Experimental Physiology

Dynamics of a transgene expression in acute rat brain slices transfected with adenoviral vectors

C. E. L. Stokes, D. Murphy *, J. F. R. Paton and S. Kasparov †

*Department of Physiology, School of Medical Sciences, University of Bristol and * Molecular Neuroendocrinology Research Group, University Research Centre for Neuroendocrinology, University of Bristol, Bristol, UK*

(Manuscript received 14 January 2003; accepted 11 March 2003)

We present a quantitative account of the expression dynamics of a transgene (enhanced green fluorescent protein, EGFP) in acute brain slices transfected with an adenoviral vector (AVV) under control of the human cytomegalovirus (HCMV) promoter. Micromolar concentrations of EGFP could be detected in brainstem and hippocampal slices as early as 7 h after *in vitro* transfection with a viral titre of 4.4×10^9 plaque-forming units (pfu) ml^{-1}. Although initially EGFP appeared mainly in glia, it could be detected in neurones with longer incubation times of 10–12 h. However, fluorescence was never detected within some populations of neurones, such as hippocampal pyramidal cells, or within the hypoglossal motor nucleus. The density of cells expressing EGFP peaked at 10 h and then decreased, possibly suggesting that high concentrations of EGFP are toxic. The age of the animal significantly affected the speed of EGFP accumulation: after 10 h of incubation in 30-day-old rats only 4.88 ± 0.51 cells/10 000 μm^2 were fluorescent compared to 7.28 ± 0.39 cells/10 000 μm^2 in 12-day-old rats ($P < 0.05$). HCMV promoter-driven transgene expression depended on the activity of protein kinase A, and was depressed with a cAMP/protein kinase A antagonist (20 μM Rp-cAMPS; $P < 0.0005$). This indicates that expression of HCMV-driven constructs is likely to be skewed towards cellular populations where cAMP-dependent signalling pathways are active. We conclude that acute transfection of brain slices with AVVs within hours causes EGFP expression in micromolar concentrations and that such transfected cells may remain viable for use in physiological experiments. *Experimental Physiology* (2003) **88.4**, 459–466.

The adenoviral vectors (AVVs) have been increasingly used for gene transfer in physiological research since pioneering studies in 1993 (Akli *et al.* 1993; Davidson *et al.* 1993; Le Gal *et al.* 1993). However, despite extensive application of AVVs for gene delivery in numerous cellular systems (Griesbeck *et al.* 1997) knowledge regarding influential factors of adenoviral-mediated gene expression (such as preferential transfection of some cell types) is mainly based on these early reports. A variety of transgenes have been expressed *in vivo* and *in vitro*. Such genes can act as reporters, for example enhanced green fluorescence protein (EGFP; Smith *et al.* 2000; Ehrengruber *et al.* 2001), or influence physiological activity, such as dominant/negative transgenes (Kantor *et al.* 1996; Paton *et al.* 2001). In both cases it is essential for the transgene to reach sufficient concentrations: for a reporter it must be detectable and for a dominant/negative it must block the bulk of its target.

AVV gene manipulation in acute slices would be most attractive because these are commonly and easily prepared in many laboratories. However, in this system the dynamics of transgene concentration is critical because such slices have a limited lifespan, and in most cases progressively deteriorate after 12–24 h (depending on the brain region and age of the animal). Hence, questions arise as to whether: (i) physiologically relevant concentrations of a transgene could be reached during the time when brain slices are still viable; (ii) AVVs in acutely transfected slices target all cell types or is there a preference for neurones of a particular type and/or glia; (iii) there is a difference in AVV-mediated gene expression between different regions of the brain since previous studies have concentrated nearly exclusively on the hippocampus; and finally (iv) which factors affect transgene accumulation?

The human cytomegalovirus (HCMV) immediate-early promoter has been extensively used to drive the expression of transgenes. This promoter is often referred to as 'constitutively active', a reflection of the plethora of response elements and enhancer sequences, including the calcium/cAMP response element (CRE). Although this

† Corresponding author: sergey.kasparov@bris.ac.uk

promoter is expected to be active in most cell types in the brain, it remains unclear whether its function is affected by the transcriptional activity of the host cell, in particular the activity of the CRE binding protein (CREB) pathway. If this pathway is essential for HCMV promoter function the acute transgene expression may be discriminative in favour of certain cell types where this cascade is highly active.

In this study we have attempted to quantitatively analyse the dynamics of transgene expression in acute brain slices prepared from two regions of the rat brain (i) at the level of the nucleus of the solitary tract (NTS) and (ii) the hippocampus. Using a confocal microscope to provide a direct measure of its concentration in individual living neurones, we sought to determine which factors affect the dynamics of EGFP accumulation (Stokes *et al.* 2002).

METHODS

Adenoviral constructs

A human type-5 AVV (with deletions in E1A part of the genome) encoding enhanced green fluorescent protein (EGFP) under control of the HCMV promoter was used (AdHCMV-EGFP; a gift from Professor James Uney, University of Bristol).

