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Efficient infection of organotypic hippocampal slice cultures with adenovirus carrying the transgene REST/NRSF

Ruth Butler-Ryan and Ian C. Wood*

School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK

RBR: bsrsb@leeds.ac.uk and ICW: i.c.wood@leeds.ac.uk
*Corresponding author

Declarations of interest: none

Abstract:
Organotypic hippocampal slice cultures provide a useful platform maintaining hippocampal structure and synaptic connections of the brain over weeks in culture with ease of in vitro manipulations. Gene transfer is a particularly desirable tool for using with them but current difficulties with transformation of transgenes into these cultures is a barrier to their use in research. Previous quantifications of viral infections have shown low transformation rates and have relied upon invasive microinjections. In this paper we present an efficient way of infecting organotypic cultures with adenovirus at the acute slice stage that does not require injection. We use the adenoviral delivery system to introduce the transcription factor REST and a GFP marker, providing around 41% cellular infection spread throughout the entire slice culture and promoting transgene expression for weeks *in vitro*. GFP expression was observed most intensely in the slices when they were infected just a few hours after plating and was shown to infect neurons and microglia. We decided to use the transcription factor REST/NRSF as an example transgene which was delivered into cells via the adenoviral construct, conferring overexpression of REST in addition to the GFP marker. This outlines a technique whereby adenoviral infection of organotypic cultures can infect neurons with good efficiency and confer successful manipulation of genetic factors within the cell.

Keywords: Organotypic, hippocampus, adenovirus, REST, NRSF

Highlights:
- Adenoviral transduction efficiency in organotypic cultures is greatest at acute slice stage
- Adenovirus infects neurons and microglia in organotypic cultures
- Adenoviral infection does not cause cytotoxicity in organotypic cultures

1. Introduction:

Organotypic hippocampal slice cultures provide a convenient way of maintaining hippocampal tissue in culture conditions in vitro long-term. Hippocampal architectural morphology and perforant pathway connections are retained from their organisations in the donor brain, while allowing ease of environmental alterations and analysis through imaging, quantification of gene and protein expression, and electrophysiological recordings (Li, Han et al. 2016). Gene transfer into organotypic cultures allows for characterisation of effects of specific genes in living brain tissue, but difficulties in transformation impede the use of the technique. Methods of gene transfer into organotypic cultures include electroporation, biolistics and viral infection (Murphy and Messer 2001). Electroporation targets individually identified neurons for which it can be up to 80% efficient, but it is unsuitable for uniform gene transfer across a whole slice (Keener, Cheung et al. 2020). Biolistics can result in "hundreds or thousands" of transfected cells in a slice culture (Arnold, Feng et al. 1994), but entails the shooting of heavy metal particles into the slice. Viral gene delivery into organotypic cultures has

great capability for gene transfer into neurons throughout the whole slice, but most characterised viral infections of organotypic slices have used microinjections at multiple sites throughout the slice, which result in infection localised to the injection site only and are invasive and could damage tissue integrity. Quantification of infection has been limited to around 100 cells around the microinjection site (Ehrengruber, Lundstrom et al. 1999, Ehrengruber, Hennou et al. 2001). Estimates show the CA1 region of a 9 day old rat holds around 150,000 pyramidal neurons alone (Smith, Pappalardo et al. 2008), highlighting room for improvement over current infection strategies. Advances so far demonstrate the potential for the technique, but improved infection efficiency and reduction in invasive delivery would enhance the prospects of future investigations into gene modulation in organotypic cultures.

The adenoviral constructs used in this paper were produced by the protocol by He and colleagues and incorporate a GFP marker driven by a CMV promoter to allow analysis of the infectivity of the virus (He, Zhou et al. 1998). One viral construct contains the GFP marker only, and one contains the gene for the Repressor Element 1-Silencing Transcription factor (REST)/Neuron-Restrictive Silencer Factor (NRSF) in addition to the GFP marker. REST is a Kruppel-type zinc finger transcriptional repressor which binds to the Repressor Element 1 site of target genes (Ooi and Wood 2007). Up to 1,892 potential RE1 sites have been identified in the human genome by in-silico analysis (Bruce, Donaldson et al. 2004, Johnson, Gamblin et al. 2006). REST represses expression of neuronal genes in non-neuronal tissues and is an important regulator of neurogenesis and ageing in the brain (Schoenherr and Anderson 1995, Lunyak and Rosenfeld 2005). REST dysregulation is implicated in a number of brain disorders, such as epilepsy, stroke, Huntington's disease, brain tumours and neurodegenerative diseases (Hwang and Zukin 2018). However, much is still unknown about its target genes and pathways, which could have the potential for outlining targets for gene therapy for these conditions. The ability to manipulate REST through viral infection of organotypic hippocampal cultures allows for simple characterisation of the effects of this transcription factor on its target genes in preserved hippocampal networks. In this study we aimed to optimise infection of organotypic hippocampal cultures grown in normal culture conditions with adenovirus without the use of microinjection. Characterisation of infection is achieved through analysis of expression of the GFP marker and REST transgene introduced by adenoviral infection.

2. Materials and Methods:

2.1 Organotypic hippocampal slice culture preparation

Slice cultures were prepared as previously described (Stoppini, Buchs et al. 1991), with dissection protocol modified according to (Grabiec, Hohmann et al. 2017). Postnatal day 6-9 (P6-9) rat pups were anaesthetised with isoflurane, sacrificed by cervical dislocation, and decapitated. The brains were removed, sliced into 400μm coronal sections using a Leica VT1200S vibratome (Leica Biosystems, UK) and placed into freshly made artificial cerebrospinal fluid (aCSF containing 124mM NaCl, 26mM $NaHCO_3$, 3mM KCl, 1.25mM NaH_2PO_4, 1.5mM $MgCl_2$, 1.5mM $CaCl_2$, 10mM Glucose) bubbled with carbogen. Hippocampal regions were dissected out of each slice and placed onto semiporous membrane inserts (Fisher Scientific) in a 6-well tissue culture plate containing 1ml medium consisting of 50% Eagle's minimum essential medium, 25% heat-inactivated horse serum, 25% Hank's Balanced Salt Solution (HBSS), 1% penicillin-streptomycin (Sigma), 0.5% B-27 supplement (Gibco) and supplemented with 3g/L glucose (Fisher Scientific), and 3.5g/L HEPES (Melford) (pH 7.2). Cultures were maintained at 37°C under room air + 5% CO_2, and medium was replaced twice a week thereafter.

