Application of viral gene transfer in studies of neurogenic hypertension

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INTRODUCTION

In spite of recent advances in the treatment of hypertension, many patients fail to respond to standard therapies targeted mainly at regulating blood volume, electrolyte balance and the peripheral renin-angiotensin system. In a recent review, S.J. Mann writes: “One such form of hypertension is the often overlooked entity of neurogenic hypertension. These implications *underscore the need for further clinical and basic research attention concerning neurogenically mediated hypertension*” [1]. The purpose of this chapter is to renew attention to this overlooked entity by considering novel molecular strategies to disentangle the complexities of neurogenic hypertension.

The lack of understanding of the mechanisms of neurogenic hypertension at which S.J. Mann alludes justifies interest in new methods of analysis of this disease, which would allow us to study these mechanisms with a new level of precision. Many such methods have evolved during the last decade or so and currently join under the umbrella of physiological genomics. Availability of fully sequenced genomes and the vast numbers of readily accessible clones greatly facilitates the use of genetic manipulations in order to reveal the roles of individual genes and their families under normal as well as pathological conditions.

We have chosen to employ viral vectors to manipulate genes within the central nervous system structure regulating blood pressure. Virally mediated gene delivery to the brain is a powerful research tool, which can be used to address a wide range of questions including central cardio-vascular control mechanisms. So, what are the advantages of the viral approach?

Why Viral Vectors?

First, it allows evaluation of the long-term outcomes of various experimental manipulations on blood pressure control. It is not a secret that many conclusions about central mechanisms of blood pressure control have originated from acute experiments in anaesthetised animals. Meanwhile, human hypertension is a chronic condition and, thus, it becomes imperative to also look at long term manipulations within specific brain structures for a more complete understanding of this condition. In addition, acute experimentation usually occurs under anaesthesia which has very profound effects on nearly every transmitter system relevant to blood pressure control (reviewed in: [2]. Nowadays chronic recording of arterial blood pressure using radio-telemetry in rats or mice has negated these problems. However, one problem that remains is the chronic central delivery of pharmacological agents directly into specific brain nuclei. Here, viral gene manipulation is of great advantage because following a single microinjection it enables chronic alteration of brain signalling in a chosen structure that can last for many days or even weeks [3-8]. Moreover, in experiments of this kind it is possible to obtain an intra-experimental control (e.g. monitor blood pressure before and after the genetic manipulation in the same animal). This is important as it greatly facilitates interpretation of the results (see below). In addition, pharmacological approaches are in many cases impossible because of the lack of suitable drugs, especially for recently discovered targets and for those awaiting discovery.

Second, viral gene manipulation is a flexible approach as the same vectors can be used in many species. There is little doubt for a variety of reasons that the rat will remain the species of choice for neuroscience studies including those concerned with hypertension. In the context of this chapter, it is a great advantage that this approach can be applied to the spontaneously hypertensive rat (SHR) and the outcome compared directly to its normotensive progenitor strain, the Wistar-Kyoto rat (WKY) or any other control strain [3;9]. While

germline transgenesis in the rat is extremely technically challenging and time consuming, viral gene delivery does allow effective manipulation of central signalling pathways [10]. Although the SHR is not an ideal model of essential hypertension in humans, it has nevertheless served for many years as a valuable model of this disease and, with its normotensive progenitor control (WKY) remains an important resource. Moreover, in addition to SHR there many other models of hypertension in the rat.

Third, viral gene transfer enables both site-specific and, in some cases, cell-type specific genetic manipulation. Viral vectors can be delivered stereotaxically into any area of the brain and, if needed, retrograde transduction performed with some types of vectors[11-13], see also Fig 1A). However, all brain nuclei are heterogeneous in terms of their histochemistry and connectivity. Many nuclei harbour excitatory glutamatergic and inhibitory GABAergic neurones and changes in both of these transmitter systems have been implemented in pathological hypertension[14-18]. 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 all been implicated in pathological hypertension [19-23]. Moreover, each group of neurones is embedded in a network of glia and blood vessels containing endothelial and vascular smooth muscle cells. Thus, it now becomes necessary to selectively target a cell type with a specific phenotype within a heterogeneous population using modified viral delivery vectors with cell-specific promoters and hence analyse their exact roles in pathological hypertension.

Finally, we should not forget that a large volume of information about the biochemical changes in central signalling mechanisms in hypertension has been obtained in highly reductionistic models, such as cell lines. For example, the PC12 line is derived from chromocytoma and used as a model of central catecholaminergic neurones. Cell lines such as these are convenient in particular

because DNA can be easily introduced using well-established methods such as calcium phosphate transfection or liposome-based carriers. However, central neurones are very different from these simple models and to make a definitive conclusion about the function of brain circuits, genetic manipulation must also be made *in vivo*. Central neurones are generally very resistant to chemical methods of gene delivery and here viruses again offer a powerful alternative. For example, if a hypothesis links hypertension to a change in a signalling cascade in catecholaminergic neurones of a particular cell group within the brainstem, viral vectors make it possible to perform a variety of experiments specifically on this cell type.

Non-uniform Transductional and Transcriptional Efficacy of Viral Vectors

We would like to draw the readers' attention to one very important feature of viral gene transfer experiments: the non-uniformity of transgene expression. Pharmacological analytical tools injected into the central nervous system affect all cells that have the appropriate binding site or receptor. In contrast, virally delivered transgenes may or may not be effective in some cells depending on the efficiency of the virus to both enter the cell and induce expression of the transgene once it has reached the nucleus. The ability of the virus to enter the cell and the subsequent transcriptional activity of its expression cassette are two critical rate-limiting steps. Indeed, this gave rise to the concept of targeted viral gene delivery, which can occur at either a transductional (i.e. at the stage of viral entry) or transcriptional level. Detailed analysis of viral targeting is outside of the scope of this chapter and more information can be found in recent reviews [24-27]. It is essential, however, to realise that due to the natural tropism of viral capsids (and/or the protein coat of enveloped viruses) and the striking variability of activity of promoters in different neuronal populations (reviewed in more details in [27], the transgene probably is *never* expressed at the same level in all cells in the brain area in which the viral vector was injected. Indeed, a "false

negative outcome" can occur if the transgene is expressed but at very low, physiologically insufficient levels. On the other hand, expression of a biologically active molecule in cells that normally do not express this molecule may alter their function in an unpredictable manner. These considerations are important for interpretation of viral gene transfer experiments and led us to perform a more thorough characterisation of the expression profile of a number of viral vectors in cardiovascular areas of both the brainstem and diencephalon [27;28].

In this chapter we will give an overview of currently available viral gene delivery systems and illustrate how they can be used to advance our understanding of the central mechanisms of hypertension.

VIRAL VEHICLES FOR GENE DELIVERY INTO CENTRAL NEURONES

Vectors for gene delivery into the brain have been derived from a number of viral genomes, such as adenoviruses, adeno-associated viruses, retroviruses and related lentiviruses, herpes simplex virus, and some others. So far, studies of central mechanisms of cardio-vascular control mainly employed adenoviral vectors (AVV) and retroviral vectors including lentiviral vectors (LVV) [3;5;10-12;29-33]. These viruses can infect various cell types present in the brain (i.e. neurones, glia, endothelial and vascular muscle cells) and result in long lasting gene expression without noticeable adverse effects. Vectors derived from the adeno-associated virus (AAV) have been successfully used for gene delivery to peripheral targets [34;35] but they are also well equipped for gene delivery into central neurones [36]. Finally, a replicating pseudorabies virus has been used to retrogradely transfect vagal cardioinhibitory neurones [13].

General properties of AVV and LVV

AVV and LVV are very different in respect to their genomic organisation, structure and life cycle. While AVV vectors are descendants of the widespread

pathogens associated with mild human infections of the respiratory tract and eyes, LVV vectors have been derived from either a deadly human immunodeficiency virus (HIV) or a feline immunodeficiency virus (FIV). From the practical point of view, the most important features of any viral gene delivery system include its safety in use (i.e. non-virulent), cellular tropism in the brain, transgene carrying capacity, rapidity, longevity and stability of gene expression, the degree of an immune response induced, the ability for retrograde transduction, and the speed and cost of production.

In contrast to AVV, LVV are able to stably insert the transgene into the host genome while the transgenes delivered by AVV remain episomal. Thus, AVV delivered transgenes may be relatively rapidly eliminated (2-3 weeks), especially in dividing cells such as endothelial cells, whereas LVV induce expression for months. In central neurones this difference might be less evident as the longevity of expression seems to be largely dependent on the promoter used. Some AVV constructs have been reported to drive expression for at least 9 months [37]. In terms of capacity, currently the majority of laboratories use AVV with deletions in E1 region (with additional deletions in the E2 and/or E3 and E4 regions). With these deletions, which render the virus replication deficient, AVV can accommodate approximately 7 kb of a transgene. This is enough for one or even two expression cassettes. Various versions of AVV have been made, some of which express two different proteins from two separate promoters [38]. In addition, high capacity AVV lacking most of their genome have been generated [39], but production of these vectors remains too complicated for them to replace the currently used E1-E2/E3 deleted AVV. In contrast, HIV-derived self-inactivating LVV, such as those developed by [40] and further modified by [41], has most of the viral genome deleted and in principle can accommodate ~ 11 – 12 kb. Size limitations within the viral backbone are important particularly when large transgenes are inserted or when cell specific expression is desirable. To achieve cell specificity in transgenic animals researchers commonly use long

promoter sequences (several kb) upstream of a particular gene to drive the expression [42] or even bacterial artificial chromosomes (tens of kb; [43]. Clearly, viral vectors have restrictions imposed by the size of viral particles but it proves possible to target expression to at least some subsets of cells relevant to central cardio-vascular control (see below). Parenthetically, in contrast to AVV and LVV, AAV have even smaller capacity. Even the most advanced versions of these vectors accommodate inserts no larger than ~4 kb, which limits their use for expression of large cassettes [44].

