



Imaging living central neurones using viral gene transfer

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Abstract

Studies of central neurones and other cellular components of the brain, such as glial and vascular cells, can be greatly advanced by the use of the modern optical techniques such as confocal live cell imaging. Fluorescent proteins have allowed imaging of particular cell types or intracellular elements to be visualised and distinguished from irrelevant background structures. To introduce the genetic information encoding for fluorescent proteins into relevant cellular targets, molecular tools are required. Viral vectors are one of the best ways of gene delivery into differentiated postnatal brain neurones and glia. Current progress in this field allows targeting of various cell types and therefore makes it possible to express a variety of fluorescent constructs in selected subpopulations of neurones, for example. In this review, we will discuss and compare the properties of the most popular viral gene delivery systems and the advantages of different brain cell preparations to illustrate how they can be used for high-resolution live cell confocal imaging in order to study new aspects of central nervous system (CNS) structure and function.

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1. Introduction

Immunofluorescence has been used extensively to visualise various cellular compartments and the distribution of specific proteins in sections cut from fixed nervous tissue. In neuroscience, it has been used to image the expression of proteins such as receptors, ion channels and enzymes, for example. This technique is powerful but has its limitations as the processing of tissue may introduce artefacts and neuronal structure and function cannot be studied in real time. The discovery of the jellyfish green fluorescent protein and its adaptation for expression in mammalian cells was followed by a whole host of fluorescent variants from this and other progenitors. This has laid the road to development of a wide range of powerful techniques all based on fluorescence detection in living cells. For successful expression of a fluorescent protein in the target cell, be it neurone, glia or a brain blood vessel cell, the relevant gene has to be introduced into the cellular nucleus. Essentially, there are two ways of achieving this goal: germline transgenesis and somatic gene transfer using one of the available vectors such as viruses. While focusing on imaging based on viral vector technology, for comparison purposes, we will also mention some data obtained by germline transgenesis.

A number of transgenic animals (typically mice), where certain cell types express enhanced green fluorescent protein (EGFP) or another fluorescent

protein, have already been produced. Examples of this approach are GAD₆₅-EGFP mice which have been reported to express EGFP in many brain areas almost exclusively in GABAergic neurones [1] or mice in which EGFP has been targeted to the astroglia using the glial fibrillary acidic protein (GFAP) promoter [2]. A major advancement in this area has been made recently by production of multiple lines of transgenic mice using bacterial artificial chromosome (BAC) vectors which permit use of very long sequences (many tens of kilobases) for gene targeting thus making expression more specific [3]. Clearly, having immediate access to tissue where the cellular phenotype of interest is fluorescent is a very attractive route for researchers interested in studying functional properties of these cells. For imaging, however, the transgenic approach has some limitations, largely in connection with the fact that many transgenic lines carry only a single copy of the transgene (cell-specific promoter driving the expression of the fluorescent protein) but in a large number of cells. The first limitation is therefore a fairly low level of EGFP expression in some of these animals. This problem may be overcome using BAC-mediated transgenesis and selecting lines carrying multiple copies of EGFP with sufficient signal-to-noise fluorescent ratio [3], but even these animals show strong variations in the level of EGFP expression [4]. The second problem is that for optimal imaging it is usually desirable to have a few brightly fluorescent objects embedded within essentially non-fluorescent tissue. A high density of overlapping fluorescent objects enor-

mously complicates imaging not only with wide-field systems but also with confocal microscopes. This parameter is very difficult to control in transgenic animals. Indeed, efficient expression of EGFP in all cells of a particular type (for example all pyramidal neurones in hippocampus) would result in a fluorescent “jungle” where imaging of the individual elements is exceedingly difficult. For this purpose, lines expressing transgene only in a minority of cells can be selected. For example, from transgenic mice with *thy1* gene promoter-driven EGFP expression [5], selected lines where only ~1% of cortical pyramidal cells were fluorescent were chosen for imaging purposes [6]. Finally, generating and characterising multiple lines of transgenic animals is a laborious and very expensive process. Alternatively, for many applications, viral transgenesis can be performed at a fraction of the cost with much speed and flexibility. For instance, it is possible to combine several viral vectors to target different cell types in the same specimen or to combine expression of fluorescent proteins with genetic manipulation of signalling pathways in a cell-specific manner.

2. Imaging of central neurones: the choice of preparation

Before discussing the range of available viral vectors, it is important to outline the preparations suitable for imaging in combination with transgenic delivery of fluorescent proteins into the brain. We will consider imaging *in vivo*, in acute and in cultured brain slices as these are the experimental models most suited to viral gene transfer into central neurones. Issues related to cultured brain slices largely apply also to dissociated primary neuronal cultures which are widely used in many laboratories but will not be specifically discussed here.

2.1. *In vivo* fluorescent imaging

In vivo fluorescent imaging can be performed in mice using two-photon confocal systems in acute and chronic experiments [6]. A recent publication shows parvalbumin-positive EGFP-expressing cortical neurones which have been successfully targeted by patch pipettes *in vivo* in BAC-transgenic mice [4]. We are not aware of comparable work using virally mediated

expression of a fluorescent protein, but believe that this would be feasible. However, multi-photon imaging *in vivo* is only available in a few laboratories around the world and is technically very challenging.

