterial genes responsible for tetracycline resistance and was converted into a convenient drug-controlled expression system suitable for both, in vivo and in vitro experiments in mammals (Gossen et al., 1995; Harding et al., 1998; Huang et al., 1999; Ralph et al., 2000). In that system expression is dependent on the presence of the Tet transactivator of either "ON" or "OFF" type. Because the transactivator expression can be controlled by a cell-specific promoter, it is possible to combine cell specificity and inducibility using this approach. Thus Ralph et al. (2000) reported neuronal-specific inducible AVV based on the synapsin I promoter and a glial specific AVV based on the GFAP promoter.

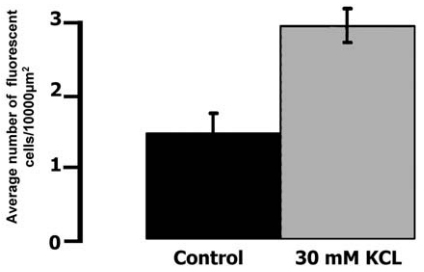


Fig. 6. cFOS promoter is an example of an activity-dependent promoter. Neurones of the paraventricular area of hypothalamus were transfected using AVV mfos-EGFP in cultured slices. Slices were then unilaterally stimulated using two drops of media containing 30 mM KCl to evoke an episode of depolarisation and high cellular activity. KCl stimulation nearly doubled the density of fluorescent cells visible on the stimulated side due to faster EGFP accumulation.

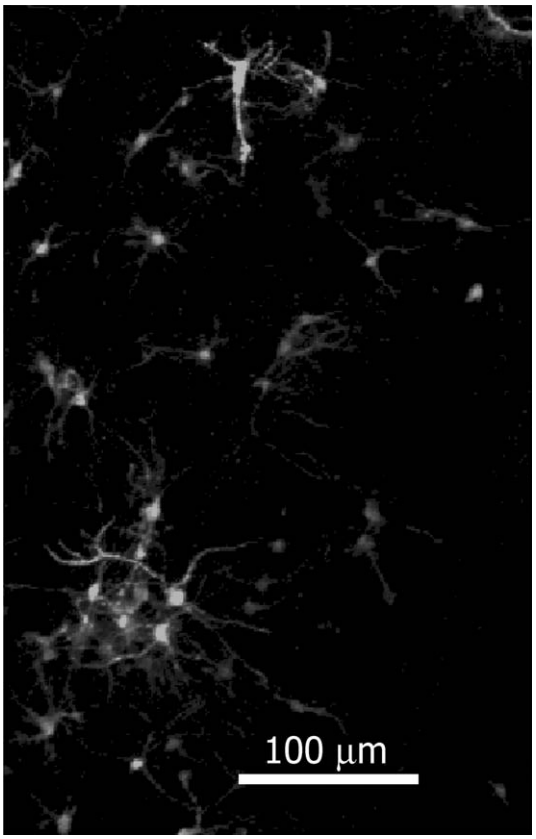


Fig. 7. "Leaky" expression with AVV tre-EGFP in hippocampus. Hippocampal slice culture was transfected in vitro with AVV tre-EGFP (10^6 PFU/ml for 1 h) and then the media was replaced to remove the excess of the virus. 3–5 days later numerous EGFP expressing cells of both, neuronal and glial phenotypes could be seen in the absence of the transactivator. This image is a maximum intensity projection of a 40 μm stack taken using $\times 10$ water immersion lens from a living hippocampal slice culture.

In our hands the use of the Tet system was limited by the significant level of background expression (or "leak"), which occurred in the absence of any transactivator both, in vivo and in vitro. Injections of tet-OFF-EGFP AVV alone in the brainstem, cortex and hippocampus using titres $>10^9$ plaque forming units/ml led to expression of EGFP in a significant number of cells, most of which seemed to be of glial appearance (Fig. 7; Kasparov, Teschemacher & Paton, unpublished observation). Dilution of the stock by 100-fold was necessary to prevent this "leaky expression". It is interesting that Clontech is currently marketing an improved version of the Tet system suggesting that this problem was not unique to this laboratory. Hopefully the new version is much "tighter" than the previous ones but some time is necessary for careful evaluation of this new development. Currently, interesting alternatives to the tet-system are being developed (Pollock and Clackson, 2002). For a further review of inducible systems, see Clackson (2000).