Transductional tropism of the AVV and LVV

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 fibres of the Ad17 serotype might be best suitable for targeting neurones [45]. Nevertheless, most of the AVV vectors currently used have been derived from 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 class I molecule and the "coxsackievirus-adenovirus" receptor (CAR). Modifications of the AVV fibre protein may change its tropism and alter its ability to infect certain cell types (for review see [26]. Recently, using this approach, Omori et al [46] have re-directed AVV to microglia which lack CAR. This was achieved 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, instead, direct it to endothelial cells [47;48]. 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, endothelium or, perhaps, neuronal subsets. Thus, alteration of vector tropism may be achieved by modification of the capsid proteins.

Axonal transport and targeting remote sites in the brain

The ability of a viral vector to transduce remote neurones via retrograde transport may have major implications for its use in experimental neuroscience. This feature is heavily dependent on the properties of the viral particle but could also be influenced by the phenotype of the targeted neurones. There has been some controversy regarding the ability of the conventional AVV (serotype 5) to transduce neurones retrogradely but this issue has been recently resolved by a number of studies. For example, using AVV with the Rous sarcoma virus long terminal repeat as the promoter, retrograde transduction was obtained after microinjections into the caudate putamen [49]. Transfected cells (predominantly dopaminergic) were found in substantia nigra compacta that was 2 mm from the site of injection ruling out viral diffusion as the method of transfection. [50] achieved retrograde transfection using intra-hippocampal AVV injections using a modified chicken β-actin promoter [51], rather than HCMV. Neurones retrogradely transduced in these experiments were located in the cerebral cortex and were likely to be glutamatergic. [12] used AVV with an HCMV-driven construct to retrogradely transduce, from the pericardial sac, cholinergic neurones in nucleus ambiguus located in the ventrolateral medulla. [11] found retrogradely transfected neurones in the subfornical organ after injection of AVV with the HCMV promoter into the supraoptical nucleus (the exact transmitter phenotype of these cells is not known). Collectively, these data indicate that AVV *are* able to retrogradely invade various types of neurones that have different neurochemical profile. Finally, we have also observed retrograde transduction of noradrenergic and adrenergic cells using AVV with the PRSx8 promoter, which is highly active in these cell types. For example, after injection of AVV PRSx8-EGFP (enhanced green fluorescent protein), into the paraventricular area of hypothalamus in the rat, noradrenergic neurones expressing EGFP were found in cardiovascular regions of the nucleus tractus solitarii (NTS) and in the A1 area of the ventrolateral medulla (Fig 1A). Moreover, injection of the same construct into

the dorsal horn of the spinal cord resulted in EGFP-expressing noradrenergic neurones in locus coeruleus and some ventral brainstem noradrenergic groups. Therefore, we believe that transgene expression in remote sites after AVV microinjections may occur within the central nervous system provided that the promoter used is active in these projection neurones.

In contrast to AVV, LVV have an additional protein coat or envelope wrapped around the capsid. The coat is associated with the capsid but consists of a separate protein encoded by separate genes. Because this coat is involved in the binding of the viral particle to the cellular membrane, modifications of the coat can alter the transductional properties of the vector, just as we described above for AVV. Most vectors used currently, irrespectively of the virus they have been derived from (i.e. HIV or feline immunodeficiency virus), are pseudotyped in that they employ a foreign protein coat such as the vesicular stomatitis virus glycoprotein G (VSVG) [11;40;41]. The VSVG coat enables LVV to deliver transgenes to a wide range of cells including neurones and glia [41]. Pseudotyping with other protein coats generates new properties. The VSVG-pseudotyped vectors seem to have negligible ability for retrograde transduction. In contrast to an AVV, for example, VSVG-pseudotyped feline immunodeficiency virus did not retrogradely transduce neurones in the subfornical organ when injected into the supraoptical nucleus [11]. This feature of VS-pseudotyped LVV may be important for functional studies when gene manipulation needs to be restricted to the injected brain area. Additionally, when an elongation factor 1α (EF1α)-EGFP LVV with the VSVG envelope was injected into the hypothalamus of the rat, no GFP-expressing neurones in remote areas such as brainstem could be found (Kasparov, Paton, Huentelman & Raizada – unpublished observation). Other coat proteins, such as the rabies-G envelope protein, however, alter this property permitting retrograde axonal transfection as demonstrated with the equine infectious anaemia virus bearing HCMV-βGal expression cassette [52]. A good example of how the type of the coat protein may change the cellular

tropism of the LVV in the brain is the Ross River virus glycoproteins changing expression by the feline immunodeficiency virus from neurones (characteristic of VS coat) to glia [53].

AAV vectors for gene delivery in the brain

AAV used currently have been derived from the serotype 2 adeno-associated virus. These viruses have a small non-enveloped capsid and are efficient in transducing central neurones [54-57]. Their tropism to other potentially interesting cell types, such as endothelial vascular cells is lower than that of the AVV but can be increased by a capsid modification [57] Recently, it has been demonstrated that as with the AVV, AAV can also transduce neurones retrogradely: for example, expression occurred in the entorinal cortex after injection into the hippocampus [58]. AAV lack almost their entire native genome and therefore do not cause expression of their capsid proteins in transduced cells. Since capsid proteins can trigger an immune response these vectors are advantaged by this property making them particularly suitable for systemic use in tissues outside of the brain-blood barrier. For some time it had been thought that AAV integrate their transgenes into the host chromosome, similarly to the wild type adeno-associated virus, but this does not seem to apply to vectors derived from it. Thus, AAV-delivered transgenes remain largely episomal. Nevertheless, the expression following AAV transduction usually lasts for months, which together with an absence of any immune response, makes these vectors one of the favourite prototypes for gene therapy research. For further information on AAV vectors, see recently published reviews [27;56;59;60].

APPLICATIONS OF SOMATIC GENE TRANSFER IN STUDIES OF CENTRAL MECHANISMS OF BLOOD PRESSURE CONTROL

Below we describe the types of experiments that can be performed using viral vectors that are capable of efficient gene delivery into central structures regulating cardio-vascular homeostasis.

Over-expression experiments

A common experiment is to over-express a certain protein in a brain structure of interest in order to increase its function and monitor the outcome chronically. This approach has been used in a number of studies by Hirooka and colleagues [5;61-65]. For example, in [5] an AVV with HCMV promoter was used to over-express the endothelial isoform of nitric oxide synthase (eNOS) in the rostral ventrolateral medulla (RVLM), a major region providing descending sympato-excitatory drive. The experiments were carried out in two strains of rats - the stroke-prone SHR and its progenitor strain, the Wistar Kyoto rat. Arterial pressure was chronically monitored using radio-telemetry. Seven days after gene transfer, mean arterial pressure and heart rate decreased in both groups but these falls were significantly larger in the SHR. In addition, baroreceptor reflex input-output function was increased in both rat groups. A control for the non-specific effects of the viral transduction was made using an AVV expressing β-galactosidase, which caused no change in the parameters measured. The authors argued that an increase in nitric oxide (NO) production in RVLM improves the impaired baroreceptor reflex control in SHR. In a previous study from the same group [65] eNOS was over-expressed in the nucleus of the solitary tract (NTS). The increased production of NO was demonstrated using microdialysis. In eNOS-transfected animals blood pressure and heart rate were significantly decreased 5 to 10 days after gene transfer.

In these studies the cellular targets to which the transgene was delivered was not established: it is not known whether the bulk of the eNOS-expressing cells were neurones, glia or vascular cells (smooth muscle or endothelium). NO is thought to freely diffuse through cellular membranes and may reach targets in

cells located some distance away from the source of release. But how far it travels under physiological conditions is not entirely clear [66]. In the experiments described above, eNOS activity will appear in the cells determined by both the transductional tropism of the AVV and the transcriptional activity of HCMV promoter. This is very likely to be a different subset of cells to the ones producing eNOS endogenously. In the NTS AVV with the HCMV promoter largely targets glial cells and transgene expression in local endothelial cells has also been documented (see below). Since NO can easily cross cellular membranes, its over-production in these experiments means that it could still reach its receptor (soluble guanylate cyclase) located in cells adjacent to site of the transfected cells. However, if the over-expressed molecule had to act on the target within the same cell, any mismatch between the expression profile of the viral vector and the distribution of the target molecule could lead to a false negative or otherwise unpredictable outcome. Moreover, the action of high local concentrations of NO on the targets *within* the cells in which the AVV causes eNOS overexpression might result in non-physiological responses.

In summary, over-expression of eNOS will produce large quantities of NO comparable to a chronic infusion of an NO donor into the structure. This provides a good way of chronic delivery of a substance without the need to make repeated injections into a single brain locus, which is technically unachievable in many brain structures. However, both over-expression of eNOS and delivery of a pharmacological agent are unlikely to accurately mimic the physiological scenario in terms of providing a site- or cell specific source of NO at a physiologically relevant concentration.