Preparation of brain slices

Male 12- or 30-day-old Wistar rats (bred in-house) were deeply anaesthetised with halothane and killed by decapitation. All experiments were performed in accordance with the UK Animals (Scientific Procedures) Act 1986. The brain was rapidly removed from the skull and immersed in cold (4 °C) oxygenated (95 % O_2–5 % CO_2) artificial cerebrospinal fluid (ACSF) containing (mM): dextrose 10, NaCl 125, $NaHCO_3$ 24, KCl 5, $CaCl_2$ 2.5, $MgSO_4$ 1.25 and KH_2PO_4 1.25. Slices (200–250 μm) were taken through the brain at the level of the NTS (from −500 μm to +200 μm relative to obex) or through the hippocampus. Slices were stored on a mesh in a beaker containing oxygenated ACSF until being transferred to a container with 3 ml of ACSF containing the AVV described above, at a final concentration of 4.4×10^9 pfu ml^{-1}. Following 1 h incubation at 36 °C, slices were transferred to a beaker containing 60 ml ACSF and left at 36 °C for incubation periods of 6, 9 and 11 h. The titre of the virus and the duration of exposure to the virus were adjusted in preliminary experiments which established that lower viral titres generally give weaker expression while higher titres become uneconomical and also may lead to severe over-expression in a large proportion of neurones. Additionally limiting exposure to high-titre virus was necessary to be able to draw conclusions as to when the process of expression actually begins. In some experiments a cAMP/protein kinase A (PKA) blocker, Rp-adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMPS; Sigma), was added to the 60 ml ACSF at a final concentration of 20 μM (Shew *et al.* 2000; Sun *et al.* 2001).

Confocal microscopy

A spectral scanning confocal microscope (Leica TCS SP, Leica, Heidelberg, Germany) was used, with a ×40 water immersion lens. Excitation was achieved using a 488 nm argon laser. EGFP fluorescence was detected within a 490 nm to 540 nm bandwidth set by the SP system. The sensitivity of the photomultipliers, the zoom and the pinhole values were maintained constant throughout the study. In brainstem slices, all sub-nuclei of the NTS were included. In hippocampal slices, all areas were measured and the data pooled.

Quantitative measurements of EGFP concentrations

Recombinant EGFP (Clontech, UK) was used as a standard for calibration. Dilutions of 1 μM, 500 nM and 250 nM were made using the storage buffer 0.01 M EDTA and loaded into a fine capillary tube, the inner volume of which was imaged using the acquisition parameters described above. Averaged intensity values were used to construct an EGFP calibration curve.

In AVV-transfected acutely prepared slices, stacks of confocal images were restricted to the upper 30 μm to minimise error relating to absorption of light in tissue. The maximal level of fluorescence throughout the *z*-plane of the soma of a cell was recorded. It is acknowledged that there might be factors that influence the EGFP fluorescence in living cells as compared to the solutions of the recombinant EGFP and therefore the concentrations measured by us in neurones might not be very precise. Nevertheless as the conditions remained the same between various experiments described here, comparisons between them are valid.

DAPI nuclear stain and cell counting

To approximate the percentage of AVV-transfected cells within NTS, it was necessary to determine the total number of cells within the same volume of brain tissue as used for counting EGFP-expressing cells. To this end the brain of a P12 rat was fixed in 4 % paraformaldehyde and 60 μm sections were mounted onto glass slides using mounting media containing DAPI (Vector Laboratories). The upper 30 μm of the slices (same as used to visualise EGFP-expressing cells) were imaged using a confocal microscope at the same magnification as in cell-counting experiments. DAPI-stained nuclei (this included both glial and neuronal cells) were imaged using 360 nm excitation and counted in five different regions within the NTS of three slices. This number was averaged to obtain an estimate of cell density per 10 000 μm^2 of slice.

Ca^{2+} and time-lapse imaging in transfected neurones

After 11 h of incubation some cells were loaded for 1 h with the membrane-permeable red-shifted Ca^{2+} indicator Calcium Orange AM (CO; Molecular Probes). CO (50 μg) was dissolved in 3 ml ACSF containing 10 μl DMSO (Sigma), 7 μl pluronic acid (Sigma) and 3 mg bovine serum albumin (Sigma). The cells were then imaged using the confocal microscope. The CO emission spectrum could be separated from EGFP using the spectral facility of the Leica SP system which was used to measure fluorescence intensity. Cells containing both EGFP and CO were imaged in time-lapse mode and challenged with 30 mM KCl or 20 μM noradrenaline applied to the bath for 30 or 40 s. In some cases the response to both drugs was tested on the same cell after a 20 min washout period.

RESULTS

The following data were obtained from brainstem slices taken through NTS of P12 rats unless stated otherwise.

Dynamics of EGFP expression

As early as 7 h after transfection, EGFP could be observed in some cells in micromolar concentrations (Fig. 1*B*). The mean density of fluorescent cells/10 000 μm^2 peaked at 10 h when it reached 7.28 ± 0.39 cells. This was equivalent to ~7.5 % of cells in this region, as detected by DAPI nuclear stain (note that in both cases only the upper 30 μm of the slice were taken into account). It is interesting that

after 12 h cell numbers were slightly decreased ($P < 0.01$; Mann-Whitney *U* test, Fig. 1*A*).