2.2. Brain slices

Brain slices provide more user-friendly conditions for imaging and do not require two-photon rigs to resolve submicrometer structures [7]. Slices can be prepared from animals previously injected with a viral vector to express such proteins in a subset of cells [8,9]. Alternatively, acutely prepared slices can be transfected using viral vectors with highly active promoters to achieve detectable levels of EGFP in some cellular populations within hours [10]. Finally, slices can be prepared using transgenic animals expressing fluorescent proteins in certain cellular populations [5]. Some of the technical issues related to the viral transgenesis in slices have been discussed previously [7]. In the context of image quality, the most important point is that of light scattering and absorbance which greatly depends on the age of the animals. In the rat brainstem imaging becomes significantly more difficult after postnatal week 3 due to the dramatic increase in myelination. For sufficient expression of the transgene, viral vectors are delivered by stereotaxic microinjection into a brain structure several days prior to the preparation of slices [9,11]. As stereotaxic surgery is difficult to perform in very young animals (although it can be done, see Ref. [11]) this approach is somewhat limited to experiments on mature animals. High optical density of the mature brain tissue is clearly more of a problem for single photon imaging and restricts the depth at which one can visualise cells without damaging tissue with excessive laser power. Considering these difficulties, the use of transgenic animals for imaging in acute brain slices [5] has some advantage because slices can be prepared from animals of all ages including neonates. Nevertheless, imaging in acute slices prepared from a pre-transfected animal can be very useful for targeting neurones of known physiological identity. For example, Irnaten et al. [8] have retrogradely transfected cardiac parasympathetic neurones by injecting CMV-EGFP adenoviral vector (AVV) into the pericardial sac in P 4–10 rats. Two days later, fluorescent vagal cardioinhibitory neurones were

identified and recorded in acute brainstem slices. We have recently developed means of retrogradely transducing noradrenergic neurones projecting to various parts of the central nervous system (CNS, Fig. 1A),

see also Ref. [12]. This will allow imaging experiments on identified populations of noradrenergic neurones which are important for placing any observation into functional context.