AVV with the HCMV promoter was also used to over-express mitochondrial or cytoplasmatic superoxide dismutase isoforms to demonstrate involvement of reactive oxygen species (ROS) in the pressor and dipsogenic responses induced by intracerebroventricular (icv) administration of angiotensin II (ANGII) in mice

[8]. ROS have been implicated in the pathogenesis of neurodegenerative disease but in addition are currently thought to act as second messengers in some signalling systems. In the [8] study, AVV were administered in the lateral ventricle of the mouse brain and the outcome evaluated 3 days later. Arterial pressure and heart rate were monitored before and after intraventricular administration of ANGII. Over-expression of both mitochondrial and cytoplasmatic isoforms strongly antagonised responses to i.c.v. injections of ANGII. The dipsogenic action of ANGII was also significantly antagonised. These studies have established a role for ROS in an AT_1 receptor mediated signalling pathway regulating the cardiovascular system. The transgene in these studies was found predominantly in the brain tissue surrounding the ventricles and the subfornical organ, a circumventricular organ with a high density of AT_1 receptors known to be involved in blood-to-brain communication. Thus, AT_1 receptors in the subfornical organ may trigger hypertension via signalling cascade involving ROS. More recently the same group has found that overexpression of Cu/Zn superoxide dismutase in subfornical organ and organum vasculosum of the lamina terminalis (this was achieved using intracerebroventricular injection of AVV) reduced sympathetic tone in mice with cardiac failure triggered by myocardial infarction[67]. Interestingly, ROS scavenging did not affect the sympathetic tone in control animals without cardiac failure suggestive that ROS signalling becomes activated only under these pathological conditions. The authors conclude that in heart failure ROS within the circumventricular organs may mediate central sympathoactivation by circulating molecules such as ANGII and aldosterone [67].

We used a similar strategy to address the role for ROS in the AT_1-receptor mediated signalling in the NTS except that we over-expressed catalase instead of superoxide dismutase. We also used an AVV with HCMV promoter but in our case this approach failed to prevent the depressant action of ANGII on baroreceptor reflex mediated bradycardia in spite of the significantly elevated catalase activity

in the area of transfection [10]. Clearly this reflects differences in the signalling mechanisms employed by the same receptor in the two different brain areas. However, the methodology of viral gene transfer leaves room for alternative interpretations that have to be considered. First, is it possible that in the NTS AVV with the HCMV promoter did not lead to sufficient catalase expression in the cells which mediate the AT_1 receptor mediated baroreceptor reflex attenuation? The precise nature of these cells is still unclear but our current evidence suggests that they could be the eNOS-bearing endothelial cells. From our experiments where eNOS dominant negative protein expressed using an AVV with HCMV promoter was able to antagonise AT1 receptor-mediated effects in NTS (see below) we reckon that this is unlikely to be the case. Second, it is not impossible that the catalase expressed was inefficient in inactivating the ROS generated by AT_1 receptor activation in the NTS. This argument is more difficult to dismiss, but we could argue that the lack of the effect of the catalase over-expression was consistent with the inability of a number of pharmacological blockers to antagonise various steps in the cascade signalling downstream of ROS generation [10].

In summary, these studies illustrate some of the potential experimental applications of virally mediated transgenesis for over-expression of a protein in different central nervous structures to evoke chronic alterations in cardiovascular function.

Expression of antisense oligonucleotides and siRNA–mediated gene suppression

Viral vectors have also been used in “loss of function” experiments in order to chronically decrease expression of a certain gene. There is more than one way to achieve this. [68] successfully used a retroviral vector to express an antisense sequence to the -132 to +1128 bases of the AT_{1B} receptor gene. The vectors were applied to primary cultures from brainstem to down-regulate AT_1 receptor

expression. This experiment resulted in down-regulation of all measured responses to ANGII including c-fos and noradrenaline transporter expression.

Although the antisense approach has been used successfully by some groups [35;68;69] the more recent reports about small interfering RNAs (siRNA) being able to selectively target specific mRNA species into the degradation pathway have evoked huge interest in the scientific community [70;71]. Most published studies to date have employed synthetic double-stranded RNA oligonucleotides [72-74]. However, it is also possible to use viral vectors to express siRNA to the same effect [75-78]. These vectors are designed in such a way that transcription results in generation of hairpin-like RNA which acts similarly to the conventional siRNA. Viral vectors for expression of siRNA have been made on the basis of polymerase II promoters such as HCMV [78] and polymerase III promoters such as U6 [75] and H1 [79]. Polymerase III promoters are currently thought to be optimal for siRNA hairpin expression because they provide a high rate of transcription of small RNA molecules in a wide range of cells. Moreover, the mechanisms of the initiation and termination of transcription by polymerase III allow production of precise lengths of transcripts that do not undergo mRNA-specific post-transcriptional modifications which interfere with siRNA function. Some systems of that kind are available commercially. Viral vectors with polymerase III-derived inducible siRNA expression systems have also been developed [79]. At this point this technology has not been applied in studies of central cardio-vascular control, but this is just a matter of time.

Expression of dominant negative proteins

It is possible to suppress the function of certain proteins by expression of dominant negative proteins. This principle usually employs protein-protein interaction, for example between the dominant negative protein and a catalytic subunit of an enzyme. We were interested in the role of NO in baroreceptor reflex control at the level of the NTS. A number of pharmacological tools were

used [32] to demonstrate that various blockers of NO production prevented a well-documented inhibitory action of ANGII on this reflex in the NTS [80;81]. Viral gene transfer of a dominant negative form of eNOS was then employed 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. Second, it was important to evaluate the role of NO in NTS chronically and long-term pharmacological blockade of eNOS in NTS is technically not 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 HCMV promoter [82]. Although TeNOS lacks catalytic activity it is correctly localised to the membrane. TeNOS acts as a dominant negative inhibitor of wild-type eNOS activity through heterodimerisation with the native protein [83]. We demonstrated 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. 2A, B & C; [32]. Similarly, the NO precursor L-arginine, which also inhibited the baroreceptor reflex when injected into the NTS of naïve rats, failed to modulate the baroreceptor reflex in TeNOS 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 baroreceptor reflex function and multiple other physiological variables measured.

More recently, the same construct was used chronically in normotensive conscious freely-moving rats instrumented with radio transmitters to record their blood pressure and heart rate. This approach gives a continuous definitive measure of arterial pressure. After the implantation of transmitters the basal level of blood pressure, heart rate and spontaneous baroreceptor reflex gain were determined by a time-series method. Subsequently, the animals were then re-anaesthetised and the AVV-TeNOS microinjected into the NTS. TeNOS

expression evoked a gradual increase in baroreceptor reflex gain between days 7 to 21 after gene transfer, peaking at day 21 (1.68 +/- 0.20 ms/mmHg). This value was significantly higher compared to that before gene transfer (1.13 +/- 0.09 ms/mmHg). It was also significantly elevated compared to two groups of control animals: those which received an NTS microinjection of an AVV expressing EGFP, which acted as a viral control, and those that received a microinjection of saline. In addition, heart rate decreased 14-21 days after TeNOS gene transfer. However, blood pressure was not affected. These results indicate that eNOS is constitutively active within the NTS and acts to regulate baroreceptor reflex gain and heart rate but not resting blood pressure in normotensive WKY rats [3]. In addition, these data suggest that baroreflex gain (at least the cardiac component) is controlled independently from arterial pressure by the NTS. This prompted the question as to whether eNOS activity in NTS of the SHR was important for the known depressed baroreceptor reflex gain in this animal model. Our preliminary data indicate that when TeNOS was expressed in 10-11 week old SHR, baroreceptor reflex sensitivity increased, while systolic blood pressure decreased [84]. These results suggest that in this model of pathological hypertension endogenous eNOS activity in the NTS is elevated compared to normotensive rats and plays a major role in determining levels of *both* arterial pressure and baroreceptor reflex gain. 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.

We have also used AVV expressing dominant negative proteins to dissect out the intracellular signalling pathway utilised by ANGII to suppress the baroreceptor reflex within the NTS [10]. In contrast, 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 in NTS, strongly antagonised the depressant action of ANGII on the baroreceptor reflex (Fig 3A). This was an important

finding because Gq is one of the established intracellular links between G-protein coupled receptors (such as the AT_1 receptor) and intracellular Ca^{2+} stores. These data were consolidated using conventional pharmacological approaches to block intracellular signalling pathways [10]. In the same study, some AVV with HCMV-driven construct were also used. One of them expressed a dominant form of Akt (or protein kinase B). However, this intervention was without effect on ANGII inhibition of the baroreceptor reflex, which was consistent with the outcome of pharmacological experiments [85]. In summary, AVV expressing various dominant negative proteins helped us to unravel the functional significance of eNOS in the NTS for long-term control of baroreceptor reflex sensitivity and arterial pressure in conscious normotensive and hypertensive animals as well as the intracellular signalling pathway that links AT_1 receptor to baroreflex inhibition.

Three methodological aspects of using dominant negative proteins

First, expression of the dominant negative protein might give a more accurate portrait of the function of endogenous molecules than their over-expression, provided that the dominant negative protein is specific and potent. Indeed, if a biologically active transgene appears in different cellular phenotypes that normally do not express it (as is the case with most over-expression experiments), this may give a wrong idea about the function of this transgene. In contrast, a dominant negative protein is not supposed to have any functional consequence in cells other than to block the activity of the native protein, if it is present. Theoretically at least, it could be argued that the use of dominant negative proteins should be somewhat more selective and any effect more reliably interpreted than responses that occur following over-expression of proteins in the “gain-of-function” experiments.