2.2 Adenoviral infection quantification of organotypic cultures

Virus stocks were diluted in medium to final stated concentrations, 100μl of which was placed on top of each slice in culture at stated time points. The vectors used were previously described by us (Wood, Belyaev et al. 2003, Bruce, Krejcí et al. 2006). They are based on an Ad5 adenovirus originally described (He, Zhou et al. 1998). Within the vectors, expression of each of the REST and GFP

transgenes is driven by a separate and independent CMV promoter sequence. The two copies of the CMV are identical. We would expect the levels of GFP and REST mRNA to be similar. Organotypic cultures which had been infected with AdGFP or AdGFP-REST were fixed in 4% PFA for 1 hour, washed with PBS, exposed to 0.05µg/ml DAPI (Calbiochem) in PBS for 30 seconds, washed with PBS and mounted onto slides with Fluoromount with DAPI (Sigma), then imaged with an Evos FL Auto 2 fluorescent microscope. GFP-expressing and DAPI-stained cells were counted manually on ImageJ from images taken at 20X magnification.

2.3 Viability staining of organotypic cultures

Cultures were infected with $1x10^7$vp/ml AdGFP and stained with ethidium homodimer after 3, 6 or 9 days in culture. Cultures were incubated at 37°C for 30 minutes in 4µM ethidium homodimer solution, washed in PBS, and fixed in 4% PFA for 30 minutes. They were then washed, exposed to DAPI (Calbiochem) for 30 sec, washed again and mounted with Fluoromount with DAPI (Sigma). Slices were imaged with an Evos FL Auto 2 fluorescent microscope. Three images were taken from each region (CA1, CA3 and dentate gyrus) per slice culture, and 2-4 slices per time point were imaged. Red ethidium homodimer-stained cells were calculated as a proportion of GFP expressing cells, using ImageJ software.

2.4 Immunohistochemistry

Organotypic cultures were cut from the membrane and placed individually into wells of a 24 well plate. They were fixed for 1 hour in 4% PFA, then incubated at 37°C for 5 mins in trypsin working buffer (0.05% trypsin, 0.1% $CaCl_2$ in dH_2O) to assist antigen retrieval, and permeabilised overnight in 0.1% Triton-X100 in PBS. Cultures were blocked for 2 hours in donkey serum blocking buffer (5% donkey serum, 0.25% Triton X100 and 0.05% Tween20 in PBS) or BSA blocking buffer (200mg/ml BSA and 0.4% Triton X-100 in PBS), and primary antibody (diluted in PBS containing 50mg/ml BSA) applied at 4°C for 4 days. Anti-GFAP (rabbit polyclonal antibody Z0334 from DAKO) at 1:300, anti-REST (rabbit polyclonal antibody 07-579 from Upstate) at 1:500, anti-Iba1 (rabbit polyclonal antibody PA5-27436 from Invitrogen) at 1:300, and anti-Oligodendrocyte Specific Protein [OSP (rabbit polyclonal antibody Ab53041 from Abcam)] at 1:2000, were all used with Alexa Fluor donkey anti-rabbit 555 secondary antibody. Anti-HUC/D (HuA/B/C/D rabbit polyclonal antibody 13032-1-AP from Proteintech) was used at 1:1000, with Alexa Fluor goat anti-rabbit 405 secondary antibody. Cultures were washed 3X in PBS and secondary antibody (diluted 1:1000 in PBS containing 50mg/ml BSA) applied at 4°C for 4 days. Cultures were washed, mounted and imaged with the Zeiss LSM880 Inverted confocal microscope, and analysed with Fiji software.

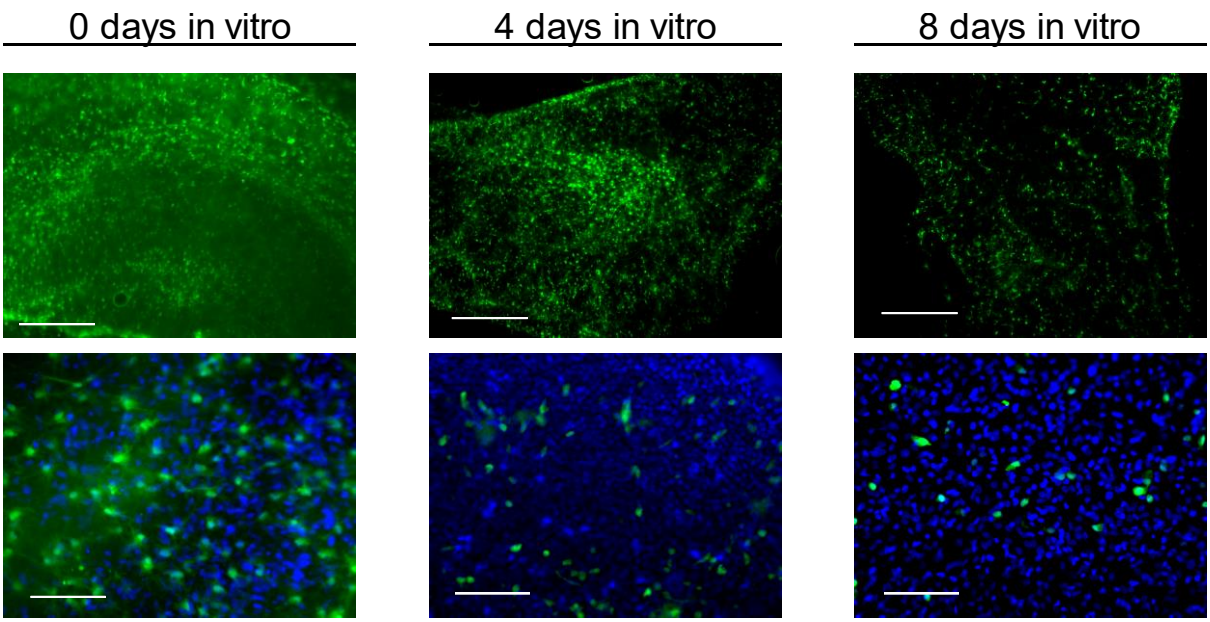
3. Results:

3.1 Adenoviral infection of organotypic cultures is up to 41% efficient when performed at 0 days in vitro with high viral titre