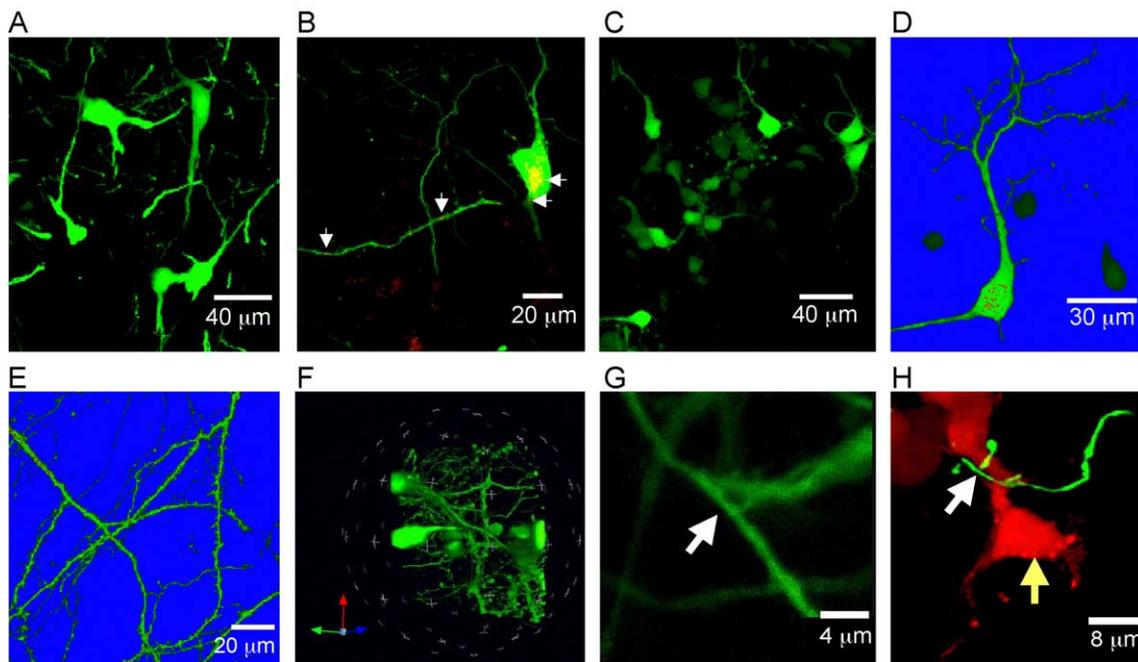


Fig. 1. Examples of application of virally delivered expression of fluorescent constructs in vitro and in vivo. (A) Physiologically distinct populations of noradrenergic neurones can be imaged using retrograde transfection with AVV. In this example, a population of spinally projecting noradrenergic neurones within the locus coeruleus (A6 group) were transfected with AVV PRSx8-EGFP injected into the dorsal horn of the spinal cord in vivo. Seven days later, a distinct cluster of EGFP-expressing A6 neurones could be imaged in brainstem slices. Visualisation of this specific population opens up a way for a number of experiments to clarify their physiological roles (S. Kasparov, A.E. Pickering and A.G. Teschemacher, unpublished observation). (B) A noradrenergic neurone within the brainstem A1 area was transfected using AVV PRSx8-EGFP in a slice co-culture of brainstem and hippocampus. Five days later, a small crystal of a lipophilic membrane dye DiI was applied to the hippocampus. The red “patches” in the cell soma (appear yellow due to superimposition of colours) and the processes of the noradrenergic neurone indicate that it has formed projections into the adjacent hippocampal explant. This approach can be used to identify specific populations of neurones based on both their transmitter phenotype and projection area. (C) A hippocampal slice culture transduced with 5×10^6 TU/ml end-point dilution of LVV containing an EF1 α -EGFP expression cassette. EGFP-expressing cells (neurones and glia) were imaged 7 days following preparation and transduction. (D and E) Slice cultures of hippocampus were transfected with 5×10^6 TU/ml of AVV driving EGFP expression under synapsin promoter control and enhanced by a woodchuck hepatitis virus post-transcriptional regulatory element (Syn1-EGFP-WHE) (a kind gift of Professor J. Uney, University of Bristol). Seven to 10 days later, numerous fluorescent neurones could be identified within C1–C3 areas, some of which had the characteristic morphology of pyramidal cells (D). At higher magnification, dendritic spines could be easily resolved (E). Note the good spatial separation of fluorescent objects that is an essential pre-requisite of high quality imaging. (F) A cluster of noradrenergic neurones with characteristic beaded axons in organotypic slice culture transfected with AVV PRSx8-EGFP. This image is a 3-D reconstruction of the 35- μ m-high confocal stack recorded with a Leica SP confocal microscope and Volocity software (Improvision®). Optimal conditions provided by the AVV-transfected slice cultures allow collection of high density confocal stacks suitable for visualisation of finest details in a 3-D-resolved image. (G and H) Inter-cellular contacts can be visualised using viral expression of fluorescent proteins in slice cultures. In (G), putative GABAergic neurones were transfected to express EGFP using AVV GAD₆₇-EGFP. Tight T-shaped oppositions could be visualised between processes (presumably dendrites in this case) of these neurones. In (H), AVV PRSx8-EGFP was used to express EGFP in noradrenergic neurones, while the second AVV, HCMV-DsRed, caused expression of red fluorescent protein in glial cells. Numerous tight contacts of noradrenergic axons (white arrow) with glial cells (yellow arrow) could be found. These oppositions are consistent with the well-established role of noradrenaline in control of glial function [68–71].

2.3. Cultured organotypic slices

Cultured organotypic slices offer ideal conditions for both viral gene transfer and high-resolution imaging [7]. Slices prepared from any region of the brain from young rodents can be transfected using viral vectors and maintained for weeks providing instant access to various cellular populations that express fluorescent proteins.

2.3.1. Preparation of organotypic slice cultures

The culturing of brain slices has been used since the 1940s by many groups throughout the world each having customised their own protocols. In the original ‘roller tube’ technique, explants are fixed by a plasma clot to a glass substrate and are alternatively exposed to medium and incubating atmosphere by a set schedule [13]. We currently use the ‘static’ way of slice culturing, adapted from Ref. [14]. This method employs commercially available membranes from Millipore™ on which slices are kept stationary at an interface between medium and humidified, carbonated air. We have further developed the technique to satisfy our requirements of slice cultures from various rat brain areas (in particular, hippocampus, hypothalamus and brainstem), which can express fluorescent constructs *in vitro* for several weeks.

Rat pups (p8–p11) are terminally anaesthetised with halothane, quickly immersed in cold 70% ethanol for sterilisation purposes and decapitated. The head is then transferred into a laminar flow hood. Using sterile technique, the skull is opened, the desired brain areas are dissected out and bathed in ice-cold dissection saline [Hanks Balanced Salt solution (HBSS) without Ca^{2+} (Gibco 14175) with added 20 mM glucose (total 25.6 mM), 10 mM MgCl_2 , 1 mM HEPES, 1 mM kynurenic acid, 0.005% phenol red, 100 U/ml penicillin, and 0.1 mg/ml streptomycin]. The tissue block is then fixed onto a Teflon stage of a vibratome (Campden Instruments) using cyanoacrylate adhesive (Permabond). The stage is mounted in the slicing chamber which is filled with cold (4 °C) sterile dissection saline, and 250- μm -thick slices are cut while supporting the tissue block with a fine, soft brush. Slices are transferred into a Petri dish with fresh dissection saline using a sterile ‘slice pipette’ (Fig. 2A) and kept covered on ice for 1 h in the hood. Millicell®-CM organotypic culture inserts (Millipore™) are placed in six-well culturing plates (Fig. 2B) and moistened by the addition of 1.05 ml plating medium [50% Optimem-1 (Gibco 31985), 25% fetal bovine serum, 21.5% HBSS; 2.5% 1M glucose, and 1% Penicillin and streptomycin 100 \times (sigma P-0781)] to the side of each well. Two or three slices are deposited on each insert and the excess saline is aspirated from