A graph showing EGFP concentration distribution in transfected cells (Fig. 1*B*) revealed that although at 7 h 55 % of all fluorescent cells expressed EGFP at concentrations $\leqslant 1$ μM there was a second small group of cells containing 4–5 μM EGFP. After a 10 h incubation period these peaks appeared skewed towards increased concentrations and encompassed a broader range of concentrations. Again, the first peak occurred between 1 and 2 μM representing a large proportion (28 %) of cells but there was also a second peak at concentrations greater than 10 μM. Although the distribution of fluorescence was more evenly spread, three peaks were evident at 12 h. The first peak was established between concentrations of 3 and 4 μM and a second between 7 and 8 μM; a further peak emerged for cells with an EGFP concentration greater than 10 μM. The size of the cell soma did not correlate with the concentration of EGFP at any time point (correlation coefficient: at 7 h, 0.02; at 10 h, 0.04; at 12 h, 8×10^{-5}; Fig. 1*C*).

Determining the phenotype of transfected cells

Most (although not all) of the cells, which first became visible after 7 h incubation were of glial appearance. It is generally agreed that the characteristics of astroglial cells are their motility and small cell soma size (< 10 μm). Figure 2*A* shows a sequence of images taken through a single plane of one such cell. It can be seen that within seconds filopodia appear and disappear indicating movement into and out of view. Figure 2*B* shows a high-power image of another glial cell after 7 h incubation, where the soma diameter is $\leqslant 10$ μm and there are many randomly orientated processes. At the same time (7 h) cells with a clearly neuronal phenotype were also seen (Fig. 2*C*; soma in excess of 15 μm with asymmetric dendritic trees). With longer incubation periods increasing numbers of neurones became fluorescent.

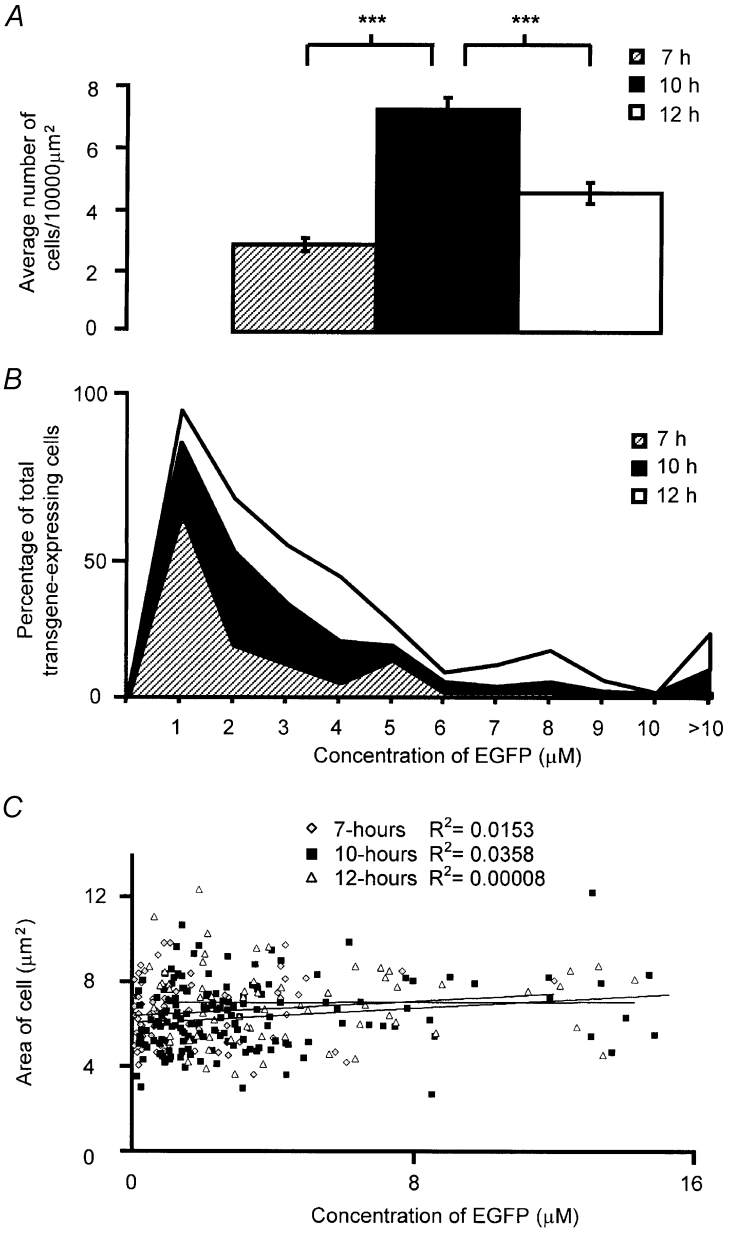

Figure 1

Dynamics of EGFP expression. *A*, increasing slice incubation time affects the number of transgene-expressing cells. Slices were acutely prepared from 12-day-old rat brainstem and incubated with an AVV-expressing EGFP under control of the HCMV promoter for increasing time periods (indicated on the graph). The numbers of fluorescent cells/10 000 μm^2 were counted (± S.E.M.). The *P* values (Mann-Whitney *U* test) are indicated on the graph. *** $P < 0.005$. *B*, increasing slice incubation time affects the concentration of EGFP within the cells. EGFP concentration values were grouped into 11 bins of 1 μM range. Slices from 12-day-old rats were taken through the brainstem. *C*, the size of the soma of the cell does not correlate with the transgene product concentration within it. For each time point, 7, 10 and 12 h incubation with AVVs, the cell surface area (in μm^2) of EGFP-expressing cells was plotted against transgene concentration. At no time point was any correlation found, as indicated by the low regression coefficient values. Slices were taken through the brainstem of 12-day-old rats.