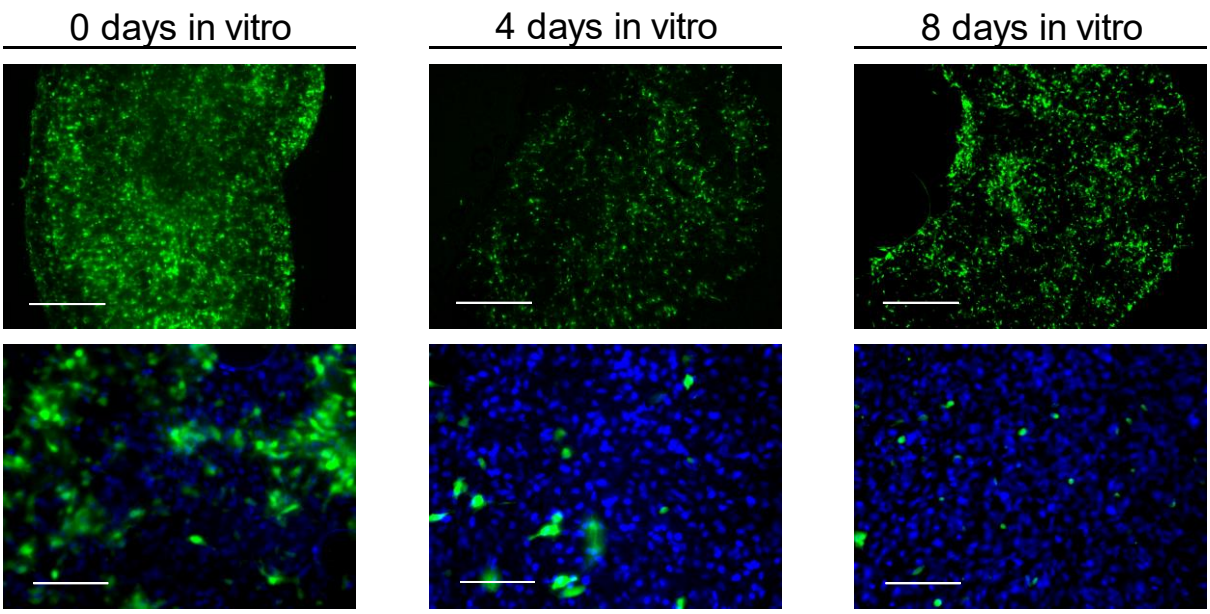
To determine whether time point of exposure to the virus or viral titre would affect infection rate, we infected a total of 22 slice cultures with Ad-GFP at $5x10^6$ and $5x10^7$vp/ml, and Ad-GFP-REST at $2x10^7$vp/ml on days 0, 4 and 8 of culture. The two titres were chosen to try to maximise infection rate while avoiding cytotoxicity. Slices were fixed and imaged after 3 days as adenovirally delivered transgene expression is reported to plateau at 2-3 days (Teschemacher, Wang et al. 2005). DAPI was used to stain all cell nuclei to determine proportion of total cells expressing GFP in each image. Infection efficiency in cultures infected with $5.0x10^7$vp/ml AdGFP at 0 days in culture (41±2%, n=12) was greater than in cultures infected with the same titre at 4 days in culture (17±5%, n=12, p=0.001, anova with Tukey's post-hoc) and 8 days in culture (6±1%, n=12, p=0.001, anova with Tukey's post-hoc). Similarly, infection efficiency in cultures with $2.0x10^7$vp/ml AdGFP-REST at 0 days in culture (41±3%, n=24) was greater than infection efficiency of the same titre viral construct at 4 days in culture (6±2%, n=12, p=0.001, anova with Tukey's post-hoc) or 8 days in culture (13±1%, n=12, p=0.001, anova with Tukey's post-hoc; See Figure 1). This demonstrates that infecting organotypic cultures at 0 days in culture rather than 4 or 8 days in culture has a strong positive effect on infection

efficiency. However, this effect is not seen in cultures infected with the lower titre $5.0x10^6$vp/ml AdGFP: efficiency of infection at 0 days (17±1%, n=23) was not significantly greater than efficiency of infection at 4 days (18±4%, n=12, p=0.9, anova with Tukey's post-hoc) or 8 days (6±1%, n=12, p=0.087, anova with Tukey's post-hoc; See Figure 1) in culture, suggesting a combination of a viral titre of 2-$5x10^7$vp/ml as well as infection at 0 days in culture is necessary for optimal viral infection efficiency. Comparison of cultures infected with differing viral titres at 0 days in culture supports this statement. Infection efficiency was higher in the higher titre $5.0x10^7$vp/ml AdGFP (41±2%, n=12) infected cultures compared to the lower titre $5.0x10^6$vp/ml AdGFP (16±1%, n=12) infected cultures (p=0.001, anova with Tukey's post-hoc; See Figure 1), demonstrating the importance of using adequate viral particles per slice to optimise infection success. The effect of higher titre resulting in greater infection was not observed in cultures infected at later time points. Cultures infected at 4 days in culture with $5.0x10^7$vp/ml AdGFP (17±5%, n=12) did not show greater infection than cultures infected with $5.0x10^6$vp/ml AdGFP (18±4%, n=12, p=0.9, anova with Tukey's post-hoc). Similarly, cultures infected at 8 days in culture with $5.0x10^7$vp/ml AdGFP (6±1%, n=12) did not show greater infection than cultures infected with $5.0x10^6$vp/ml AdGFP (6±1%, n=12, p=0.9, anova with Tukey's post-hoc; See Figure 1). Again, this supports the notion that both adequate viral titre and infection on day 0 of the culture process is necessary for optimal infection efficiency

A


0 days in vitro
4 days in vitro
8 days in vitro

B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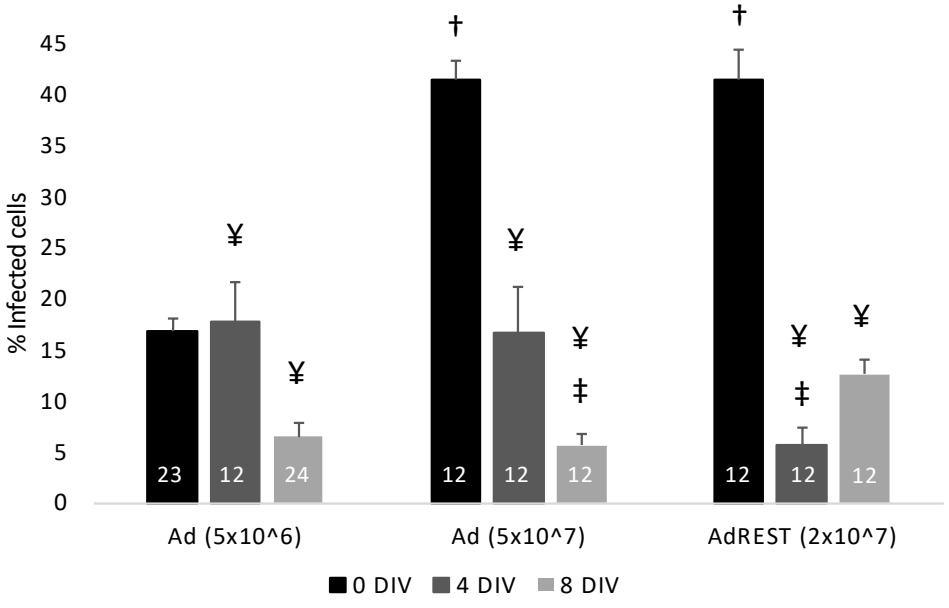
C