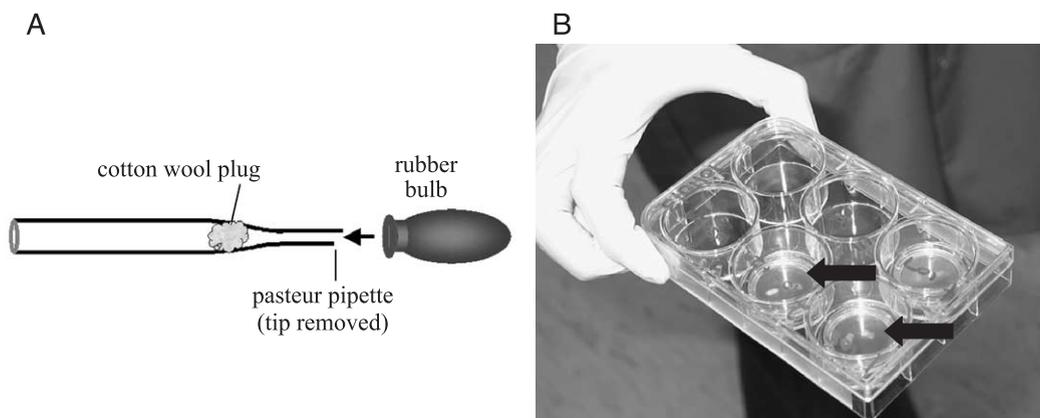


Fig. 2. (A) Glass ‘slice pipettes’ for slice handling are constructed from glass Pasteur pipettes the tips of which are shortened. Tapers are plugged with cotton wool; pipettes are singly wrapped in aluminium foil and autoclaved. To transfer slices, a rubber ball is attached to the remainder of the tip and slices are gently manipulated by suction through the large, fire-polished opening. (B) Slice cultures plated on the Millicell® membranes are kept in six-well plates. Several cultures may be plated on the same membrane (black arrows). The same principle may be used for co-culturing of explants from different brain areas. For imaging, a section of the membrane with the slice culture can be cut out of the membrane with a pointed scalpel blade.

the membrane surface. Cultures are incubated at 37 °C in a 5% CO₂ atmosphere. After 3 days, the plating medium is exchanged for Neurobasal culturing medium [94.5% Neurobasal (Gibco 21103), 2% B-27 media supplement (Gibco 17504), 1% L-glutamine (sigma G-7513), 2.5% 1 M glucose, and optionally 1% Penicillin and streptomycin 100× (sigma P-0781)] which is subsequently replaced twice a week. Sterile technique is crucial throughout the preparation and maintenance of cultures. The vibratome is kept in a designated clean area. All liquids are purchased sterile or are sterile-filtered. Surgical instruments used for dissection, including blades, brushes, 'slice pipettes', glassware and the vibratome cutting chamber, are autoclaved, whereas non-autoclavable items are treated with UV light for 20 min. We do not irradiate slices or treat with antimetabolites [1].

2.3.2. *Imaging of slice cultures*

Cultured slices flatten over time, but the overall position of nuclear groups remains very similar to that found in an acute slice. The preparation is particularly flexible for imaging purposes as the membranes used to support the cultures are optically transparent while wet, and the tight layer of glial cells which covers the culture surface after a few days in vitro does not impede high-resolution confocal imaging. Furthermore, the absence of a plasma clot [13] allows experimental manipulation at any time after plating. For imaging, a section of the Millicell® membrane with a slice culture is excised using a pointed scalpel blade and transferred into the recording chamber where it is perfused with oxygenated bicarbonate-buffered artificial cerebro-spinal fluid, as in Ref. [15]. The chamber has a glass bottom and allows combination of incident light fluorescent imaging (including confocal) and transmitted light differential interference contrast optics.

2.3.3. *Viral transduction of slice cultures*

Preparation of high-quality slices is essential for achieving optimal viral transduction. It appears that suboptimal slice preparations contain a mass of degrading neurones on their surface, which drastically reduces the efficiency of viral transduction, besides obstructing imaging. Slice cultures prepared and maintained using the protocol described above are viable and routinely contain numerous fluorescent cells for more than 3 weeks. The method of admin-

istration of viral vectors is also critical for successful transduction in cultured slices. Ehrenguber et al. [16] used hippocampal slices cultured according to the roller-tube technique and injected high titre suspension of various viral vectors into approximately 10 sites in the CA1 and CA3 regions for successful transgene delivery and expression [16]. We have evaluated a variety of protocols for viral transduction in 'static' slice cultures and concluded that exposure to a viral vector needs to take place during the initial culturing stages, before a glial layer surrounds the slice. Two protocols have been employed successfully. Slices are either incubated with adenoviral suspension in the dissection medium (end-point dilution 10⁷–10⁹ transducing units (TU) per ml) for 1 h at 4 °C and rinsed in plating medium prior to plating or, alternatively, viral suspension is added to the plating medium directly (10⁷–10⁹ TU/ml) immediately before plating. When vectors were used to express fluorescent constructs, the first fluorescent cells could be detected as early as 24 h after the transfection. However, we usually image preparations from 6 to 7 days in vitro onward, by which time expression has reached a plateau with fluorescent cells appearing close to the surface of the slice culture due to its flattening. As the vectors are not cytotoxic, it is possible to combine viral gene expression with conventional vital fluorescent techniques such as DiI membrane staining or fluorescent beads to retrogradely label neuronal cell bodies (Fig. 1B).

3. Viral vectors for imaging applications

The success of viral gene delivery experiments depends primarily on two factors: (i) the ability of the viral vector to deliver its genome into the target cell and (ii) the activity of the promoter used for transgene expression. In addition, viral invasion of a cell may or may not lead to cytotoxicity resulting in cell death, which may put the validity of any observations in question. Several types of viral vectors derived from various genomes are currently in use. We will review and compare the features of AVVs and LVVs that have proven particularly amenable to imaging studies in our hands. Further, adeno-associated virus-derived vectors (AAVVs) and alphavirus genus vectors will be briefly discussed.