7. Targeting specific neuronal phenotypes using AVV and LVV

In our research we have faced the need to evaluate the roles of noradrenergic and GABAergic neuronal populations in brainstem areas involved in cardio-vascular homeostasis. Since pharmacological analytical tools cannot selectively act on a subgroup of neurones, viral gene delivery may be indispensable for investigating their functions. Therefore we have: (i) evaluated the recently developed artificial promoter PRSx8 (Hwang et al., 2001) as a means for confining transgene expression to noradrenergic neurones and (ii) generated a novel AVV for targeting GABAergic neurones based on the available information about the promoter of glutamate decarboxylase (GAD67), the GABA-synthesising enzyme.

7.1. PRSx8 promoter

Pharmacological, physiological and genetic evidence all point to the importance of the noradrenergic system of the brain for the long-term control of blood pressure and a possible role in essential hypertension. Therefore, our laboratory has been looking for ways to genetically manipulate various populations of noradrenergic neurones in key brain areas involved in cardio-vascular homeostasis. PRSx8 was developed in the laboratory of K.-S. Kim (Hwang et al., 2001) and represents a novel and completely different approach for gene targeting, compared to all other systems discussed so far in this review. PRSx8 is a synthetic promoter, non-existent in nature. It consists of 8 binding sites for the transcription factor Phox2a/2b, fused with a TATA box and the transcription initiation site of the dopamine-β-hydroxylase (DBH) gene. Phox2a/2b are transcription factors thought to be essential for neuronal differentiation into the noradrenergic phenotype and in particular in control of DBH expression (Hwang et al., 2001). PRSx8 is a very attractive promoter because it is both very short and powerful with a predictable mechanism of transcriptional control. In the original publication the promoter activity of PRSx8 was characterised in detail at the level of the locus coeruleus, the main noradrenergic cell group of the brain. In order to assess its effectiveness in other areas of the brainstem which contain noradrenergic and adrenergic neurones, we performed a number of in vivo and in vitro experiments.

In vivo, a PRSx8-EGFP AVV was injected into various brainstem areas containing catecholaminergic cell groups. In the locus coeruleus (A6 group, $n = 3$) essentially all ($96 \pm 3\%$) GFP-expressing neurons were DBH-immunoreactive (Fig. 8) in full agreement with previously published results (Hwang et al., 2001). On the other hand, a smaller proportion of EGFP expressing neurons in the A1 and A2 areas were DBH-positive ($56 \pm 11\%$ and $26 \pm 3\%$, respectively). PRSx8-driven EGFP expression was robust and allowed visualisation of the finest neuronal details using a Leica SP confocal microscope. We also noted that within the region of the A2 group, EGFP-expressing DBH-negative neurones tended to appear ventral to NTS, in regions overlapping with the location of the dorsal vagal motor nucleus. In the ventral brainstem areas adjacent to A1 such cells could also be found in the region of the nucleus ambiguus which contains vagal preganglionic neurones. However, at this point it is not clear whether the PRSx8-EGFP expressing cells were indeed cholinergic vagal motor neurones.