% Infected cells
45
40
35
30
25
20
15
10
5
0
23
12
24
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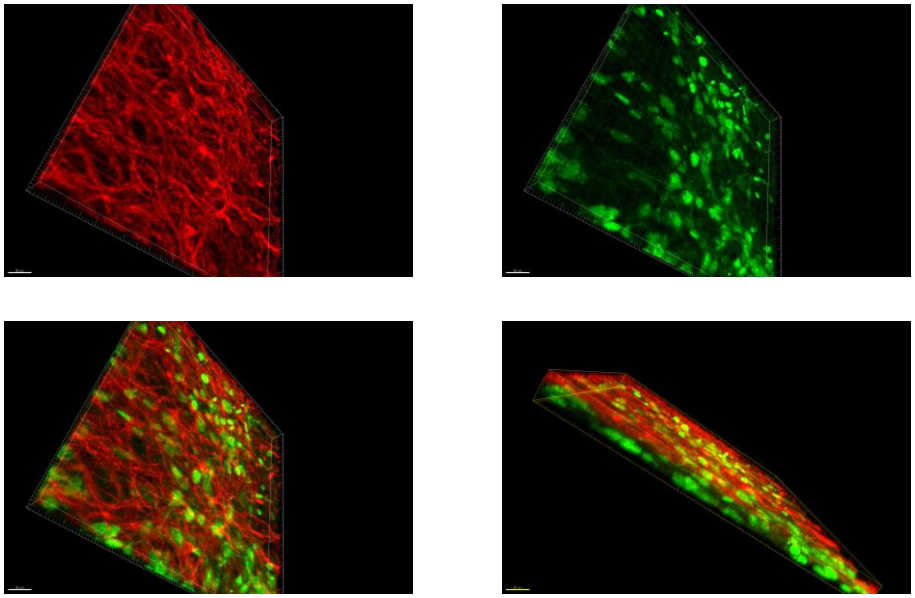
Ad (5x10^6)
Ad (5x10^7)
AdREST (2x10^7)
0 DIV
4 DIV
8 DIV

Figure 1. Assessment of adenoviral infection efficiency of organotypic hippocampal slice cultures. Organotypic hippocampal slice cultures infected with AdGFP or AdGFP-REST at either 0, 4 or 8 days in vitro and co-stained with DAPI. Top row scale bars = 500μm. Bottom row scale bars = 100μm. A) Representative images of cultures infected with AdGFP ($5x10^7$vp/ml) at 0 days in vitro and imaged after 3 days; whole slice (top row) and CA1 region (bottom row) are presented. B) Representative images of cultures infected with AdGFP-REST ($2x10^7$vp/ml) at 0 days in vitro and imaged after 3 days; whole slice (top row) and CA1 region (bottom row) are presented. C) GFP positive cells in AdGFP and AdGFP-REST infected cultures were quantified and presented as a proportion of total DAPI-stained nuclei per image. † $p<0.01$ compared to AdGFP ($5.0x10^6$vp/ml) at 0 days in vitro. ‡ $p<0.05$ compared to AdGFP ($5.0x10^6$vp/ml) at 0 days in culture. ¥ $p<0.01$ compared to AdGFP ($5.0x10^7$vp/ml) at 0 days in culture and to AdGFP-REST ($2.0x10^7$vp/ml) at 0 days in culture. One way anova was used with Tukey's post-hoc test. N images written on bars. 2-4 slices used per condition.

3.2 Adenovirus infects cells throughout the culture, beneath the glial layer

To observe the spread of adenoviral infection, organotypic hippocampal slice cultures which had been infected at 0 days in vitro with $2.0x10^7$vp/ml Ad-GFP-REST and cultured for 5 days were immunostained for astrocytes (GFAP) and co-stained with DAPI to mark all cell nuclei. The 5 day mark was chosen for evaluation to allow potential structural effects from the slicing procedure such as astrocyte proliferation to occur. Z stack images (Figure 2B) of GFP, DAPI and GFAP (red) and 3D reconstruction (Figure 2A) of GFP and GFAP (red) show strong adenoviral infection, marked by bright GFP expressing cells, to be evenly distributed throughout the region imaged. A layer of glial cells known as the 'glial scar' is observed as GFAP positive cells on the top surface of the culture. No GFP expression is observed to colocalise with the GFAP positive cells here. This could be due to a lack of infection of astrocytes by the virus. Alternatively, the virus may have infected the astrocytes, but the astrocyte proliferation during reactive gliosis could have diluted the virus in each cell to such a degree that GFP expression is no longer visible by day 5 in culture.