3.1. AVVs and LVVs

AVVs hold DNA genomes which remain episomal in the host cell nucleus, while lentiviruses, such as HIV, are RNA-containing viruses which insert their genomes into the host chromosomes following reverse transcription. In the context of this review, the most important differences between these two vectors are their capacity, the structure of the viral particle and the method of their production.

3.1.1. Capacity

The capacity is one of the critical characteristics of any vector. The AVVs which have been used in our laboratories over the last few years [7,10,12,17,18] have major deletions in the E1 region of their genome [19,20]. This makes them replication-incompetent and they can in theory accommodate approximately 7 kb of a transgene, which is enough space for a sufficiently long promoter and 1–2 genes or even two different expression cassettes [21]. We have successfully inserted up to about 5 kb of transgene (AVV GAD67-EGFP, see below). The HIV-derived LVVs we use retain only a small fraction of the viral genome [22,23] and can, in principle, accommodate 11–12 kb. However, such theoretical limits may be very hard to reach in practice. Nevertheless, these differences in size limitation are important when cell-specific expression is desired because currently the most common route to achieve cell specificity lies through use of long promoter/enhancer sequences [24].

3.1.2. Structure

The structure of AVV particles is very different to LVVs in that they are non-enveloped and their ability to invade cells depends entirely on the proteins present in their fibres and capsid. It has been demonstrated that the serotype affects the neuronal tropism of AVVs and vectors with the Ad17 fibre may be best for targeting neurones [25]. Nevertheless, most of the currently used AVVs are derivatives of serotype 5 adenoviruses. Adenoviral fibres interact with a range of proteins present on the membranes of many cells such as major histocompatibility complex molecules and the coxsackievirus–adenovirus receptor. Modifications of the AVV fibre protein may

change vector tropism which can be used for targeting transgene expression to certain cell types (for review, see Ref. [26]). With this approach, Omori et al. [27] have re-directed an AVV to microglia (which lack the coxsackievirus–adenovirus receptor) by incorporating an Arg–Gly–Asp motif containing peptide into the HI loop of the fibre knob. This motif enables interaction with α V integrins, ubiquitously expressed on the surface of mammalian cells. The recent identification of the platelet-derived growth factor (PDGF) receptor as the receptor responsible for transduction by AAVs [28] might give clues for further improvements in neuronal targeting.

In contrast to AVVs, LVV virions have a coat, or envelope, which is associated with the capsid but is constituted of a separate protein encoded by a separate gene. The envelope interacts with the cellular membrane and therefore modifications in its structure can alter the tropism of the vector. Most vectors used currently, irrespectively of the genome they have been derived from (i.e., HIV or feline immunodeficiency virus), are pseudotyped and employ a foreign protein coat such as the one from vesicular stomatitis virus, VS glycoprotein G (VSVG) [22,23,29]. VSVG enables LVVs to deliver transgenes to a wide range of cells including neurones and glia [22]. The envelope clearly affects the LVV properties. For example, VSVG-pseudotyped vectors seem to have negligible ability for retrograde transfection in nervous tissue [29] (Kasparov, Paton, Huentelman and Raizada, unpublished observation). However, a rabies-G envelope provided the ability for retrograde axonal transfection to the equine infectious anaemia virus [30]. Other envelopes confer LVV tropism to different cellular targets such as epithelial tissues [31].

3.1.3. Production

Of all vector systems mentioned in this review, AVV takes the longest to make (several months), while a batch of LVV, AAVV or alphaviruses can be prepared in less than a week. However, for AVVs and LVVs, speed and yield of production are inversely proportional. Typically, a single round of AVV production yields 2.5 ml of viral suspension of roughly 10^{10} TU/ml. In contrast, production of large quantities of LVV remains difficult and expensive. Using improved protocols [22] and starting from a

comparable amount of helper cells, we obtain $\sim 25 \mu\text{l}$ of LVV particles at about 10^{10} TU/ml.

Yield becomes an issue if a considerable amount of vector particles is required for an extensive series of imaging experiments. This will be even more significant if only a subset of neurones has to be targeted, because the probability of successfully transducing this particular phenotype is directly proportional to the number of available targets per preparation. In our experiments, we typically use 10^7 – 10^8 TU/ml medium for slice cultures and $0.5 \mu\text{l}$ of 10^9 – 5×10^9 TU/ml for each stereotaxic injection. Fig. 1C shows a hippocampal slice culture transduced with a LVV inducing EGFP expression driven by the non-selective elongation factor 1α (EF1 α) promoter.