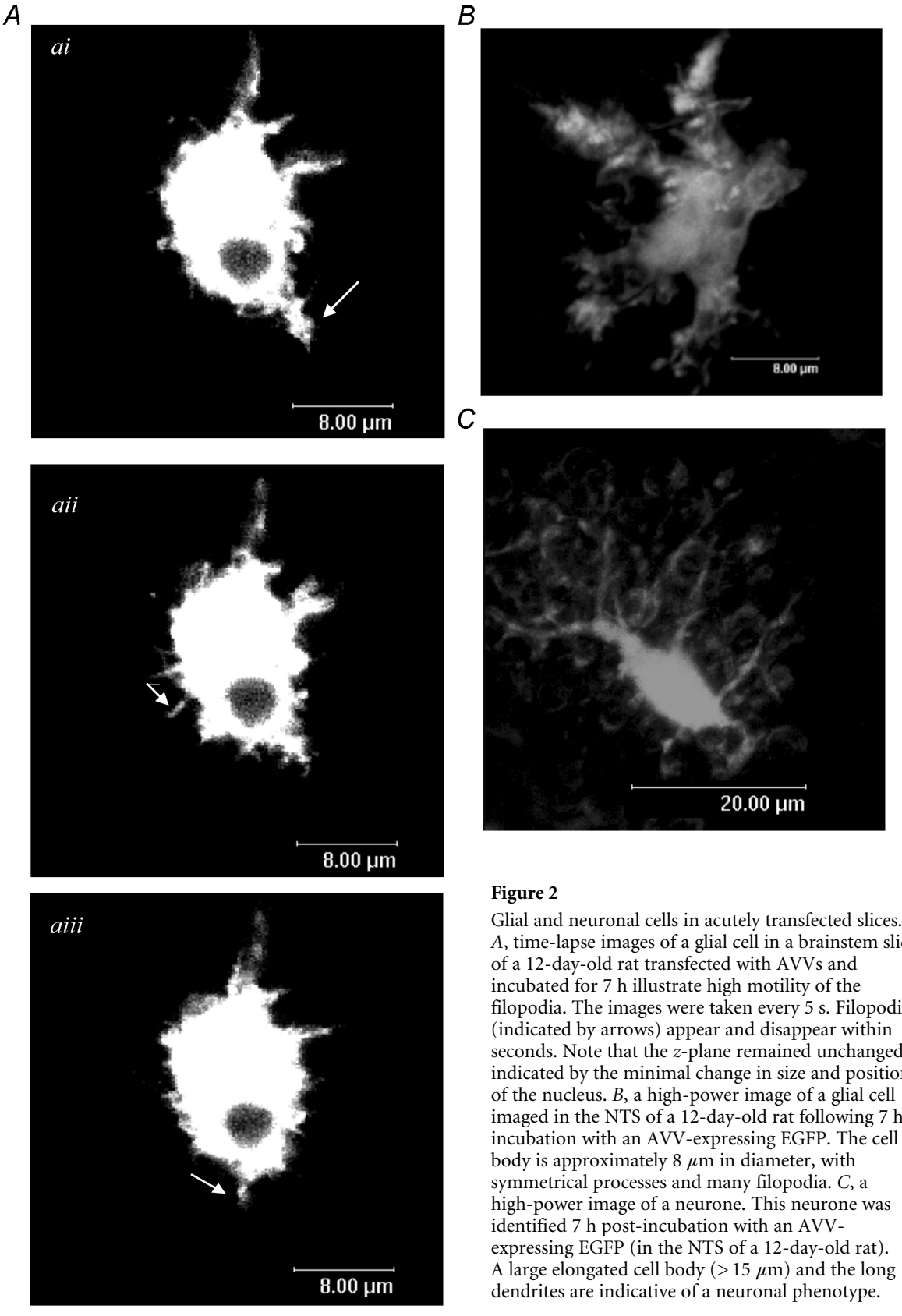

Figure 2

Glial and neuronal cells in acutely transfected slices. *A*, time-lapse images of a glial cell in a brainstem slice of a 12-day-old rat transfected with AVVs and incubated for 7 h illustrate high motility of the filopodia. The images were taken every 5 s. Filopodia (indicated by arrows) appear and disappear within seconds. Note that the *z*-plane remained unchanged as indicated by the minimal change in size and position of the nucleus. *B*, a high-power image of a glial cell imaged in the NTS of a 12-day-old rat following 7 h incubation with an AVV-expressing EGFP. The cell body is approximately 8 μm in diameter, with symmetrical processes and many filopodia. *C*, a high-power image of a neurone. This neurone was identified 7 h post-incubation with an AVV-expressing EGFP (in the NTS of a 12-day-old rat). A large elongated cell body (>15 μm) and the long dendrites are indicative of a neuronal phenotype.