Second, as mentioned above we have used largely AVV with the HCMV promoter with one exception being the EF1α-driven construct. We want to draw attention to this issue because this is an example of the case where viral transgenesis

differs radically from the use of a pharmacological blocker: expression of a transgene might result in its inadvertent targeting of a sub-set of cells with a specific phenotype. As mentioned above, we have recently found that HCMV-driven expression is not as ubiquitous in the brain as sometimes thought [27]. A striking result was obtained in experiments in organotypic brainstem slice cultures co-transfected with two AVV at the same time: the first AVV contained an HCMV-DsRed expression cassette (DSRed is a far-red shifted fluorescent protein from coral), while in the second AVV expression of EGFP was controlled by the PRSx8 artificial promoter, which is highly active in noradrenergic, adrenergic and some vagal preganglionic neurons. In several brainstem noradrenergic cell groups no co-localisation of DsRed and EGFP could be detected in numerous (>50) cells using spectral confocal microscopy (for details see [86]. In contrast, co-infection with HCMV-DsRed AVV and HCMV-EGFP AVV resulted in >90% of double labelled cells (many of which had the characteristic glial morphology, unpublished observation). These results were consistent with the outcome of *in vivo* experiments where noradrenergic neurones in the locus coeruleus did express EGFP but did not express DsRed after injection of a mixture of PRSx8-EGFP and HCMV-DsRed AVV, although red fluorescent cells were visible outside this nucleus [27]. Interestingly, HCMV-EGFP AVV caused robust expression in brainstem cholinergic motor nuclei of the hypoglossal nerve. (Fig 1 D&E). The ability of AVV with the HCMV promoter to efficiently target glia has also been documented before both *in vivo* [87] and in acute brain slices [88]. In addition, AVV with HCMV-EGFP can transduce local vascular endothelium after brainstem microinjections *in vivo* [89]. Therefore, it is possible, and even likely, that HCMV-controlled vectors discriminate against some cellular phenotypes in favour of others and the physiological outcome of these experiments may be due to expression in non-neuronal cellular compartments such as the local vascular cells or glia.

A dominant negative approach has also been employed to analyse the role of Rho/Rho kinase pathway in the NTS [6]. Rho is a small GTPase present in many cells which can be activated by a number of extracellular signals. In the GTP-bound state, Rho binds to numerous intracellular targets to activate them. One such target is Rho kinase. The dominant negative Rho kinase bears mutations in its ATP-binding domain and effectively inhibits the Rho-mediated phosphorylation of its numerous downstream targets. AVV with HCMV promoter to drive expression of this dominant negative protein were injected into the NTS of both WKY and SHR from which blood pressure was measured using radio-telemetry. In both strains a transient drop in heart rate and blood pressure was observed between day 4 and day 7 but this was more dramatic in the SHR. The authors also found that in the SHR Rho re-distributes to the plasma membrane from the cytosol and that phosphorylation of one of the downstream targets of Rho kinase in SHR is enhanced. In addition, a small but statistically significant decrease in both eNOS and nNOS protein was found after Rho-kinase dominant negative protein expression in the NTS. In this study an AVV with the HCMV promoter was used [6]. Thus, based on our evidence [27] it would be predicted that only a small minority of targets transduced by this vector in the NTS could be neurones. What then could be the cellular target of the Rho-kinase dominant negative expression in the NTS described by [6]? Clearly, in contrast to the earlier discussed eNOS overexpression experiments the Rho-kinase dominant negative could only act on Rho signalling within the same cells which were expressing the transgene. Given that the effect was very transient (3 days only) it is possible that the local endothelial cells were responsible for it: these cells can express transgenes by injection of AVV with HCMV promoter in the brain [89]. They are exposed to the immune system and are likely to be rapidly eliminated due to the residual expression of the adenoviral proteins. A change in eNOS expression reported in that study could be consistent with this suggestion [6].

In conclusion, the examples presented illustrate the usefulness of expressing dominant negative proteins in unravelling neuronal mechanisms in central regulation of cardio-vascular function. These have allowed identification of ligand mediated intracellular cascades *and* the simultaneous physiological consequences of perturbing such pathways on blood pressure regulation. There is no doubt that viral gene transfer coupled with radio-telemetry provides a powerful tool for the cardio-vascular neuroscientist. However, further refinement of viral constructs is required. As exemplified by the AVV with HCMV-driven trangenes, caution is required when interpreting data and ascribing functional effects to a specific cell type within the brain.

Tracking cardio-vascular circuits using retrograde viral transfection

Certain types of viruses (e.g. the pseudorabies virus) can be used to track neuronal pathways due to their retrograde transport and ability to express transgenes, such as EGFP, in connected central neurones, which presumably form a neuroanatomical circuit. [13] used the Bartha strain of swine pathogenic presudorabies virus in a study on cardio-respiratory networks. Pseudorabies virus is an attenuated replicating pathogen and is therefore different to all other vectors mentioned in this chapter because it is *not* replication deficient. It enters neurones via their peripheral axonal terminals and invades the soma by retrograde transport. Once in a neuronal soma, the virus replicates within a few hours and becomes highly concentrated in dendritic processes and then leaves its host at sites of synaptic contact. It is thought that glial processes limit the lateral spread of virions to unrelated nerve cells and contribute to a predominantly trans-synaptic pattern of viral spread (see [90;91]. [13] engineered a Bartha virus mutant to express EGFP using an endogenous gG promoter present in that virus. Two days after the injection of the virus into the pericardial sac of rat pups, EGFP fluorescent cardio-vagal neurones could be identified in the nucleus ambiguous. However, an adjacent population of motoneurones with their axons in the superior laryngeal did not express EGFP. Electrophysiological analysis revealed that the EGFP-expressing neurones could generate action potentials and had currents similar to those in nearby non-transfected neurones. If pups were sacrificed 3 days after pericardial injections, EGFP labelling was also found in the periambigual area and NTS. However, *in vivo* analysis of transfected pups revealed that viral infection caused a clear shift in the baroreceptor reflex input-output curve measured at day 3, suggestive of a change in the functional state of the reflex pathway [13]. In conclusion, this viral approach allows clear identification of cardiac vagal motoneurones and in this regard is similar to that achieved using conventional fluorescent retrograde tracers such as rhodamine beads. It appears, however, that inter-neurones antecedent to the motoneurones are much more difficult to study. It is highly

unlikely that the infected neurones retain normal neurophysiological characteristics for any length of time due to the detrimental effects of rapidly replicating virus.

AVV can also be used for retrograde tracing but they do not pass transynaptically. Moreover, as AVV do not cause any deleterious effects in the neurones they infect, they can also be used to co-express a functional protein along with a fluorescent reporter. As an example, cardiac parasympathetic pre-ganglionic neurones in nucleus ambiguus were retrogradely transfected with AVV after injection of this virus into the pericardial sac of P4 – P10 rat pups [12]. The AVV contained two transgenes in a head-to head orientation, both under control of the HCMV promoter. One of these cassettes expressed EGFP and the other – the ε-subunit of $GABA_A$ receptor. Two to four days after viral injection medullary slices were prepared. Retrogradely transduced fluorescent neurones could be identified and their electrophysiological characteristics studied using the patch clamp technique. It was found that expression of the ε-subunit in cardiac vagal motoneurones blocked the pentobarbitone-induced potentiation of GABAergic currents.

Finally, and as mentioned above, we have developed a method for selective retrograde transduction of subsets of brainstem noradrenergic neurones which may be used to analyse their function [27].

Future potential applications of viral gene transfer

There may be several other applications of viral gene transfer in studies of central cardio-vascular control. We wish to describe two: First, our laboratory recently demonstrated direct recording of transmitter release from individual visualised varicosities of brainstem noradrenaline-containing neurones. An AVV was constructed to express EGFP under control of the PRSx8 promoter, which is highly active in noradrenergic neurones (see above). In slice cultures,

catecholaminergic neurones displayed characteristic beaded axons that were readily identifiable using either conventional fluorescent or confocal microscopy (Fig 1F). Moreover, these axons could be traced for hundreds of microns. Using fine carbon electrodes microamperometric recordings of noradrenaline release from these varicosities can be made (Fig. 4). Thus, this novel approach will allow for the first time a study of the modulation of noradrenaline release from characterised central neurones of normotensive and hypertensive rat strains. Second, the "vigilant vector" introduced by [92] and colleagues has a number of unique characteristics. It has both a cardiac myocyte specific promoter (a truncated version of MLC2v) and an enhancer - the hypoxia response element (HRE) operating within a single AAV. This construct drives expression of antisense RNA to the AT_1 receptor in the heart in hypoxia-inducible manner. The motivation for such a construct was based on the problem of myocardial ischaemia, which initially is a progressive asymptomatic condition. Following a hypoxic episode in the heart, AT_1 receptor antisense expression could help alleviate consequences of ischaemia since this receptor contributes to this condition. Such vigilant vectors have not yet been applied to the central nervous system but it is conceivable that expressing transgenes to depress AT_1 receptors in specific cell types could be most beneficial in neurogenic hypertension. Possibly cascades which link neuronal activity to c-fos gene expression could be exploited to achieve inducibility as in [93].