3.2. *Alphaviruses*

So far, the majority of studies where viruses were employed to express fluorescent proteins in nervous tissue were using alphavirus genus vectors: Sindbis Virus (SV) and Semliki Forest Virus (SFV). Alphaviruses are closely related RNA-containing enveloped viruses originally obtained from African mosquitoes. The total size of their genomes is ~ 12 kb and of that up to ~ 6.5 kb can be used for placement of transgenes [32]. Their production is relatively rapid using commercially available kits and they have been reported to preferentially target neurones. The transgene expression is controlled by the sequences provided by the SFV and SV viral genomes. As with other viral vectors, SFV and SV used for transgene delivery lack genes encoding for structural proteins, which prevents formation of intact viral particles in transduced cells. However, in contrast to all other vectors mentioned later in this review, alphaviral vector genomes *do* replicate in the invaded cell and this requires the presence of the replicase complex part of the viral genome. Consequently, each viral transduction event leads to formation of a large number of templates for protein synthesis. In primary neurones and brain slice cultures both SFV and SV lead to a rapid expression of transgenes within 4 to 6 h [32] which is slightly faster than that of AVV (>6 h) [10]. Alphaviruses hijack the cellular protein synthesis machinery and are well known to cause rapid apoptosis in transduced cells leaving a very short time window for experimentation before obvious

signs of deterioration develop [33,34]. Typically, recordings from cells transduced with these vectors were made between 12 and 24 h after application of the virus. Recently, new versions of both SFV and SV have been developed which bear mutations in their replicase complex to decelerate their proliferation, prolonging the life-span of transfected neurones for up to 7 days [33,34]. According to Lundstrom et al. [34], the titres obtainable using the modified SFV are lower than with the wild-type virus. Altogether, it is likely that alphaviruses with mutated replicase complexes will prove to be less toxic at the price of lower expression efficiency. Interestingly, mutants of the SFV have been constructed which change their cellular tropism dependent on the temperature of incubation: at 37°C , one such mutant induced expression nearly exclusively in glia, while at 31°C , the majority of expression occurred in neurones [35].

In spite of the known ability of alphaviruses to rapidly kill neurones and the lack of targeting potential, the ease and speed of production of vectors have attracted a number of groups who use them for rapid expression of fluorescent constructs in nerve cells. For example, the hippocampus of adult rats was injected with SV for EGFP expression; 18 h later, acute hippocampal slices were prepared and fluorescent neurones in the CA1 field were analyzed optically and electrophysiologically [36]. The authors found that the transfected slices still retained characteristic electrophysiological responses such as long-term potentiation. They used two-photon imaging to visualize fine processes and dendritic spines of pyramidal cells which was facilitated by expressing EGFP-actin fusion protein. In a study of the role of trafficking and assembly of metabotropic GABA_B receptors, SFV was applied to express a fluorescent fusion protein of activating transcription factor 4 in primary dissociated hippocampal cultures [37]. Cultures were prepared from 1-day-old rat pups, maintained for 14 days and then incubated in a viral suspension for 2 h. Transfected cells were imaged 12–14 h later and exhibited punctate fluorescence which co-localised with GABA_BR1 immunostaining [37]. SFV has also been employed to express tagged GABA_A receptor subunits in superior cervical ganglion cells [38]. In this study, measurements were made 18 h after incubation with the virus.

The majority of studies employing SV to express fluorescent fusion proteins originate from Malinow's group. For example, SV-driving expression of fluorescent AMPA receptor subunits, such as GluR1-EGFP, was injected into the barrel cortex in P12 rats and acute slices were prepared 2 days later [11]. Transfected neurones could be visualised with fluorescence microscopy and their electrophysiological characteristics were analysed in order to evaluate activity-dependent recruitment of GluR1 subunits to synapses. Expression of EGFP alone was reported not to alter neuronal behaviour. SV was also utilised by that group to express fluorescent fusions of Ras and Rap proteins in hippocampal slices from 6- to 7-day-old rats which were incubated for 15 h and then studied electrophysiologically [39]. In a recent study, SV with reduced cytotoxicity was employed to express fluorescent fusions of a zinc finger transcription factor Zic2 in explants of embryonic retina. Eighteen to twenty-four hours after transduction with EGFP alone, the outgrowth of neurites was preserved, but it was inhibited by Zic2 expression [40].

It is worth noting, however, that with the exceptions mentioned above, most studies used alphaviruses to express fluorescent proteins simply as markers of successful transduction, rather than for imaging fine morphological details of live cells or for investigating functional aspects of signalling mechanisms.

3.3. AAVVs

Adeno-associated viruses are small non-enveloped particles containing a single strand of DNA. Their wild-type genomes are <5 kb in length, and, in AAVVs, these are almost completely deleted with only short inverted terminal repeats remaining which are required for packaging and integration [41,42]. These vectors are considered to be rather safe, as they are not associated with any human disease. They lead to very long-lasting expression in neurones without any signs of cell deterioration. AAVVs were shown to have natural tropism to neurones via their recently identified entry pathway, the platelet-derived growth factor (PDGF) receptor [28]. AAVVs are produced by a cell line co-transfected with one shuttle and two helper plasmids. For this reason, production of significant quantities of pure viral stock remains a

problem. To date, AAVVs have been used largely in studies related to *in vivo* protein expression for gene therapy [43–47], but in spite of their comparatively small transgene capacity, they certainly hold promise as non-cytotoxic vectors for imaging applications.

4. Promoters for targeted gene expression in brain tissue

Imaging of selected cellular populations and multi-colour imaging of several cellular phenotypes opens new perspectives for studies of brain functions. This may be achieved by using targeted viral gene expression. Once the transgene has been delivered into the target cell, its expression level will depend on the activity of the promoter elements incorporated into the vector.