Expression of EGFP is affected by the age of the rat

In order to determine whether the developmental age of an animal affects the rate of transgene expression, we used slices ($n = 8$) from 30-day-old rats and incubated them for 10 h with AVVs. The mean number of cells expressing the transgene was 4.88 ± 0.51 cells/10 000 μm^2 and was significantly lower ($P < 0.05$) than the number in 12-day-old rats (7.28 ± 0.39 cells/10 000 μm^2; Fig. 3). In addition, the concentration of transgene in cells of the older rats was lower; whilst 80 % of fluorescent cells in 12-day-old rats expressed more than 1 μM EGFP, only 52 % of fluorescent cells in 30-day-old rats expressed in excess of 1 μM EGFP. Together these findings suggest that the rate of AVV-expressed transgene *in vitro* is decreased in rats at a later developmental stage.

Dynamics of expression of EGFP is similar in NTS and hippocampus

The expression of AVV-delivered transgenes has not been directly compared between different brain regions. Therefore, we compared the number of transgene-expressing cells in NTS and hippocampal slices (of different rats) following an incubation period of 10 h with 4.4×10^9 pfu ml^{-1} AdHCMV-EGFP (Fig. 3). No obvious difference was found in the density of cells expressing EGFP suggesting that these two areas are not differentially permissive to AVV transfection and/or transgene expression. In the NTS the mean number of cells was 7.28 ± 0.39 cells/10 000 μm^2 and in the hippocampus, 6.18 ± 0.30 cells/10 000 μm^2; this was not significantly different ($P > 0.05$; Mann-Whitney). The overall mean size of EGFP-expressing cells was 63.21 μm^2 and 84.79 μm^2 in NTS and hippocampus, respectively. It should also be mentioned that no cells with pyramidal characteristics expressed EGFP within the hippocampus. Similarly, no cells typical of large hypoglossal motoneurones were seen at any time throughout the experiment. However, motoneurone-like cells with a dorsal vagal motor nucleus could be detected, which is consistent with our earlier findings (Kasparov & Paton, 2000).

Using Rp-cAMPS to block endogenous cAMP reduces HCMV promoter-driven transgene expression

Blocking the cAMP/PKA intracellular signalling pathway using Rp-cAMPS decreased the number of fluorescent cells/10 000 μm^2 from 7.28 ± 0.39 to 4.38 ± 0.23 (Fig. 3, $P < 0.001$; Mann-Whitney).

Physiological responses in transfected cells

To test whether transfected cells were viable, slices were loaded with CO following an 11 h incubation period and, after 1 h, those cells containing both EGFP and CO were imaged using the spectral facility of the Leica microscope. Not all cells that were fluorescent at this time point were considered to be viable as evidenced by swelling of their somata and the appearance of vacuoles. However, in healthy-looking EGFP-expressing neurones it was possible to obtain clear physiological responses with 30 mM KCl and/or 20 μM noradrenaline. Three examples of intracellular Ca^{2+} responses to application of KCl and/or noradrenaline, as indicated by an increased fluorescence in the red channel, are given in Fig. 4. The red fluorescence due to CO increased by 55 % in cell 1 and by 167 % in cell 2, following a KCl

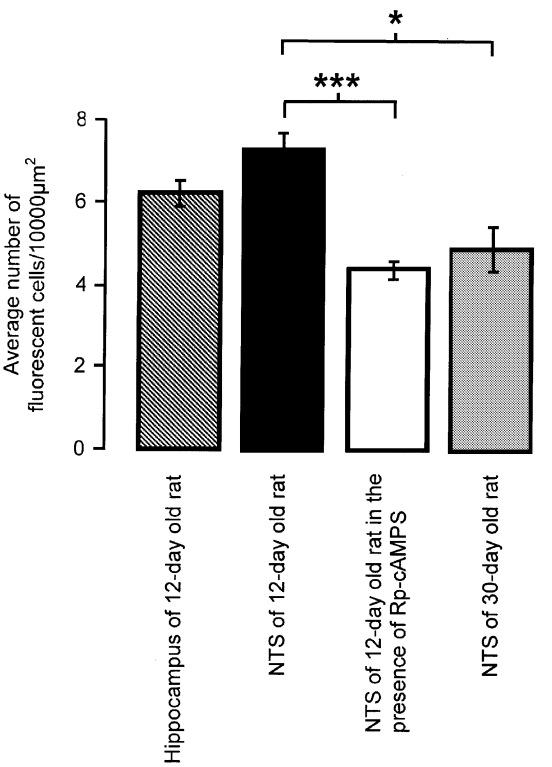

Figure 3

Developmental age of rat and activity of intracellular signalling pathway (PKA) both influence transgene expression. The number of transgene-expressing cells/10 000 μm^2 (± S.E.M.) in NTS slices taken from 12-day-old rats was compared to the number in hippocampus, in the NTS in the presence of a PKA blocker (20 μM Rp-cAMPS) and in the NTS of older (30-day-old) rats. In each case the slices were incubated for a period of 10 h in AVVs. Statistical significance was tested using the non-parametric Mann-Whitney *U* test. $^*P < 0.05$, $^{***}P < 0.005$.

challenge (Fig. 4*A*). In another example (Fig. 4*B*), application of 20 μM noradrenaline resulted in a 35% increase in fluorescence in the red channel while fluorescence in the EGFP channel remained unchanged. Thus, EGFP-expressing neurones in acute slices after 12 h remain viable for neuropharmacological analysis.