CONCLUSIONS

There is no question that genetic manipulation to address biological questions has recently become a universal research tool in all areas of physiology. As illustrated herein, viral vectors offer an efficient way of gene transfer and can be used to perform a variety of experiments in order to address mechanisms of central cardio-vascular control. So far, AVV and LVV have been used mainly in these studies but AAV also has very attractive features. All available viral vectors

target (albeit sometimes inadvertently) the transgene to one or more cell types in the brain (glia, different components of blood vessels, various neuronal phenotypes). This occurs at both the transductional (e.g. at the stage of viral entry into the cells) and transcriptional levels (e.g. via the mechanisms which control gene expression) and needs to be taken into account when interpreting the functional outcome of such experiments. It is expected that in the near future viral vector production will become faster and cheaper and that viruses will be completely non-immunogenic with targeted cellular tropism in which expression is controllable and suitable for both *in vivo* and *in vitro* applications. It may also become important to further refine protocols for production of large quantities of vectors, such as LVV for systemic applications. Finally, further research should focus on developing short and powerful promoters suitable for directing virally mediated expression to selected populations of cells. Viral gene manipulation in the brainstem has already been successfully used to identify mechanisms relating to cardio-vascular control as well as the alterations that occur and which may contribute to hypertension. In this type of research, viral gene transfer is particularly valuable because it is fully compatible with other established conventional experimental strategies, such as hypertensive rat models and long-term telemetric monitoring of blood pressure.

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Fig. 1

A – Retrogradely transfected noradrenergic neurones in the A1 brainstem area. AVV PRSx8-EGFP (1μl of 2.2*10^8 PFU) was injected in the paraventricular area of the hypothalamus. 5 days later sections were prepared and immunostained for dopamine β-hydroxylase (DBH; red fluorescence of the CY3 conjugated secondary antibody). This image in an overlay of the enhanced green fluorescent protein (EGFP) and CY3 channels obtained separately using spectral confocal microscope. Note that both EGFP expressing neurones are also DBH-positive and therefore appear yellow. (reproduced from Kasparov et al 2004 with permission)

B&C – AVV PRSx8-EGFP was injected into either Locus Coeruleus (A6) or dorsal vagal complex (including the A2 group). 6 days later the tissue was fixed and processed for DBH. The images are overlays of EGFP (green) and DBH (red) channels of confocal images. Note that in the A6 all transduced neurones appear yellow (arrows) due to co-localisation with the DBH stain. In contrast, in the dorsal vagal complex while the EGFP expressing cells in NTS were DBH-positive A2 cells (yellow colour, thick arrows), the underlaying neurones of the dorsal vagal motor nucleus (below the dotted line) also expressed high levels of EGFP (green colour, DBH-negative). Data contributed by D. Lonergan (University of Bristol).

D&E - AVV HCMV-EGFP expression profile in the dorsal vagal complex. 2 injections of 0.5 μl of 2.7*10^8 PFU of the vector were made at 300 & 400 μm from the dorsal surface of the medulla. The tissue was then processed for DBH. The NTS is devoid of EGFP-expressing neurones, no EGFP-expressing cells in NTS area were DBH-positive. Some glial cells can bee seen. In contrast there are numerous EGFP-expressing cells (both, glia and neurones) in the deeper cholinergic motor nuclei. In particular, many of the neurones of the motor nucleus of the hypoglossal nerve expressed EGFP. These cells could be easily

identified on low-power images (right panel) by their long axons projecting towards the ventral edge of the medulla.

F – AVV PRSx8-EGFP was used to visualise catecholaminergic neurones and their beaded axons in organotypic slice cultures of the rat brainstem. Vectors were applied to plating media and fluorescent neurones visualised > 3 days using conventional and confocal fluorescent microscopy. Further details in [27].

Fig 2

Examples of the usefulness of AVV-mediated expression of a dominant negative protein in the nucleus tractus solitarii (NTS) for cardiovascular control. Angiotensin II (ANGII) injections into the NTS attenuate the reflex bradycardia to baroreceptor stimulation (A). This was shown to be dependent on endothelial nitric oxide synthase (eNOS) as adenoviral transfection with a dominant negative "TeNOS" (a truncated form of eNOS) prevented the effect of angiotensin II (B). Transfection sites (indicated by grey shading) were localised within the NTS as revealed by expression of EGFP using a second adenovirus (C). Chronic expression of TeNOS in rats fitted with telemetry devices to record arterial pressure revealed a significant elevation in baroreceptor reflex sensitivity in normotensive rats (D). Note that neither saline injections into NTS or expression of EGFP in this nucleus altered baroreceptor reflex gain. Abbreviations: AP. Area postrema; DVM, dorsal vagal motor nucleus; HR- heart rate in beats per min (bpm) and PP – perfusion pressure; TS, solitary tract. Data taken from [32] and [3].

Fig 3

Determining the intracellular signalling pathway activated by angiotensin II (ANGII) to inhibit the baroreceptor reflex. Two possible pathways were tested (A & B). Using adenoviral gene transfer (and pharmacological antagonists – see Wong et al. 2002) to disrupt pathway A prevented the ANGII-induced

attenuation of the baroreceptor reflex. Two adenoviruses were used to express dominant negative proteins to block Gq (AVV 1) and eNOS (AVV 2). In contrast, reducing reactive oxygen species (ROS) by overexpressing catalase (AVV 3) or expressing a dominant negative to block protein kinase B activity (Akt; AVV 4) were unable to prevent the baroreceptor reflex attenuation by ANGII. It has been hypothesised that pathway A is responsible for the ANGII-induced activation of eNOS. Data taken from [32] and [10].

Fig 4

Detection of noradrenaline release from visualised varicosities of A1 neurones.

A – Schematic to illustrate the principle of microamperometric catecholamine detection. Oxidation of the catecholamine at the pre-set electrode potential generates current, as shown in panel B. In these experiments a 5 μm carbon fibre electrode was positioned directly on the visualised EGFP-fluorescent axonal varicosities.

B – A current trace illustrates bursts of vesicular noradrenaline release from a single varicosity.

Reference List

1. Mann,S.J., Neurogenic essential hypertension revisited: the case for increased clinical and research attention, American Journal of Hypertension, 16 (2003) 881-888.

2. Keeton,T.K. and Campbell,W.B., The pharmacologic alteration of renin release, Pharmacological Reviews, 31 (1980) 81-227.

3. Waki,H., Kasparov,S., Wong,L.-F., Murphy,D., Shimizu,T., and Paton,J.F.R., Chronic inhibition of eNOS activity in NTS enhances baroreceptor reflex in conscious rats, Journal of Physiology, 546 (2003) 233-242.

4. Paton,J.F., Waki,H., and Kasparov,S., In vivo gene transfer to dissect neuronal mechanisms regulating cardiorespiratory function, Canadian Journal of Physiology & Pharmacology, 81 (2003) 311-316.

5. 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 (2003) 255-260.

6. Ito,K., Hirooka,Y., Sakai,K., Kishi,T., Kaibuchi,K., Shimokawa,H., and Takeshita,A., Rho/Rho-kinase pathway in brain stem contributes to blood pressure regulation via sympathetic nervous system: possible involvement in neural mechanisms of hypertension, Circulation Research, 92 (2003) 1337-1343.

7. Kishi,T., Hirooka,Y., Sakai,K., Shigematsu,H., Shimokawa,H., and Takeshita,A., Overexpression of eNOS in the RVLM causes hypotension and bradycardia via GABA release, Hypertension, 38 (2001) 896-901.

8. Zimmerman,M.C., Lazartigues,E., Lang,J.A., Sinnayah,P., Ahmad,I.M., Spitz,D.R., and Davisson,R.L., Superoxide mediates the actions of angiotensin II in the central nervous system., Circulation Research, 91 (2002) 1038-1045.

9. Waki,H., Kasparov,S., Katahira,K., Shimizu,T., Murphy,D., and Paton,J.F., Dynamic exercise attenuates spontaneous baroreceptor reflex sensitivity in conscious rats, Experimental Physiology, 88 (2003) 517-526.

10. Wong,L.F., Polson,J.W., Murphy,D., Paton,J.F., and Kasparov,S., Genetic and pharmacological dissection of pathways involved in the angiotensin II-mediated depression of baroreflex function, FASEB Journal, Online. 16 (2002) 1595-1601.

11. Sinnayah,P., Lindley,T.E., Staber,P.D., Cassell,M.D., Davidson,B.L., and Davisson,R.L., Selective gene transfer to key cardiovascular regions of the brain: comparison of two viral vector systems, Hypertension, 39 (2002) 603-608.

12. Irnaten,M., Walwyn,W.M., Wang,J., Venkatesan,P., Evans,C., Chang,K.S., Andresen,M.C., Hales,T.G., and Mendelowitz,D., Pentobarbital enhances GABAergic neurotransmission to cardiac parasympathetic neurons, which is prevented by expression of $GABA_A$ ε subunit, Anesthesiology, 97 (2002) 717-724.

13. Irnaten,M., Neff,R.A., Wang,J., Loewy,A.D., Mettenleiter,T.C., and Mendelowitz,D., Activity of cardiorespiratory networks revealed by transsynaptic virus expressing GFP, Journal of Neurophysiology, 85 (2001) 435-438.

14. Chalmers,J., Brain, blood pressure and stroke, Journal of Hypertension, 16 (1998) 1849-1858.

15. de Wardener,H.E., The hypothalamus and hypertension, Physiological Reviews, 81 (2001) 1599-1658.

16. Haywood,J.R., Mifflin,S.W., Craig,T., Calderon,A., Hensler,J.G., and Hinojosa-Laborde,C., γ-Aminobutyric acid $(GABA)_A$ function and binding in the paraventricular nucleus of the hypothalamus in chronic renal-wrap hypertension, Hypertension, 37 (2001) 614-618.

17. Arnolda,L., Minson,J., Kapoor,V., Pilowsky,P., Llewellyn-Smith,I., and Chalmers, Amino acid neurotransmitters in hypertension, Kidney International - Supplement, 37 (1992) S2-S7.