A



B

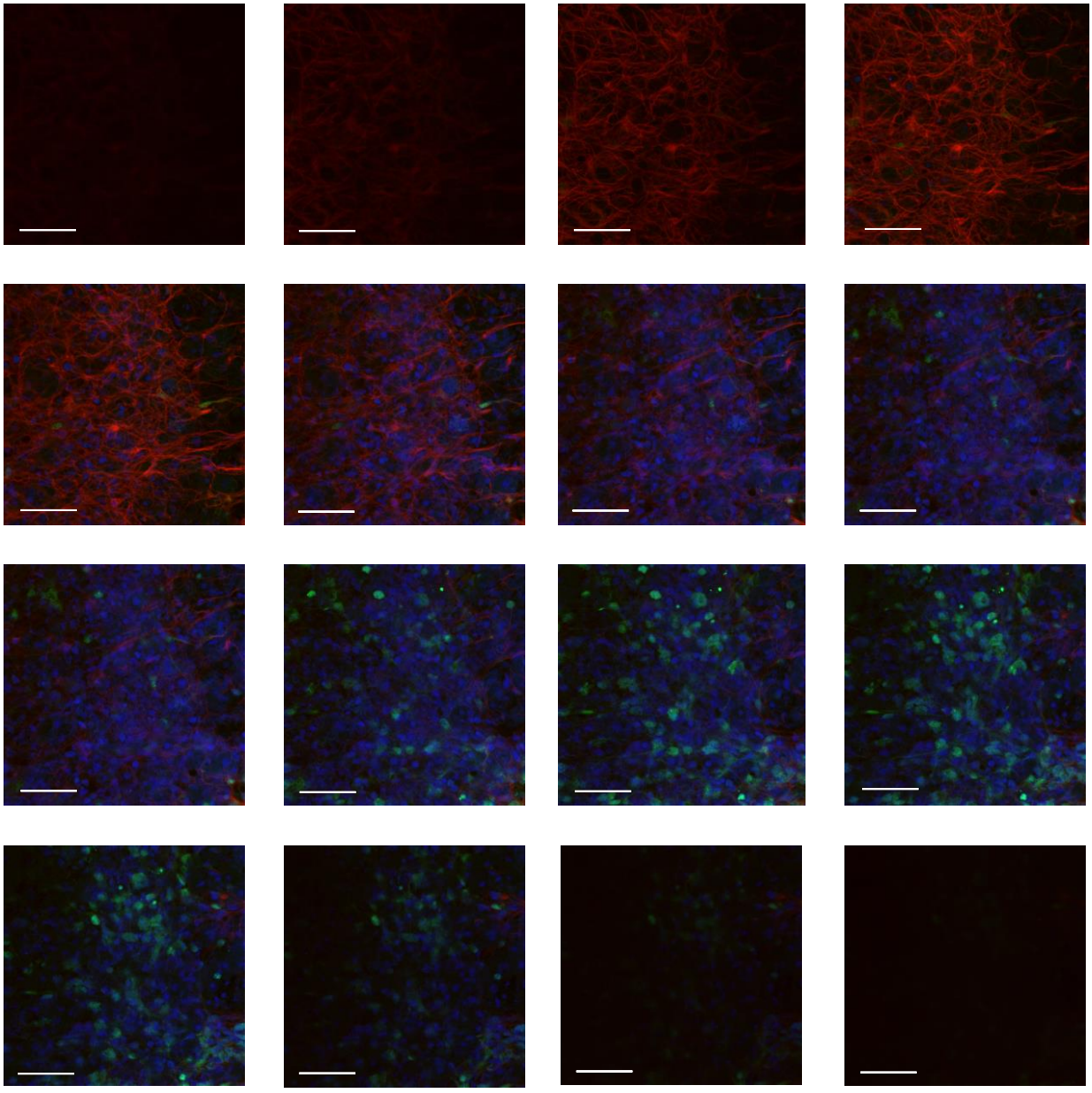


Figure 2. Adenovirus infects cells throughout the organotypic hippocampal slice culture, beneath the surface glial layer. Organotypic hippocampal slice cultures infected with AdGFP-REST ($2x10^7$vp/ml) and stained with antibodies against astrocytes (GFAP), and co-stained with DAPI. A) 3D reconstruction showing GFP-expressing cells (green) with GFAP-positive cells (red). Scale bars = 20µm. B) A representative z-stack image set top to bottom from CA1 region of an organotypic hippocampal slice culture, merging channels of GFP (green), GFAP (red) and DAPI (blue). Z stack images are 1µm apart on the z axis for a total depth of 16µm. Depth is lower than an organotypic hippocampal slice in culture due to a compression effect from the mounting procedure. Scale bars = 50µm.

3.3 GFP expression persists for weeks and does not cause significant cell death

Viral infection of cells has previously been noted to cause toxicity, cell death and loss of gene expression in vitro and in vivo (Cregan, MacLaurin et al. 2000). Therefore, we used ethidium homodimer to quantify cell death in infected cells, as well as imaging GFP expression in cultures over time. Slices were stained with ethidium homodimer and evaluated for cell death at 3, 6 and 9 days following infection. These time points were proposed to evaluate early signs of toxicity over time. Some cells die from axotomisation from the slicing procedure involved in organotypic culture preparation, so toxicity was analysed specifically in adenoviral infected GFP-expressing cells. In cultures infected with AdGFP, less than 5% of GFP expressing cells were colocalized with ethidium homodimer fluorescence at 3, 6 and 9 days in culture (Figure 3A, B). The CA1 region showed 1.9±0.8% colocalization at 3 days in culture, 0.33±0.3% colocalization at 6 days in culture, and 0.18±0.2% colocalization at 9 days in culture (n=12 per group). The CA3 region showed 1.65±1.2% colocalization at 3 days in culture, 0.60±0.6% colocalization at 6 days in culture, and 4.08±2.0% colocalization at 9 days in culture (n=12 per group). The dentate gyrus region showed 0.56±0.4% colocalization at 3 days in culture, 0.23±0.2% colocalization at 6 days in culture, and 0.44±0.3% colocalization at 9 days in culture (n=12 per group). Adenovirally delivered transgene expression has been reported to persist for weeks in vitro (Miyaguchi, Maeda et al. 1999, Teschemacher, Wang et al. 2005). GFP expression in AdGFP-REST infected organotypic cultures was present at 2 (Figure 3A), 4 (Figure 3B) and 5 (Figure 3C) weeks in culture, though a gradual diminishment of expression can be observed over time. This demonstrates adenoviral delivered GFP expression to persist for weeks without need for concern over toxicity-induced cell death.