DISCUSSION

This study provides the first quantitative account of AVV-mediated transgene expression in acutely transfected brain slices. Using the protocol described here, we obtained micromolar concentrations of EGFP in some cells (glia and neurones) as early as 7 h after incubation with AVV. Although it has previously been suggested that AVV-mediated expression in brain slices is rapid (Kantor *et al.* 1996; Griesbeck *et al.* 1997) the actual dynamics of this process have not been documented. Such information is important since, on the one hand, acute slices do not remain viable indefinitely and, on the other, transgene concentration must reach a critical level to evoke an appropriate response. For comparison, a ubiquitous cellular signalling protein such as calmodulin is present in cells at approximately 10 μM concentration (Tansey *et al.* 1994). Therefore, a dominant negative mutant of a signalling molecule under control of the HCMV promoter and expressed using AVVs in acute slices, can reach concentrations sufficient to compete for its substrate within just a few hours.

It is not surprising that the mean number of cells/10 000 μm^2 significantly increased between 7 and 10 h, consistent with previous reports (Griesbeck *et al.* 1997). Interestingly, it then declined between 10 and 12 h of incubation possibly suggesting breakdown of some cells expressing the reporter gene. This is unlikely to arise from exposure to the adenovirus, as the virus is thought to be non-cytotoxic (Le Gal *et al.* 1993; Davidson *et al.* 1993), but rather reflects a toxic effect of high concentrations of EGFP (Davidson *et al.* 1993). We did not find any significant differences in the density of transfected cells in NTS *versus* hippocampus ($P > 0.05$ after 10 h incubation). At all observed time points in both NTS and hippocampus, EGFP concentration in cells was unevenly spread across the population of cells, revealing distinct peaks.

This multinomial distribution may be a consequence of transfection by either one or multiple viral particles per cell as proposed earlier (Akli *et al.* 1993; Smith *et al.* 2000). It is

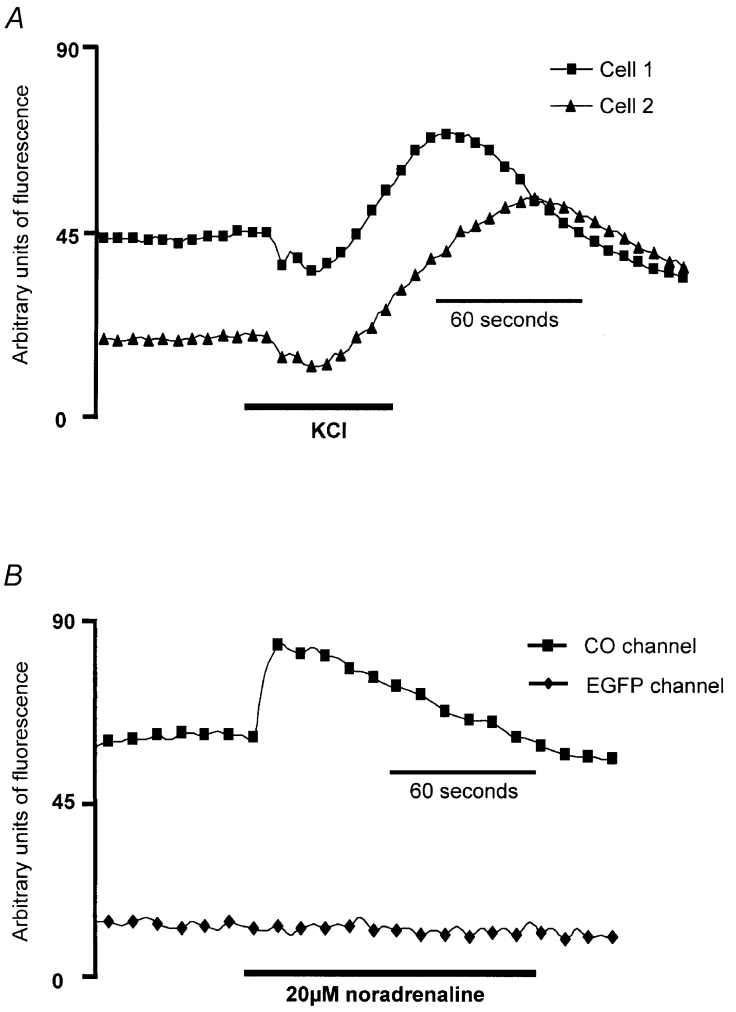

Figure 4

Viability of acutely transfected EGFP-expressing neurones in brain slices. *A*, a depolarising stimulus of 30 mM KCl applied to transgene-expressing cells causes a rise in intracellular calcium, as detected by calcium orange (CO). Examples are shown of the responses of two NTS neurones, loaded with CO, to a 40 s bath application of 30 mM KCl. *B*, 20 μM noradrenaline applied to transgene-expressing cells causes the release of intracellular calcium, detected by CO. An example is shown of the change in fluorescence in an NTS neurone following a 2 min application of noradrenaline. One channel detects the fluorescence change of CO (bandwidth, 560–600 nm) and the other channel detects fluorescence of the EGFP (bandwidth, 495–525 nm). Slices were taken from the NTS of 12-day-old rats incubated with AVVs for 10 h.