18. Yamada,K., Moriguchi,A., Mikami,H., Okuda,N., Higaki,J., and Ogihara,T., The effect of central amino acid neurotransmitters on the antihypertensive response to angiotensin blockade in spontaneous hypertension, Journal of Hypertension, 13 (1995) 1624-1630.

19. Dampney,R.A.L., Goodchild,A.K., and Tan,E., Identification of cardiovascular cell groups in the brain stem, Clinical & Experimental Hypertension, 6 (1984) 205-220.

20. Dampney,R.A.L., Functional organization of central pathways regulating the cardiovascular system, Physiological Reviews, 74 (1994) 323-364.

21. Lu,D., Yu,K., Paddy,M.R., Rowland,N.E., and Raizada,M.K., Regulation of norepinephrine transport system by angiotensin II in neuronal cultures of normotensive and spontaneously hypertensive rat brains, Endocrinology, 137 (1996) 763-772.

22. MacLean,M.R., Raizada,M.K., and Sumners,C., The influence of angiotensin II on catecholamine synthesis in neuronal cultures from rat brain, Biochemical & Biophysical Research Communications, 167 (1990) 492-497.

23. MacLean,M.R., Phillips,M.I., Summers,C., and Raizada,M.K., α1-Adrenergic receptors in the nucleus tractus solitarii region of rats with experimental and genetic hypertension, Brain Research, 519 (1990) 261-265.

24. Baker,A.H., Adenoviral vectors for gene therapy, Molecular Biotechnology, 25 (2003) 101-102.

25. Nicklin,S.A., Dishart,K.L., Buening,H., Reynolds,P.N., Hallek,M., Nemerow,G.R., Von Seggern,D.J., and Baker,A.H., Transductional and transcriptional targeting of cancer cells using genetically engineered viral vectors, Cancer Letters, 201 (2003) 165-173.

26. Wickham,T.J., Targeting adenovirus, Gene Therapy, 7 (2000) 110-114.

27. Kasparov,S., Teschemacher,A.G., Hwang,D.-Y., Kim,K.-S., Lonergan,T., and Paton,J.F.R., Viral Vectors as Tools for Studies of Central Cardiovascular Control, Progress in Biophysics and Molecular BIology, 84 (2004) 251-277.

28. Lonergan,T., Teschemacher,A.G., Paton,J.F.R., and Kasparov,S., Expression profile of adenoviral vectors incorporating hCMV, synapsin-1 and PRSx8 promoters in brainstem centres of cardiovascular control, Journal of Physiology, http://www.physoc.org/publications/proceedings/archive/index.asp (2004).

29. Katovich,M.J., Reaves,P.Y., Francis,S.C., Pachori,A.S., Wang,H.W., and Raizada,M.K., Gene therapy attenuates the elevated blood pressure and glucose intolerance in an insulin-resistant model of hypertension, Journal of Hypertension, 19 (2001) 1553-1558.

30. Wang,H., Lu,D., Reaves,P.Y., Katovich,M.J., and Raizada,M.K., Retrovirally mediated delivery of angiotensin II type 1 receptor antisense in vitro and in vivo, Methods in Enzymology, 314 (1999) 581-590.

31. Stec,D.E., Davisson,R.L., Haskell,R.E., Davidson,B.L., and Sigmund,C.D., Efficient liver-specific deletion of a floxed human angiotensinogen transgene by adenoviral delivery of Cre recombinase in vivo, Journal of Biological Chemistry, 274 (1999) 21285-21290.

32. Paton,J.F.R., Deuchars,J., Ahmad,Z., Wong,L.-F., 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, Journal of Physiology, 531 (2001) 445-458.

33. Hirooka,Y., Sakai,K., Kishi,T., and Takeshita,A., Adenovirus-mediated gene transfer into the NTS in conscious rats. A new approach to examining the central control of cardiovascular regulation, Annals of the New York Academy of Sciences, 940 (2001) 197-205.

34. Phillips,M.I., Gene therapy for hypertension: the preclinical data, Hypertension, 38 (2001) 543-548.

35. Phillips,M.I., Gene therapy for hypertension: sense and antisense strategies, Expert Opinion in Biology and Therapy, 1 (2001) 655-662.

36. Kugler,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 Therapy, 10 (2003) 337-347.

37. Glover,C.P., Bienemann,A.S., Hopton,M., Harding,T.C., Kew,J.N., and Uney,J.B., Long-term transgene expression can be mediated in the brain by adenoviral vectors when powerful neuron-specific promoters are used, Journal of Gene Medicine, 5 (2003) 554-559.

38. Hwang,D.-Y., Carlezon,W.A., Jr., Isacson,O., and Kim,K.-S., A high-efficiency synthetic promoter that drives transgene expression selectively in noradrenergic neurons, Human Gene Therapy, 12 (2001) 1731-1740.

39. Thomas,C.E., Schiedner,G., Kochanek,S., Castro,M.G., and Lowenstein,P.R., Peripheral infection with adenovirus causes unexpected long-term brain inflammation in animals injected intracranially with first-generation, but not with high-capacity, adenovirus vectors: Toward realistic long-term neurological gene therapy for chronic diseases, Proceedings of the National Academy of Sciences of the United States of America, 97 (2000) 7482-7487.

40. Iwakuma,T., Cui,Y., and Chang,L.-J., Self-Inactivating lentiviral vectors with U3 and U5 modifications, Virology, 261 (1999) 120-132.

41. Coleman,J.E., Huentelman,M.J., Kasparov,S., Metcalfe,B.L., Paton,J.F.R., Katovich,M.J., Semple-Rowland,S.L., and Raizada,M.K., Efficient Large-Scale Production and Concentration of HIV-1-Based Lentiviral Vectors For Use *In vivo*, Physiological Genomics, 12 (2003) 221-228.

42. Jin,X., Mathers,P.H., Szabo,G., Katarova,Z., and Agmon,A., Vertical bias in dendritic trees of non-pyramidal neocortical neurons expressing GAD67-GFP in vitro, Cerebral Cortex, 11 (2001) 666-678.

43. Gong,S., Zheng,C., Doughty,M.L., Losos,K., Didkovsky,N., Schambra,U.B., Nowak,N.J., Joyner,A., Leblanc,G., Hatten,M.E., and Heintz,N., A gene expression atlas of the central nervous system based on bacterial artificial chromosomes, Nature, 425 (2003) 917-925.

44. Kugler,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 Therapy, 10 (2003) 337-347.

45. Chillon,M., Bosch,A., Zabner,J., Law,L., Armentano,D., Welsh,M.J., Davidson, and BL., Group D adenoviruses infect primary central nervous system cells more efficiently than those from group C, Journal of Virology, 73 (1999) 2537-2540.

46. Omori,N., Mizuguchi,H., Ohsawa,K., Kohsaka,S., Hayakawa,T., Abe,K., and Shibasaki,F., Modification of a fiber protein in an adenovirus vector improves in vitro gene transfer efficiency to the mouse microglial cell line, Neuroscience Letters, 324 (2002) 145-148.

47. Nicklin,S.A., Von Seggern,D.J., Work,L.M., Pek,D.C., Dominiczak,A.F., Nemerow,G.R., and Baker,A.H., Ablating adenovirus type 5 fiber-CAR binding and HI loop insertion of the SIGYPLP peptide generate an endothelial cell-selective adenovirus, Molecular Therapy, 4 (2001) 534-542.

48. White,S.J., Nicklin,S.A., Sawamura,T., and Baker,A.H., Identification of peptides that target the endothelial cell-specific LOX-1 receptor, Hypertension, 37 (2001) 449-455.

49. Bilang-Bleuel,A., Revah,F., Colin,P., Locquet,I., Robert,J.-J., Mallet,J., and Horellou,P., Intrastriatal injection of an adenoviral vector expressing glial-cell- line-derived neurotrophic factor prevents dopaminergic neuron degeneration and behavioral impairment in a rat model of Parkinson disease, Proceedings of the National Academy of Sciences of the United States of America, 94 (1997) 8818-8823.

50. Nishimura,I., Uetsuki,T., Dani,S.U., Ohsawa,Y., Saito,I., Okamura,H., Uchiyama, Y, and Yoshikawa,K., Degeneration in vivo of rat hippocampal neurons by wild-type Alzheimer amyloid precursor protein overexpressed by adenovirus-mediated gene transfer, Journal of Neuroscience, 18 (1998) 2387-2398.

51. Niwa,H., Yamamura,K., and Miyazaki,J., Efficient selection for high-expression transfectants with a novel eukaryotic vector, Gene, 108 (1991) 193-199.

52. Mazarakis,N.D., Azzouz,M., Rohll,J.B., Ellard,F.M., Wilkes,F.J., Olsen,A.L., Carter,E.E., Barber,R.D., Baban,D.F., Kingsman,S.M., Kingsman,A.J., O'Malley,K., and Mitrophanous,K.A., Rabies virus glycoprotein pseudotyping of lentiviral vectors enables retrograde axonal transport and access to the nervous system after peripheral delivery, Human Molecular Genetics, 10 (2001) 2109-2121.

53. Kang,Y., Stein,C.S., Heth,J.A., Sinn,P.L., Penisten,A.K., Staber,P.D., Ratliff,K.L., Shen,H., Barker,C.K., Martins,I., Sharkey,C.M., Sanders,D.A., McCray,P.B., Jr., and Davidson,B.L., In vivo gene transfer using a nonprimate lentiviral vector pseudotyped with Ross River Virus glycoproteins, Journal of Virology, 76 (2002) 9378-9388.