Only partial co-localisation of EGFP expression with DBH immunostaining in the vicinity of the NTS in these experiments could be probably explained by the fact that Phox2 appears in some

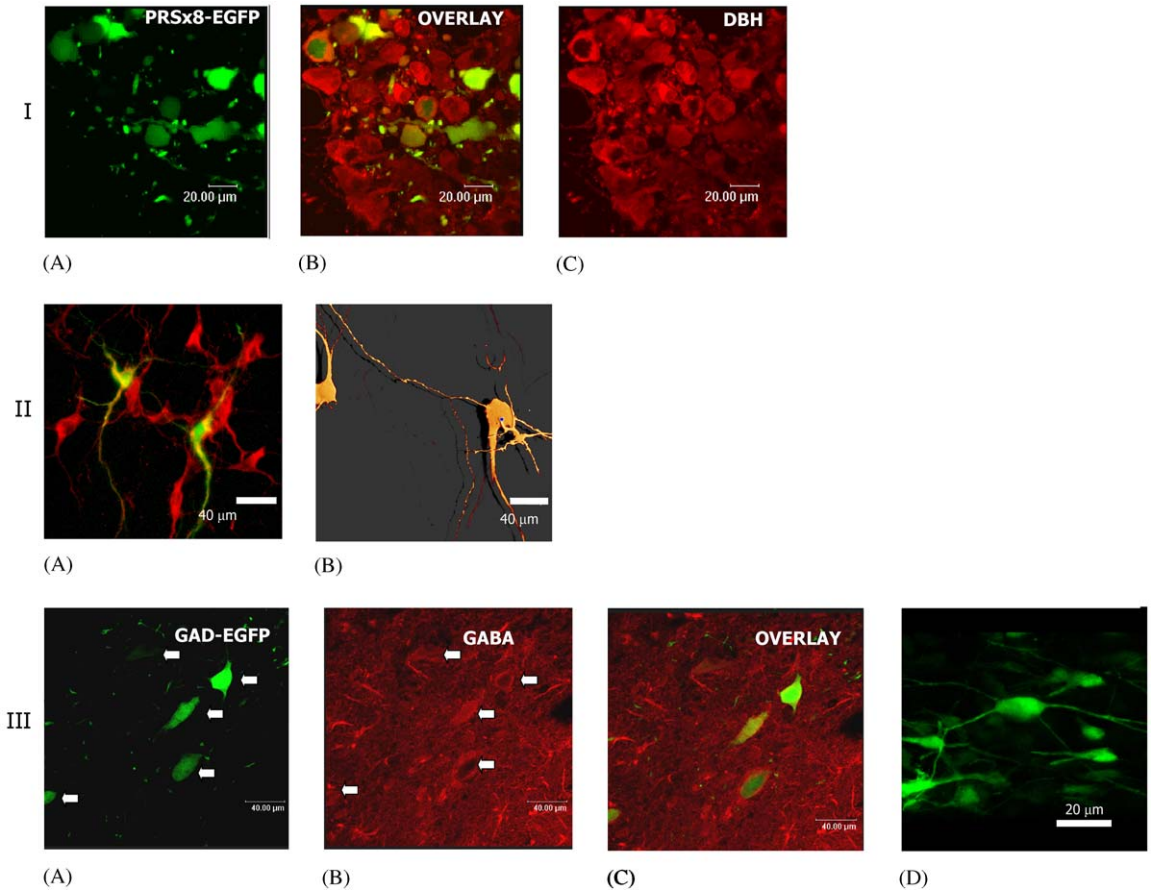


Fig. 8. Targeting selected neuronal populations using cell-specific promoters. I. AVV PRSx8-EGFP selectively targets noradrenergic neurones in LC. Panel A—EGFP. Panel B—staining for DBH. Panel C—overlay of A and B to demonstrate co-localisation of EGFP and DBH. Nuclei are largely devoid of DBH staining and therefore some of them appear green. Maximum projection of 10 µm confocal stacks taken separately as in Figs. 5a and b. II. AVV PRSx8-EGFP expression in vivo and in vitro. Panel A—retrogradely transfected neurones in the A1 area are noradrenergic (DBH-positive). PRSx8-EGFP was injected in hypothalamus. Five days later sections were prepared and immunostained for DBH (as in the previous images). This image in an overlay of the EGFP and CY3 channels obtained separately using spectral confocal microscope. Note that both EGFP expressing neurones are also DBH-positive and therefore appear yellow. Panel B—a "point spread function" projection with "shadow" effect made using a confocal stack of images of living noradrenergic neurones in A1 area of a cultured slice targeted with AVV PRS8x-EGFP. Note that a characteristic beaded bifurcating axon is clearly visible. III. AVV GAD-EGFP targets GABA-positive neurones in NTS. Panel A—EGFP. Panel B—staining for GABA using primary anti-GABA antibody from Chemicon and a rhodamine-coupled secondary antibody. Note that contours of all 5 EGFP expressing cells visible in A can be found in this panel. Some GABA-positive EGFP-negative cells can also be found. Panel C—overlay of A and B to demonstrate co-localisation of EGFP and GABA. Nuclei are devoid of GABA staining and therefore appear green. Maximum projection of 10 µm confocal stacks taken separately as in Figs. 5a and b. Panel D—living GABAergic neurones visualised in a cultured brainstem slice using confocal imaging. Note that fine details of neuronal structure can be visualised.