less likely that these peaks reflect the clustering of data from distinct phenotypes of cells (i.e. either glia *versus* neurones or different kinds of neurones) because there was no correlation between cell soma size and transgene expression. The latter assumes that cell soma diameter differs depending on phenotype.

We have noticed that at the earliest time point of assessment (7 h), the majority of cells had morphological characteristics of glia (i.e. small rounded cell bodies < 10 μm in diameter with highly motile processes (Fig. 2*A*). This is consistent with other studies that have reported the preferential transfection of glial cells in dissociated hippocampal neurones (Caillaud *et al.* 1993; Baboval *et al.* 2000). The reason for the high expression of transgenes in glia is still unclear. It has been suggested that this is a feature of the HCMV promoter rather than of the AVV itself (Kugler *et al.* 2001). It is also possible that glial cells simply have a higher overall transcriptional activity than neurones.

HCMV promoter is commonly referred to as 'constitutively active' but it appears that this cliché might be misleading, as the transcriptional activity of HCMV is clearly influenced by cellular second messenger systems. When slices were incubated with a cAMP-dependent competitive inhibitor of PKA, EGFP expression was significantly reduced, presumably due to the inhibition of the CREB pathway. Moreover, the impact of transcriptional (and translational) activity on AVV-mediated gene expression is likely to underlie another observation from this study, that is, the strong age dependence of EGFP expression: it is well known that young animals have a higher rate of protein synthesis (Shahbazian *et al.* 1986).

It should be emphasised that numerous fluorescent neurones, with somata in excess of 15 μm diameter were observed in this study in both NTS and hippocampus. This last observation contrasts with recent data in which injection of AVVs into hippocampal slice cultures lead to expression in glial cells alone (Ehrengruber *et al.* 2001). Apparently, delivery of the virus into an already established preparation *in vitro* (e.g. hippocampal slice culture) may prevent AVV transfection of neurones (Wilkemeyer *et al.* 1996). *In vivo*, increased myelination and areas with a high density of glia often restrict the spread of AVV transfection (Akli *et al.* 1993; Meyrelles *et al.* 1997; Hermens *et al.* 1997; Peltekian *et al.* 1997), but whether these factors are significant for transfection in acutely prepared slices is unclear. No pyramidal neurones were identified in the hippocampus throughout the course of these experiments, which is consistent with other studies that also found poor transgene expression in pyramidal cells using AVVs (Kugler *et al.* 2001). This may result from a lack of AVV receptors on these neurones, as data suggests that neuronal size does not influence AVV transfection and transgene expression (Fig. 1*C*).

Finally, we tested the viability of the EGFP-expressing neurones in NTS using the calcium indicator CO. The emission spectrum of CO is red-shifted and can be easily separated from EGFP using soft control of the spectral band implemented in the Leica SP system. As expected, the cells reliably responded to depolarisations (short bath applications of KCl; Fig. 4*A*) with increases of fluorescence and some of them also responded to application of noradrenaline (Fig. 4*B*), although the response was much smaller. While KCl-induced Ca^{2+} elevations are triggered by the influx of Ca^{2+} through the voltage-gated Ca^{2+} channels, the response to noradrenaline depends primarily on the release of calcium from intracellular stores. However, in both instances cells targeted by AVVs in acute brain slices are still fully viable for imaging the elevations in intracellular Ca^{2+} after $\geqslant 12$ h. It should be noted that there was no change in fluorescence in the green channel during the increase seen in red fluorescence. This suggests that there was no leak in fluorescence between the channels and that the change in fluorescence was not an artefact.

In conclusion we describe a method that may be used to test the actions of various transgenes in acutely prepared brain slices and provide evidence that the concentration of transgene may be sufficient to achieve biologically significant actions within ~10 h. Apparently, not all neuronal phenotypes can be targeted equally well and the expression may depend on the age of the animals and the transcriptional/translational activity of the cells. Transfected neurones expressing moderate (< 10 μM) concentrations of EGFP remain fully viable and can be used for imaging experiments with fluorescent indicators.

Akli S, Caillaud C, Vigne E, Stratford-Perricaudet D, Poenaru L, Perricaudet M, Kahn A & Peschanski MR (1993). Transfer of a foreign gene into the brain using adenovirus vectors. *Nat Genet* **3**, 224–228.