54. Davidson,B.L. and Chiorini,J.A., Recombinant adeno-associated viral vector types 4 and 5. Preparation and application for CNS gene transfer, Methods in Molecular Medicine, 76 (2003) 269-285.

55. Okada,T., Nomoto,T., Shimazaki,K., Lijun,W., Lu,Y., Matsushita,T., Mizukami,H., Urabe,M., Hanazono,Y., Kume,A., Muramatsu,S., Nakano,I., and Ozawa,K., Adeno-associated virus vectors for gene transfer to the brain., Methods, 28 (2002) 237-247.

56. Paterna,J.C. and Büeler,H., Recombinant adeno-associated virus vector design and gene expression in the mammalian brain., Methods, 28 (2002) 208-218.

57. Nicklin,S.A., Buening,H., Dishart,K.L., de Alwis,M., Girod,A., Hacker,U., Thrasher,A.J., Ali,R.R., Hallek,M., and Baker,A.H., Efficient and selective AAV2-mediated gene transfer directed to human vascular endothelial cells, Molecular Therapy, 4 (2001) 174-181.

58. Kaspar,B.K., Erickson,D., Schaffer,D., Hinh,L., Gage,F.H., and Peterson,D.A., Targeted retrograde gene delivery for neuronal protection, Molecular Therapy, 5 (2002) 50-56.

59. Büning,H., Nicklin,S.A., Perabo,L., Hallek,M., and Baker,A.H., AAV-based gene transfer, Current Opinion in Molecular Therapy, 5 (2003) 367-375.

60. Davidson,B.L. and Breakefield,X.O., Viral vectors for gene delivery to the nervous system., Nature Reviews Neuroscience, 4 (2003) 353-364.

61. 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, Hypertension Research - Clinical & Experimental, 26 (2003) 325-331.

62. Hirooka,Y., Kishi,T., Sakai,K., Shimokawa,H., and Takeshita,A., Effect of overproduction of nitric oxide in the brain stem on the cardiovascular response in conscious rats., Journal of Cardiovascular Pharmacology, 41 Suppl 1 (2003) S119-S126.

63. Kishi,T., Hirooka,Y., Ito,K., Sakai,K., Shimokawa,H., and Takeshita,A., Cardiovascular effects of overexpression of endothelial nitric oxide synthase in the rostral ventrolateral medulla in stroke-prone spontaneously hypertensive rats, Hypertension, 39 (2002) 264-268.

64. Matsuo,I., Hirooka,Y., Hironaga,K., Eshima,K., Shigematsu,H., Shihara,M., Sakai,K., and Takeshita,A., Glutamate release via NO production evoked by NMDA in the NTS enhances hypotension and bradycardia *in vivo*, American Journal of Physiology - Regulatory Integrative & Comparative Physiology, 280 (2001) R1285-R1291.

65. 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 (2000) 1023-1028.

66. Paton,J.F.R., Kasparov,S., and Paterson,D.J., Nitric oxide and autonomic control of heart rate: a question of specificity., Trends in Neurosciences, 25 (2002) 626-631.

67. Lindley,T.E., Doobay,M.F., Sharma,R.V., and Davisson,R.L., Superoxide is involved in the central nervous system activation and sympathoexcitation of myocardial infarction-induced heart failure, Circulation Research, 94 (2004) 402-409.

68. Lu,D. and Raizada,M.K., Delivery of angiotensin II type 1 receptor antisense inhibits angiotensin action in neurons from hypertensive rat brain, Proceedings of the National Academy of Sciences of the United States of America, 92 (1995) 2914-2918.

69. Kagiyama,S., Qian,K., Kagiyama,T., and Phillips,M.I., Antisense to epidermal growth factor receptor prevents the development of left ventricular hypertrophy, Hypertension, 41 (2003) 824-829.

70. Shi,Y., Mammalian RNAi for the masses., Trends in Genetics, 19 (2003) 9-12.

71. Couzin,J., Breakthrough of the year. Small RNAs make big splash, Science, 298 (2002) 2296-2297.

72. Czauderna,F., Fechtner,M., Aygun,H., Arnold,W., Klippel,A., Giese,K., and Kaufmann,J., Functional studies of the PI(3)-kinase signalling pathway employing synthetic and expressed siRNA, Nucleic Acids Research, 31 (2003) 670-682.

73. Higuchi,H., Yamashita,T., Yoshikawa,H., and Tohyama,M., Functional inhibition of the p75 receptor using a small interfering RNA, Biochemical & Biophysical Research Communications, 301 (2003) 804-809.

74. Sorensen,D.R., Leirdal,M., and Sioud,M., Gene silencing by systemic delivery of synthetic siRNAs in adult mice, Journal of Molecular Biology, 327 (2003) 761-766.

75. Miller,V.M., Xia,H., Marrs,G.L., Gouvion,C.M., Lee,G., Davidson,B.L., and Paulson,H.L., Allele-specific silencing of dominant disease genes, Proceedings of the National Academy of Sciences U. S. A, 100 (2003) 7195-7200.

76. Rubinson,D.A., Dillon,C.P., Kwiatkowski,A.V., Sievers,C., Yang,L., Kopinja,J., Rooney,D.L., Ihrig,M.M., McManus,M.T., Gertler,F.B., Scott,M.L., and Van Parijs,L., A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference, Nature Genetics, 33 (2003) 401-406.

77. Barton,G.M. and Medzhitov,R., Retroviral delivery of small interfering RNA into primary cells, Proceedings of the National Academy of Sciences of the United States of America, 99 (2002) 14943-14945.

78. Xia,H., Mao,Q., Paulson,H.L., and Davidson,B.L., siRNA-mediated gene silencing in vitro and in vivo, Nature Biotechnology, 20 (2002) 1006-1010.

79. Wiznerowicz,M. and Trono,D., Conditional suppression of cellular genes: lentivirus vector-mediated drug-inducible RNA interference, Journal of Virology, 77 (2003) 8957-8961.

80. Casto,R. and Phillips,M.I., Angiotensin II attenuates baroreflexes at nucleus tractus solitarius of rats, American Journal of Physiology - Regulatory Integrative and Comparative Physiology, 250 (1986) R193-R198.

81. Casto,R. and Phillips,M.I., Baroreflex resetting by infusions of angiotensin II into the nucleus tractus solitarius, Federation Proceedings, 44 (1985) 3645.

82. Kantor,D.B., Lanzrein,M., Stary,S.J., Sandoval,G.M., Smith,W.B., Sullivan,B.M., Davidson,N., and Schuman,E.M., A role for endothelial NO synthase in LTP revealed by adenovirus- mediated inhibition and rescue, Science, 274 (1996) 1744-1748.

83. Lee,C.M., Robinson,L.J., and Michel,T., Oligomerization of endothelial nitric oxide synthase. Evidence for a dominant negative effect of truncation mutants, Journal of Biological Chemistry, 270 (1995) 27403-27406.

84. Kasparov,S., Waki,H., Okwuadigbo,E., Murphy,D., and Paton,J.F.R., Endothelial nitric oxide synthase in the nucleus tractus solitarii (NTS) attentuates baroreflex and increases blood pressure in spontaneously hypertensive rat (SHR): evidence from *in vivo* gene transfer, Society for Neuroscience Abstracts, 28 (2002) 861.1.

85. Wong,L.-F., Kasparov,S., Murphy,D., and Paton,J.F.R., Angiotensin II-mediated signal transduction mechanisms in the nucleus of the solitary tract (NTS) that depress the baroreflex, Society for Neurocience Abstracts, 27 (2001) 837.4.

86. Kasparov,S., Teschemacher,A., and Paton,J.F.R., Dynamic confocal imaging in acute brain slices and organotypic slice cultures using a spectral confocal microscope with single photon excitation, Experimental Physiology, 87 (2002) 715-724.

87. Kugler,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 Therapy, 10 (2003) 337-347.

88. Stokes,C.E.L., Murphy,D., Paton,J.F.R., and Kasparov,S., Dynamics of a transgene expression in acute rat brain slices transfected with adenoviral vectors, Experimental Physiology, 88 (2003) 459-466.

89. Kasparov,S. and Paton,J.F.R., Somatic gene transfer: implications for cardiovascular control, Experimental Physiology, 85 (2000) 747-755.

90. Aston-Jones,G. and Card,J.P., Use of pseudorabies virus to delineate multisynaptic circuits in brain: opportunities and limitations., Journal of Neuroscience Methods, 103 (2000) 51-61.

91. Loewy,A.D., Viruses as transneuronal tracers for defining neural circuits., Neuroscience & Biobehavioral Reviews, 22 (1998) 679-684.

92. Phillips,M.I., Tang,Y., Schmidt-Ott,K., Qian,K., and Kagiyama,S., Vigilant vector: heart-specific promoter in an adeno-associated virus vector for cardioprotection, Hypertension, 39 (2002) 651-655.

93. Stokes,C.E.L., Teschemacher,A., Murphy,D., Paton,J.F.R., and Kasparov,S., Visualisation of c-*fos* activation in living neurones of the paraventricular nucleus of hypothalamus using adenoviral gene transfer, FASEB Journal, 17 (2003) Abstract# 564.4.