non-noradrenergic cells in that area (Tiveron et al., 1996). Some of those Phox2-positive cells were also cholinacetyltransferase-positive, located in the dorsal vagal motor nucleus and nucleus ambiguus. Some Phox2-positive cells in that part of the medulla were negative for both, DBH and cholinacetyltransferase and their transmitter phenotype is obscure (Tiveron et al., 1996). In addition, these neurotransmitter phenotypes may even convert between each other during development (Francis and Landis, 1999). Since the promoter activity of PRSx8 is totally dependent upon functionally active Phox2 protein, this phenotypic overlapping and/or switch may contribute to only partial co-expression of EGFP and DBH in A2.

Interestingly, as already mentioned, PRSx8-EGFP AVV transfected many neurones retrogradely (i.e. in A1 and RVLM when injected in A2, in A1 and C1 when injected in A2, in A5 when injected into LC) and these retrogradely transduced cells were all DBH-positive, similar to the cells retrogradely transfected from the hypothalamus (Fig. 8, panel IIA). Although on the one hand this property makes localised manipulation of a certain noradrenergic group difficult to achieve, it may provide a way of selectively manipulating noradrenergic projections to selected brain areas by retrograde transduction.

In vitro, the same AVV was applied to cultured brainstem slices. Organotypic brain slice cultures of the rat brainstem were developed in our laboratory following the 'static' culturing method, modified from Stoppini et al. (1991). Briefly, 250 μm thick brainstem slices were prepared from Wistar rat pups (postnatal days 9–11) by conventional methods but under sterile conditions. The slices containing the area of interest were plated on suspended membranes (Millipore) and cultured at an interface between serum-supplemented medium and 5% CO_2 at 37°C for days to several weeks. Exposure to adenoviral suspension at the time of plating results in optimal infection rates and transgene expression within 48 h, when active promoters are used. It appears

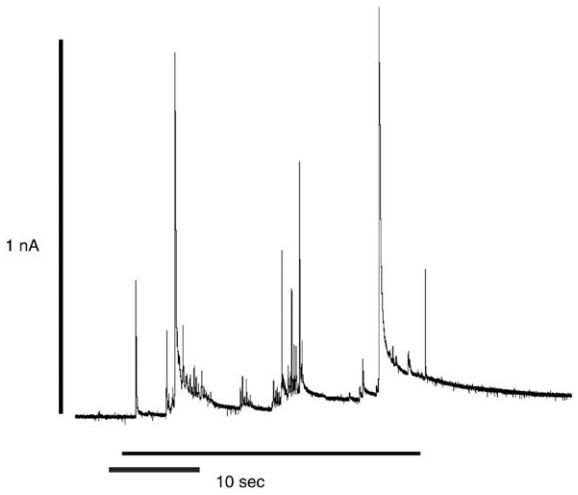


Fig. 9. Microamperometric recording of noradrenaline release from visualised axons of PRSx8-EGFP targeted neurones in slice culture. Axons of noradrenergic neurones in the A1 area were visualised using a fluorescence microscope. A carbon fibre probe with a tip diameter of 5 μm (ALA Scientific) was positioned in the vicinity of a fluorescent putative axonal varicosity. Shape and time course of these signals resemble closely previously published exocytotic events of catecholamine release measured in bovine chromaffin cells or rat substantia nigra neurones (Teschemacher and Seward, 2000; Jaffe et al., 1998).