Baboval T, Crandall JE, Kinnally E, Chou DK & Smith FI (2000). Restriction of high CD15 expression to a subset of rat cerebellar astroglial cells can be overcome by transduction with adenoviral vectors expressing the rat alpha 1,3-fucosyltransferase IV gene. *Glia* **31**, 144–154.

Caillaud C, Akli S, Vigne E, Koulakoff A, Perricaudet M, Poenaru L, Kahn A & Berwald-Netter Y (1993). Adenoviral vector as a gene delivery system into cultured rat neuronal and glial cells. *Eur J Neurosci* **5**, 1287–1291.

Davidson BL, Allen ED, Kozarsky KF, Wilson JM & Roessler BJ (1993). A model system for *in vivo* gene transfer into the central nervous system using an adenoviral vector. *Nat Genet* **3**, 219–223.

Ehrengruber MU, Hennou S, Bueler H, Naim HY, Deglon N & Lundstrom K (2001). Gene transfer into neurons from hippocampal slices: comparison of recombinant Semliki Forest Virus, adenovirus, adeno-associated virus, lentivirus, and measles virus. *Mol Cell Neurosci* **17**, 855–871.

Griesbeck O, Korte M, Gravel C, Bonhoeffer T & Thoenen H (1997). Rapid gene transfer into cultured hippocampal neurons and acute hippocampal slices using adenoviral vectors. *Brain Res Mol Brain Res* **44**, 171–177.

Hermens WT, Giger RJ, Holtmaat AJ, Dijkhuizen PA, Houweling DA & Verhaagen J (1997). Transient gene transfer to neurons and glia: analysis of adenoviral vector performance in the CNS and PNS. *J Neurosci Methods* **71**, 85–98.

Kantor DB, Lanzrein M, Stary SJ, Sandoval GM, Smith WB, Sullivan BM, Davidson N & Schuman EM (1996). A role for endothelial NO synthase in LTP revealed by adenovirus-mediated inhibition and rescue. *Science* **274**, 1744–1748.

Kasparov S & Paton JF (2000). Somatic gene transfer: implications for cardiovascular control. *Exp Physiol* **85**, 747–755.

Kugler S, Meyn L, Holzmuller H, Gerhardt E, Isenmann S, Schulz JB & Bahr M (2001). Neuron-specific expression of therapeutic proteins: evaluation of different cellular promoters in recombinant adenoviral vectors. *Mol Cell Neurosci* **17**, 78–96.

Le Gal LS, Robert JJ, Berrard S, Ridoux V, Stratford-Perricaudet LD, Perricaudet M & Mallet J (1993). An adenovirus vector for gene transfer into neurons and glia in the brain. *Science* **259**, 988–990.

Meyrelles SS, Sharma RV, Whiteis CA, Davidson BL & Chapleau MW (1997). Adenovirus-mediated gene transfer to cultured nodose sensory neurons. *Brain Res Mol Brain Res* **51**, 33–41.

Paton JF, Deuchars J, Ahmad Z, Wong LF, Murphy D & Kasparov S (2001). Adenoviral vector demonstrates that angiotensin II-induced depression of the cardiac baroreflex is mediated by endothelial nitric oxide synthase in the nucleus tractus solitarii of the rat. *J Physiol* **531**, 445–458.

Peltekian E, Parrish E, Bouchard C, Peschanski M & Lisovoski F (1997). Adenovirus-mediated gene transfer to the brain: methodological assessment. *J Neurosci Methods* **71**, 77–84.

Shahbazian FM, Jacobs M & Lajtha A (1986). Regional and cellular differences in rat brain protein synthesis *in vivo* and in slices during development. *Int J Dev Neurosci* **4**, 209–215.

Shew T, Yip S & Sastry BR (2000). Mechanisms involved in tetanus-induced potentiation of fast IPSCs in rat hippocampal CA1 neurons. *J Neurophysiol* **83**, 3388–3401.

Smith RL, Traul DL, Schaack J, Clayton GH, Staley KJ & Wilcox CL (2000). Characterization of promoter function and cell-type-specific expression from viral vectors in the nervous system. *J Virol* **74**, 11254–11261.

Stokes CE, Kasparov S, Polson JW & Paton JF (2002). Dynamics of transgene expression in acute brain slices transfected with adenoviral vectors. *Abstr Soc Neurosci* **27**, 504.20.

Sun JH, Yan XQ, Xiao H, Zhou JW, Chen YZ & Wang CA (2001). Restoration of decreased N-methyl-d-asparate receptor activity by brain-derived neurotrophic factor in the cultured hippocampal neurons: involvement of cAMP. *Arch Biochem Biophys* **394**, 209–215.

Tansey MG, Luby-Phelps K, Kamm KE & Stull JT (1994). Ca^{2+}-dependent phosphorylation of myosin light chain kinase decreases the Ca^{2+} sensitivity of light chain phosphorylation within smooth muscle cells. *J Biol Chem* **269**, 9912–9920.

Wilkemeyer MF, Smith KL, Zarel MM, Benke TA, Swann JW, Angelides KJ & Eisensmith RC (1996). Adenovirus-mediated gene transfer into dissociated and explant cultures of rat hippocampal neurons. *J Neurosci Res* **43**, 161–174.