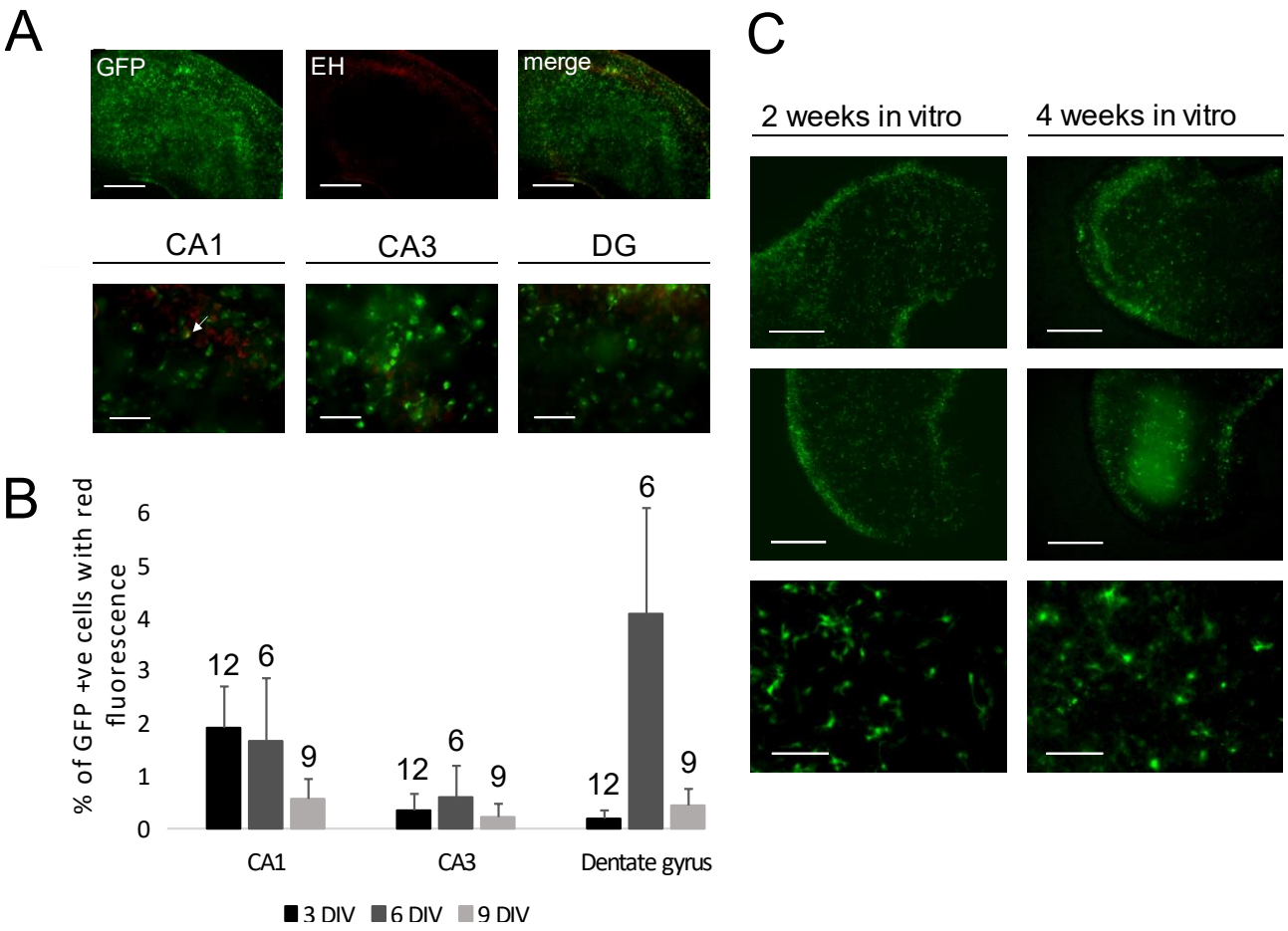


Figure 3. GFP positive cells in adenoviral infected organotypic cultures show minimal cell death and continue to express GFP for weeks in culture. A) Cultures infected with $1x10^7$vp/ml AdGFP and stained with ethidium homodimer (EH) after 3 days in culture. Top row scale bar = 500µm. Bottom row = 100µm. White arrow shows GFP positive cell stained with ethidium homodimer. B) Quantification of proportion of GFP positive cells which also show ethidium homodimer fluorescent signal in the CA1, CA3 and dentate gyrus regions at 3, 6 and 9 days in cultures infected with $1x10^7$vp/ml AdGFP. N images written above bars. Three images taken per region in 2-4 slices. C) Images shown from CA3 regions of two independent cultures infected with AdGFP-REST ($2x10^7$vp/ml) and imaged at stated time points. Scale bars for top 2 rows = 500µm. Scale bars for bottom row = 100µm.

3.4 Adenovirus carrying GFP reporter infects neurons and microglia

To assess which types of cells were infected by the adenovirus, immunohistochemical staining was performed on organotypic cultures which had been infected with AdGFP. Separate cultures were stained for neurons (HUC/D – Figure 4A, B, G), microglia (Iba1 - Figure 4C, D) and oligodendrocytes (OSP: Oligodendrocyte-specific protein – Figure 4E, F). GFP expression was observed at high levels in brightly expressing cells, and at lower levels in more dimly expressing cells. Some brightly expressing cells were observed to colocalize with HUC/D staining, and other brightly expressing cells do not appear to colocalize with HUC/D staining (Figure 4G), suggesting the brightly expressing cells are a mixture of neuronal and non-neuronal. Dimly expressing cells were also observed to colocalize with HUC/D (Figure 4A, B), Iba1 (Figure C, D), and weakly with OSP (Figure 4E, F), suggesting adenovirus infects neurons, microglia, and oligodendrocytes, respectively. The colocalization was quantified through measurement of antibody signal intensity. Signal intensity for HUC/D, Iba1 and OSP antibodies were all above background levels. The HUC/D colocalized GFP fluorescence shows a normalised 4.3-fold increase above background level, demonstrating clear neuronal GFP expression and successful viral infection (HUC/D-positive=51.21±1.5, n=65; background=11.78±1.5, n=33, t=25.2; $p<0.001$, t-test; Figure 4A, 4B). GFP colocalization with Iba1 is also visible (Figure 3C, white arrows mark Iba1-GFP colocalization) and shows a 4.4-fold increase in normalised GFP fluorescence

intensity above background level, demonstrating GFP expression in microglial cells (Iba1-positive=9.49±3.0, n=20; background=2.15±0.1, n=15, t=4.4; $p<0.001$, t-test; Figure 3D). GFP colocalization with OSP was faintly visible in some cells (Figure 4E, white arrows mark OSP-GFP colocalization) and is represented by an average of 2.1-fold increase in normalised GFP fluorescence intensity above background level (OSP=1.84±0.3, n=20; background=0.89±0.3, n=15, t=8.5; $p<0.001$, t-test; Figure 4F). The increase in GFP signal compared to background in oligodendrocytes is less than half of the difference observed in neuronal and microglial staining compared to background. The quantification of fluorescence intensity reflects the faint GFP expression observed in the images, and suggests low levels of adenoviral GFP expression. Low GFP signal in OSP-positive cells could be due to the adenoviral construct infecting oligodendrocytes poorly compared to neurons and microglia, or could reflect a weaker effect of the CMV promoter in oligodendrocytes. We can conclude from these results that adenovirus carrying the GFP reporter reliably infects neurons and microglia, but transgene expression appears to be weak in oligodendrocytes.