that in vitro slice culture offers better chances for the cell-specific transduction. In slice cultures PRSx8-EGFP AVV reliably caused expression only in areas known to correspond to the catecholaminergic cell groups such as A2, A1, A5, C1, C2 (although not all EGFP-expressing cells in the NTS area were DBH-positive, data not shown). Nevertheless, distinct neurones with characteristic beaded axons could be readily identified using conventional fluorescent and confocal microscopy (Fig. 8). Moreover, their axons could be traced for hundreds of microns and release of noradrenaline from the varicosities documented using microamperometry indicating that the cells are viable and physiologically active (Fig. 9).

It appears that PRSx8 is able to drive high levels of EGFP expression both, in vivo and in vitro. In vitro slice culture offers better conditions for cell specific expression with the current version of the PRSx8—containing AVV while in vivo retrograde transfection may be used to circumvent some of the existing problems.

7.2. GAD67

A relatively short ($\sim$1 kb) part of the GAD67 promoter has been used by Oliva Jr. et al. (2000) to generate a mouse which expressed EGFP in some subpopulations of hippocampal GABAergic neurones. Longer sequences ($\sim$10 kb) introduced using the ''gene gun'' (DNA-coated gold particles accelerated with a stream of He) enabled specific expression in GABAergic neocortical neurones (Jin et al., 2001). In both cases the first exon–intron sequence was also required to achieve specificity. We have developed an AVV with an intermediate length (3.7 kb) of this promoter to drive EGFP and evaluated its expression in several brain areas including NTS (Fig. 8). The absolute majority of EGFP-positive cells ($>$90% in NTS) were also immunopositive for GABA. Given the inherent difficulty in immunocytochemical identification of GABAergic neurones, this result suggests that this AVV is quite selective for GABAergic neurones, at least in some areas of the brain. In addition, we have never observed any EGFP expression in small glial cells many of which were brightly stained for GABA (possibly taken up from the extracellular space) which speaks on favour of neurone-specific expression profile of this novel construct. We expect that this novel AVV will greatly facilitate our studies centred on the role of GABA in pathological hypertension.

8. General discussion and conclusions

Over the last decade viral vectors have transformed from exotic exhibits of molecular expertise into reliable and very powerful tools which can be used to address physiological questions in any environment—from single cell to the whole animal level, with clear implications for gene therapy. They can be used for overexpression experiments (Kishi et al., 2002, 2003), expression of the dominant negatives (Waki et al., 2003; Wong et al., 2002; Paton et al., 2001), gene inhibition using RNA interference (Xia et al., 2002) or antisense (Pachori et al., 2001; Raizada et al., 2000), expression of fluorescent protein constructs for imaging purposes (Wang et al., 2001; Kasparov et al., 2002a), etc. Viral vectors are advantaged by the relatively short time required for their production, they are highly versatile, cheap and not restricted to any single species. They can be relatively easily directed to a particular brain nucleus and more recently to a particular cellular

type in that nucleus. They permit numerous types of experiments, from chronic manipulation of known and unknown genes to the visualisation of particular neuronal populations and their subcellular compartments. Indeed, targeting of viral vectors is one of the mainstream directions in gene therapy (Wickham, 2000; Krasnykh et al., 2000). However we should not forget that this technology is still in its infancy and the excitement about its potential should not prevent us from its scrupulous evaluation.

Acknowledgements

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