Fig 1

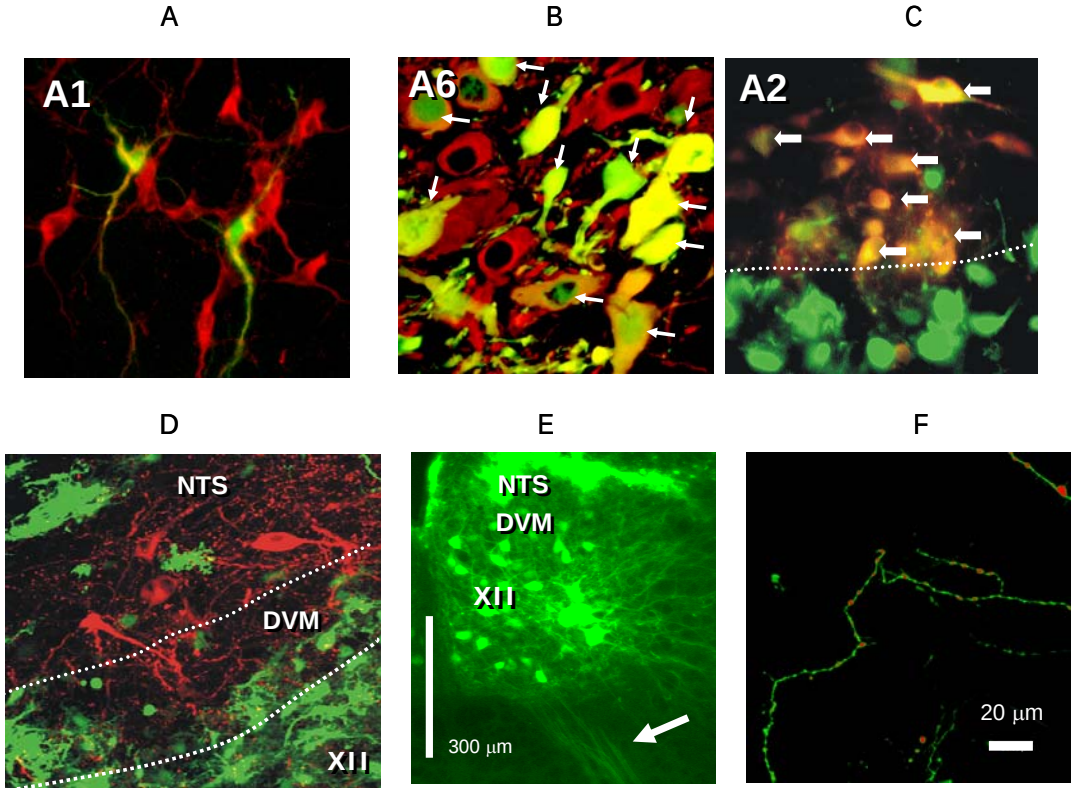
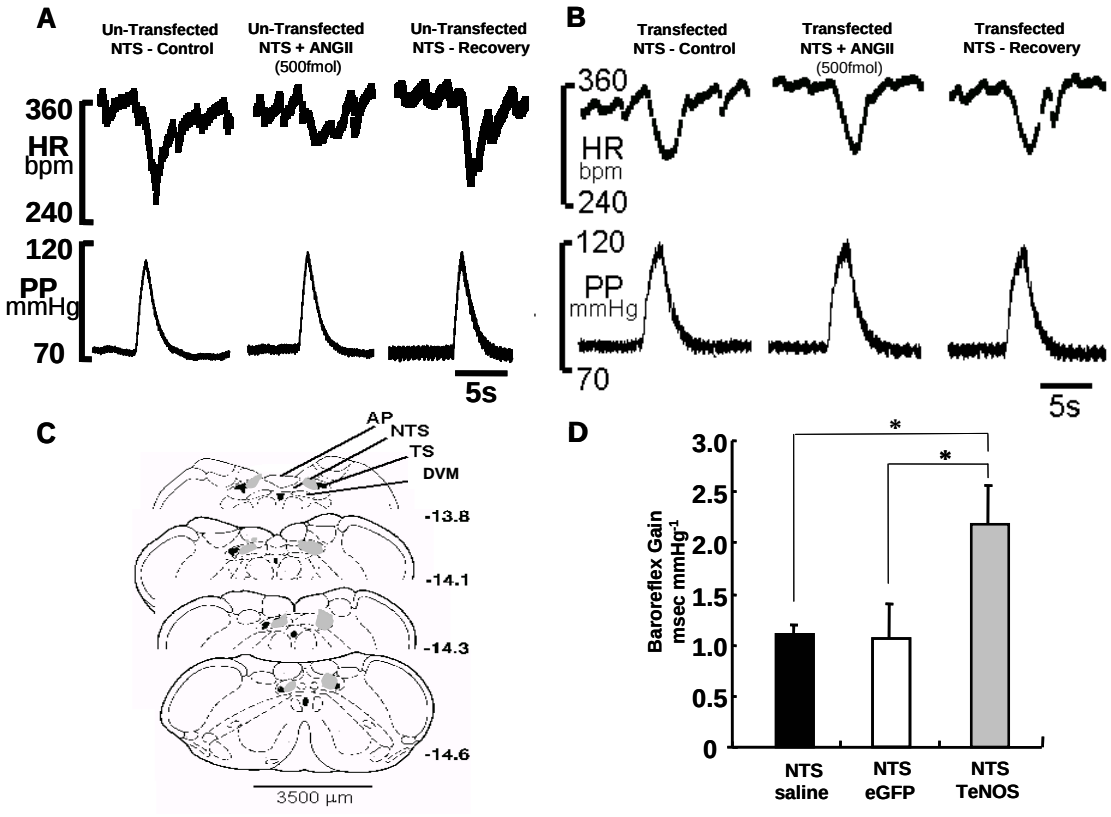


Fig 2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Un-Transfected NTS + ANGII (500fmol)
Un-Transfected NTS - Recovery
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HR
bpm
240
120
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mmHg
70
5s
B
Transfected NTS - Control
Transfected NTS + ANGII (500fmol)
Transfected NTS - Recovery
360
HR
bpm
240
120
PP
mmHg
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C
AP
NTS
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NTS saline
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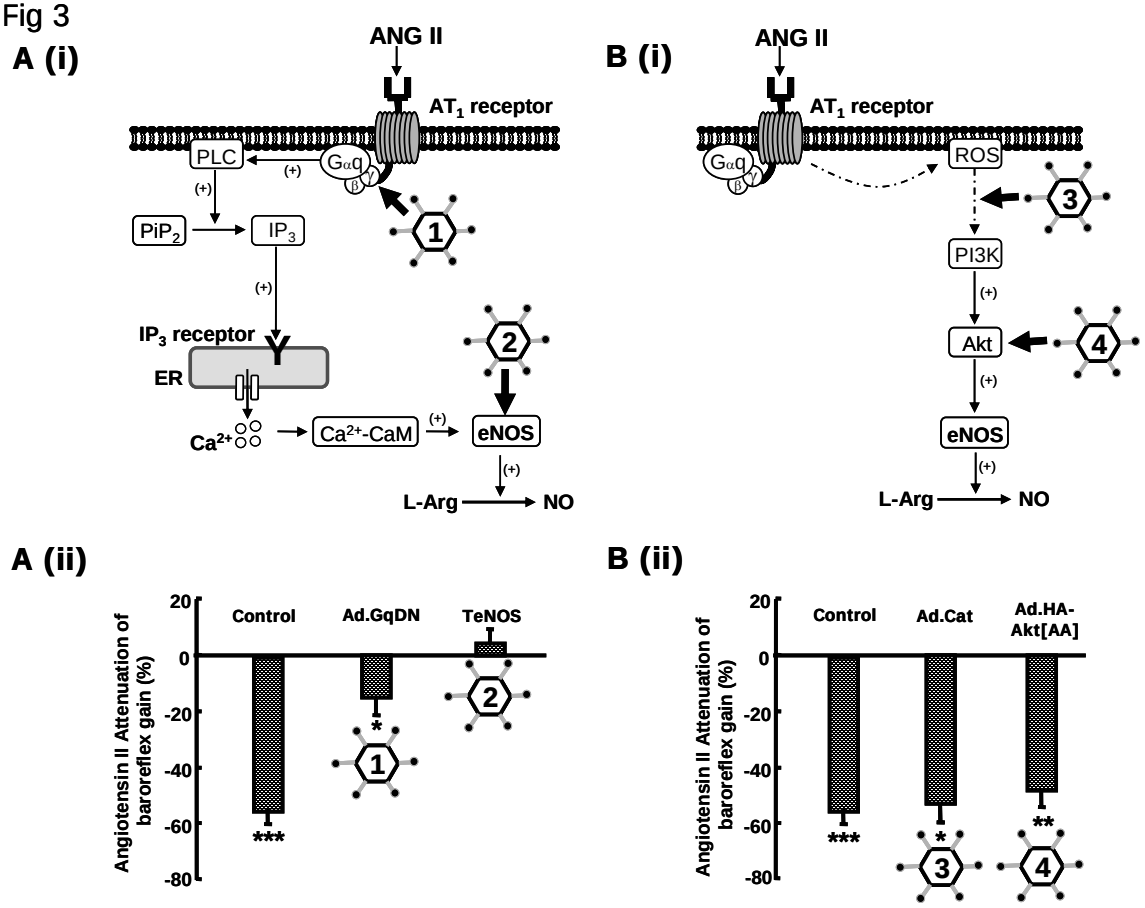
Fig 3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L-Arg
NO
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B (i)
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AT1 receptor
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βγ
ROS
PI3K
Akt
eNOS
L-Arg
NO
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4
A (ii)
Angiotensin II Attenuation of baroreflex gain (%)
20
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-60
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Control
Ad.GqDN
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Fig 4.