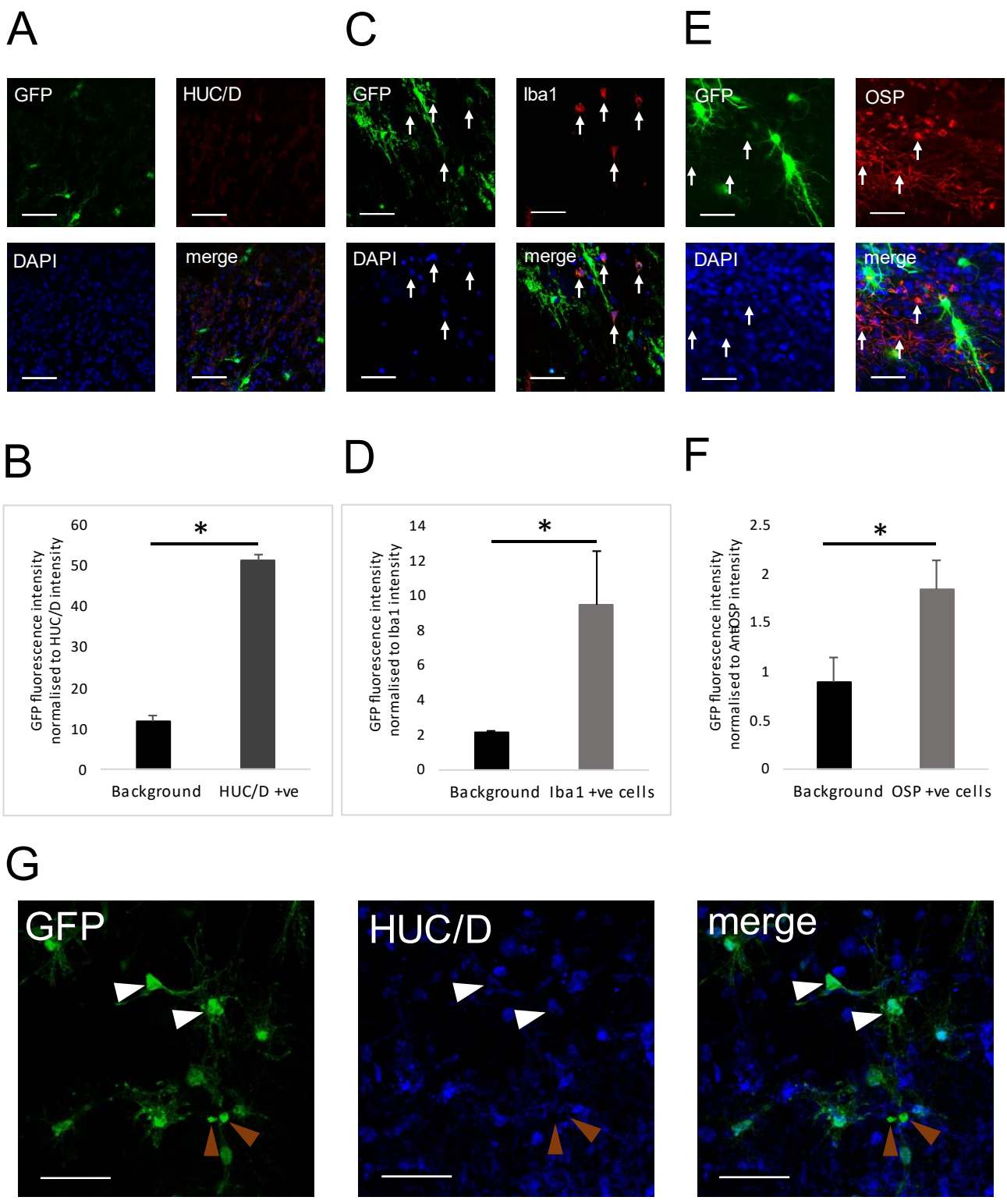


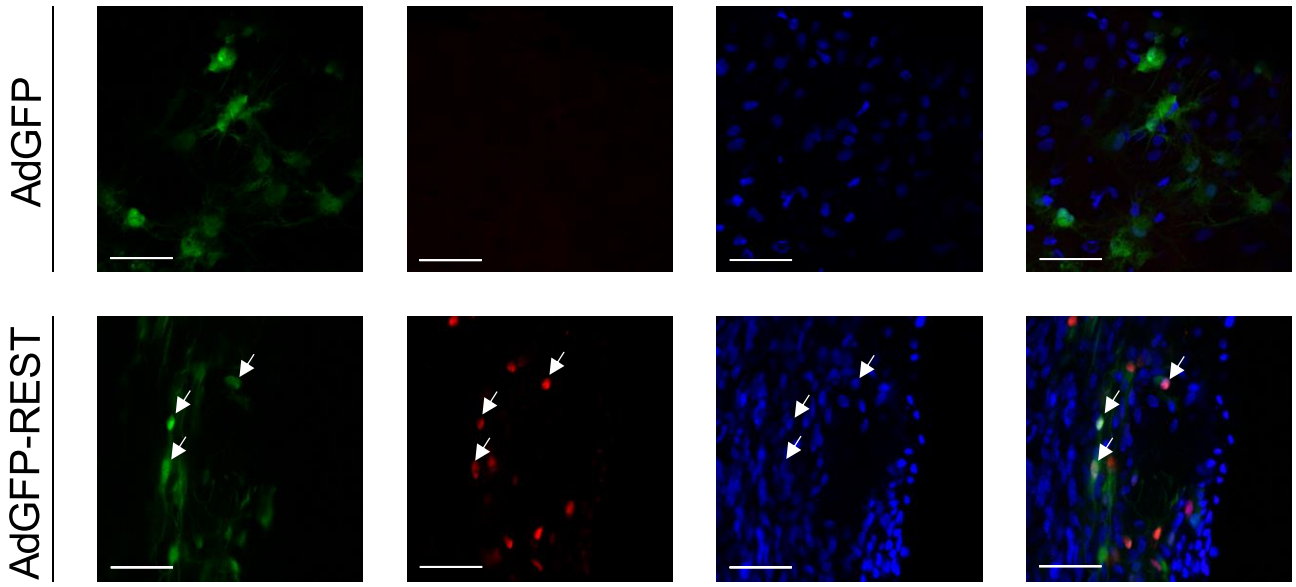
Figure 4. Adenovirus infects neurons, microglia and oligodendrocytes in organotypic hippocampal slice cultures. Cultures infected with AdGFP were immunostained with antibodies against neurons (HUC/D), microglia (Iba1) or oligodendrocytes [Oligodendrocyte-specific protein (OSP)]. A) Representative immunohistochemistry images taken in CA1 region from single HUC/D-stained culture, co-stained with DAPI to mark all cell nuclei. B) Quantification of GFP fluorescence intensity in HUC/D positive cells compared to background $p<0.001$, n=33-65 cells per condition (background or HUC/D positive cell). C) Representative immunohistochemistry images taken in CA1 region from single Iba1-stained culture, co-stained with DAPI to mark all cell nuclei. White arrows show Iba1-positive cells. D) Quantification of GFP fluorescence intensity in Iba1 positive cells compared to background $p<0.001$, n=15-20 cells per condition (background or Iba1-positive cell). E) Representative immunohistochemistry images taken in CA1 region from single OSP-stained culture, co-stained with DAPI to mark all cell nuclei. White arrows show OSP-positive cells. F) Quantification

of GFP fluorescence intensity in OSP positive cells compared to background $p<0.001$, n=15-20 cells per condition (background or OSP-positive cell). G) GFP expression and HUC/D staining show colocalization in some brightly expressing cells (white arrows) but other brightly GFP expressing cells are HUC/D negative (brown arrows). Scale bars are 50μm.