4.1. Constitutive and pan-neuronal promoters

Human cytomegalovirus (HCMV) immediate-early promoter has been extensively used to drive expression of viral transgenes since the pioneering studies of Wilkinson and Akrigg [48]. Although several versions of HCMV exist, most laboratories use the ~600-bp-long HCMV sequence, which is also commonly found in commercially available plasmids. HCMV is often referred to as “constitutively active” [48] due to the wide range of response elements and enhancers such as calcium/cAMP response elements (CRE), NF- κ B binding sites, an AP-1 site, a serum-response element, etc. Although the HCMV promoter was thought 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). It is likely that a high activity of the CREB pathway is essential for HCMV promoter function [48]. Cultured sympathetic ganglion neurones and hippocampal neurones transfected with HCMV-EGFP AVV did not express EGFP unless exposed to 40 mM K⁺ [49]. The authors argue that depolarisation opens voltage (and nifedipine)-sensitive Ca²⁺ channels and this leads to activation of the CREB pathway. Mutation of all five CREs abolished this induction. This is consistent with our observations where Rp-cAMPs, which blocks a number of cAMP-dependent

cascades including protein kinase A, significantly attenuated EGFP expression driven by HCMV-EGFP AVV in acute brain slices [10]. It is therefore possible that HCMV-driven expression may be discriminative in favour of some cell types where this cascade is highly active for some reason [12]. This might also play a role in the well-documented ability of HCMV-based AVV constructs to drive high levels of expression in glia *in vivo* [50] and in acute brain slices [10]. In slice cultures, which contain numerous glial cells on their surface, high levels of expression in glia is particularly noticeable and highly undesirable. In addition, some neuronal phenotypes, such as brainstem noradrenergic neurones, do not express any measurable amounts of EGFP when exposed to HCMV-EGFP AVV in spite of the fact that neurones in nearby cholinergic motor nuclei express high levels of EGFP when exposed to the same vector [12]. Finally, in hippocampal neurones, HCMV appears to be less suitable for long-term expression than the synapsin promoter [51].

Recent publications suggest that the lack of HCMV-driven expression in many neuronal phenotypes might only apply to AVV-based constructs. HCMV-EGFP AVV mainly transduced glial cells, while HCMV-EGFP AAVV successfully transduced nerve cells such as pyramidal neurones [16,50]. Also, an AVV designed for EGFP expression driven by the neurone-specific enolase (NSE) promoter resulted in a significant proportion of EGFP-positive glial cells, despite the neuronal profile of that promoter [50]. In contrast, when a VSVG-coated HIV-derived LVV containing the HCMV-EGFP cassette was injected into the rat striatum, 82% of cells were double labelled for NeuN, a widely used neuronal marker [52]. These observations raise an interesting possibility that the adenoviral backbone might play a key role in restricting HCMV-driven expression to some neuronal phenotypes such as cholinergic neurones, selectively. There is, however, an alternative explanation. Jakobsson et al. [52] evaluated their constructs in the striatum, which contains a high density of cholinergic neurones. It is thus possible that the high ratio of neurones transduced in that study using HCMV within LVV backbone is simply a reflection of the high activity of HCMV in cholinergic neurones [53]. Thus, the issue of HCMV tropism requires careful re-evaluation before this promoter is used in different brain areas.

The EF1 α and β -actin promoters are also commonly used to drive expression in the brain in a non-discriminative way. EF1 α has been used by a number of authors including ourselves [18,22,52,54,]. The levels of EGFP expression in transfected brain cells (many of which looked neuronal or stained for neuronal markers) were fairly high although they seem to be somewhat lower than those achieved with HCMV. The activity of the human β -actin promoter within the retroviral backbone has been documented for Schwann cells [55,56], as well as for astroglial and unidentified neurones in primary cultures [57].

Some promoters, such as the NSE promoter, synapsin-1 promoter and human PDGF β -chain promoter with CMV enhancer [58], have been used for “pan-neuronal” targeting (i.e., non-discriminative expression in all kind of neurones but not other cell types). In primary cultures transfected with plasmid DNA using lipofection, the NSE promoter resulted in GFP expression primarily in neurones [59]. In contrast, AVV-delivered NSE promoter-driving expression of EGFP provided only preferential expression in neurones while ~30% of the EGFP-positive cells did not stain for the chosen neuronal marker NeuN suggestive of a “leak” into glia in the striatum [50]. NSE was equally effective when compared to the synapsin promoter in cortical neurones [50]. Several authors have recently used the synapsin promoter in viral vectors to target neurones. A sequence of only ~500 bp is sufficient for highly neurone-selective targeting with AVV in the hippocampus [60,61] and striatum [50]. Apparently, the activity of the synapsin promoter is fairly low, but the expression can be enhanced using the woodchuck hepatitis virus post-transcriptional regulatory element [62]. It has been reported that this AVV remains transcriptionally active in hippocampal neurones for as long as 9 months [51]. AVVs with synapsin promoter provide an excellent tool for imaging of both spiny and non-spiny neurones in the hippocampus. Indeed, transduction of slice cultures with this construct makes visualisation of the finest dendritic spines a trivial task (Fig. 1D and E).

It is important to stress that the cellular tropism and the expression profile conferred by various promoters mentioned above applies to studies where these promoters were used in the AVV backbone, but does not necessarily match the results obtained using

similar sequences in other viral vector types such as the AAVV. A striking example was the use of 2.2 kb of the GFAP promoter in an AVV backbone which it targeted mainly, if not exclusively, glial cells [60] while the same promoter appeared to confer neuronal expression when delivered by an AAVV [63]. Two factors might be responsible for these discrepancies: first, the terminal repeats present in AAVV might have acted as promoters/enhancers and have altered the transcriptional regulation of the GFAP promoter; second, the AAVV discriminated for neurones vs. glia due to their internalisation mechanism which requires PDGF receptors that are abundant on neurones [28]. This might lead to a very high number of viral genomes per neurone and consecutive loss of promoter specificity.

4.2. Phenotype-specific neuronal promoters

With an interest in our laboratories as to the function and plasticity of central noradrenergic and GABAergic neuronal networks in cardiovascular regulation, we have: (i) generated an AVV PRSx8-EGFP using a recently developed artificial promoter PRSx8 [21] and evaluated it as a means for confining transgene expression to noradrenergic neurones and (ii) generated AVV GAD67-EGFP, a novel AVV for targeting GABAergic neurones based on the available information about the promoter of the glutamate decarboxylase (GAD67), the GABA-synthesising enzyme [64].

4.2.1. PRSx8

PRSx8 is an artificial promoter which consists of eight 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 [21]. PRSx8 is both very short and a powerful promoter with a predictable mechanism of transcriptional control and may be expected to operate in any cell that expresses Phox2a/2b. Recently, we performed a number of experiments with PRSx8-EGFP AVV in order to assess its suitability for in vivo and in vitro targeting of noradrenergic neurones.

In acute slices following in vivo injection, PRSx8-EGFP AVV led to high levels of EGFP expression in all tested noradrenergic and adrenergic cell groups (A1, A2, A6, C1). According to the immunocytochemical marker DBH, PRSx8-EGFP was ~100% selective for noradrenergic neurones in locus coeruleus (A6) but in some areas where noradrenergic neurones are in close proximity to cholinergic nuclei (such as in the dorsal vagal complex), many of the transduced neurones appeared to be DBH-negative [12] due to expression in cholinergic motor neurones. This can be explained by the phylogenetic relationship between noradrenergic and cholinergic neurones since some cholinergic neurones in the autonomic nervous system also express Phox2a/2b [65,66]. However, we have found that noradrenergic neurones can be selectively transduced retrogradely by injecting AVV PRSx8-EGFP into their projection areas. For example, PRSx8-EGFP AVV injection in the hypothalamus retrogradely labelled A2 noradrenergic neurones whereas spinal injections transduced A6 neurones (Fig. 1A). This opens an exciting opportunity to directly compare various properties of different populations of noradrenergic neurones by combining EGFP-based fluorescent signalling probes.

Transduction in vitro of slice culture offers better chances for cell-specific transduction because the titre of the virus can be controlled much better. In these experiments, PRSx8-EGFP AVV reliably caused expression in areas known to correspond to the catecholaminergic cell groups and distinct neurones with characteristic beaded axons which could be traced for hundreds of micrometers were readily identified using both conventional fluorescent and confocal microscopy (Fig. 1B and F). These cells persist in slice cultures for several weeks and can be imaged with superb resolution. Release of noradrenaline from the varicosities of these neurones recorded using microamperometry indicates that the cells are viable and retain their neurochemical phenotype in vitro [12].

4.2.2. GAD67

A relatively short (~1 kb) part of the GAD67 promoter has been reported to express EGFP in some subpopulations of hippocampal GABAergic neurones in a transgenic mouse line [67]. Longer sequences (~10 kb) introduced using a gene gun resulted in

specific expression in GABAergic neocortical neurones [24]. 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 the GAD67 promoter to drive EGFP and evaluated its expression in several brain areas including hippocampus and the dorsal vagal complex. The majority of EGFP-positive cells (>90% in the brainstem) were also immunopositive for GABA. Given the inherent difficulty in immunocytochemical identification of GABAergic neurones, we believe that this AVV is quite selective for GABAergic neurones, at least in some areas of the brain. Application of the GAD67-EGFP AVV in hippocampal slice culture resulted in moderate levels of EGFP expression which was, nevertheless, sufficient to visualise not only the somata of the putative GABAergic neurones but also their fine processes and inter-cellular contacts (Fig. 1G).

In conclusion, it is feasible to target selected cellular phenotypes using viral vectors with cell-specific promoters. Given that more than one vector can be used at the same time (Fig. 1H) and that the vectors can be directly applied in slice cultures prepared from animal models of human diseases (for example, the spontaneously hypertensive rat), the combination of targeted viral transgenesis with high-resolution imaging is an experimental strategy with much potential.

5. Conclusions

Viral vectors provide a highly efficient means of transgene expression in essentially any cell type of interest. There are several available viral gene expression systems which vary greatly in their cellular tropism, targeting potential, toxicity to the transduced cells, their speed and yield of production. In the context of imaging, alphaviruses have so far been employed most extensively to confirm successful transduction by fluorescent labelling. At the same time, the bulk of imaging with fluorescent proteins was performed in cell lines and other heterologous systems using transient transfection protocols such as Ca²⁺ phosphate, liposome-based carriers or DNA injection. It seems that the rapid cell death triggered by alphaviruses has somewhat limited the applicabil-

ity of viral gene transfer for imaging neuronal structure and function. From that perspective, AVVs and LVVs, although more laborious to make, offer an alternative way of more long-lasting, non-cytotoxic gene expression which should help to obtain valuable and physiologically relevant data. As discussed above, these vectors can be directed to different cellular phenotypes and used *in vivo* and *in vitro* for imaging both structure and signalling pathways in the living brain tissue.

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