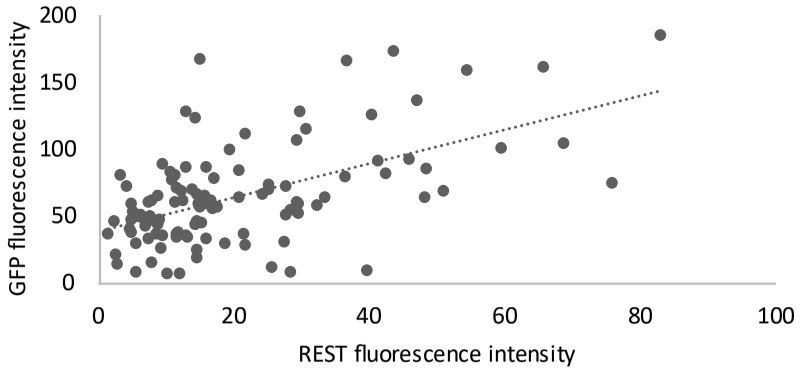
3.5 AdGFP-REST infection causes REST overexpression

We wanted to confirm that adenoviral infections with AdGFP-REST conferred overexpression of the REST transgene. Organotypic cultures infected with AdGFP and AdGFP-REST were immunostained for REST and co-stained for DAPI to mark all cell nuclei. REST staining was visible in GFP positive cells in the culture infected with AdGFP-REST, but it was not visible in GFP positive cells in the AdGFP infected culture (Figure 5A. White arrows show REST positive cells). REST signal was quantified and found to be greatly increased in GFP-positive cells from the AdGFP-REST infected culture compared to GFP positive cells from the AdGFP infected culture (Figure 5C left; AdGFP-REST=20.49±2.8, n=109; AdGFP=2.46±2.1, n=59, t=11; $p<0.001$, t-test; right; AdGFP-REST=42.16±12.2, n=109; AdGFP=5.71±7.7, n=59, t=6.4; $p<0.001$, t-test). There was a correlation between GFP and REST signal intensities in GFP positive cells of the AdGFP-REST infected culture (Figure 5B; n=109, R=0.55; $p<0.001$, Pearson). This suggests the observed increase in REST signal intensity is a result of adenoviral delivered REST overexpression. The correlation may vary somewhat across cells due to cells being infected with variable numbers of copies of the virus, and differing penetration of the REST antibody into different cell types. Examination of localization of REST and DAPI staining in the AdGFP-REST infected culture shows REST transgene expression localized to the DAPI-positive cell nuclei (Figure 5D; White arrows show REST positive cells).

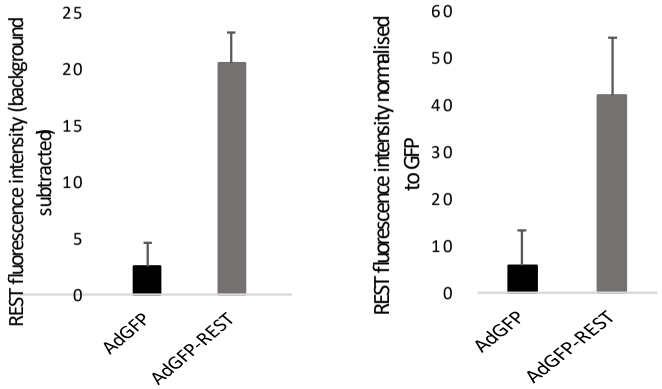
A


AdGFP
AdGFP-REST

B


GFP fluorescence intensity
200
150
100
50
0
0
20
40
60
80
100
REST fluorescence intensity

C


REST fluorescence intensity (background subtracted)
25
20
15
10
5
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AdGFP
AdGFP-REST
REST fluorescence intensity normalised to GFP
60
50
40
30
20
10
0
AdGFP
AdGFP-REST

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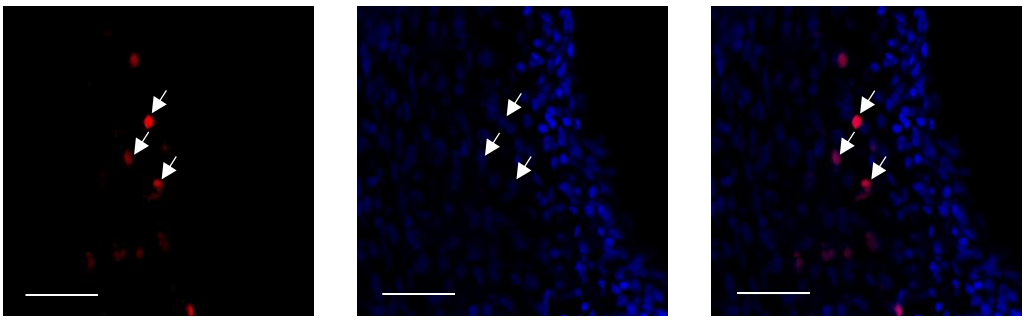

Figure 5. Adenoviral infection of organotypic hippocampal slice cultures with Ad-GFP-REST causes REST overexpression in infected GFP-expressing cells. Culture infected with AdGFP or AdGFP-REST was stained with antibodies against neurons (HUC/D) and REST, and co-stained with DAPI to mark all cell nuclei. A) Representative immunohistochemistry images are presented. Colocalization of the red, green and blue may not make yellow due to variations in how strong each colour is. Scale bars are 50μm. B) Signal intensities from REST fluorescence plotted against signal intensities from GFP fluorescence from AdGFP-REST infected culture (Pearson's correlation, $R = 0.55$, $p<0.001$). C) REST fluorescence intensity with background subtracted, and normalised to GFP fluorescence intensity per cell. * $p<0.001$. D) Representative immunohistochemistry images from an AdGFP-REST infected culture to show localization of REST expression. White arrows mark 3 cell nuclei across all images to show complete colocalization of REST expression with DAPI. N=59-109 GFP-positive cells in 1 slice per condition. Scale bars = 50μm.

4. Conclusions:

This study presents an easy technique for efficient uniform infection of cells in an organotypic hippocampal slice culture, primarily targeting neurons. We have demonstrated penetration of the virus throughout the full depth of the slice, with expression of both the GFP and REST transgenes in infected cells which persists for weeks in vitro. We observed infection of up to 41% of total DAPI-positive cells in each organotypic hippocampal slice culture in the CA1, CA3 and dentate gyrus regions, without noticeable cytotoxicity. Our study utilizes the droplet method at 0 days in vitro, which is in contrast to the more commonly used microinjection method in mature slices (Wiegert, Gee et al. 2017). Direct application of virus to organotypic hippocampal cultures on the day of plating has been used for transgene delivery by other groups with adeno-associated virus and lentivirus, but efficiency of the method was unclear (Selkirk, Stiefel et al. 2005, Avaliani, Sørensen et al. 2014, Avaliani, Andersson et al. 2016, Schätzle, Kapitein et al. 2016). Expression appears more thorough in recent work by Croft and colleagues, and specific promoters were used to successfully target adeno-associated viral infection to neurons, astrocytes, microglia or oligodendrocytes, though infection efficiency was not quantified (Croft, Cruz et al. 2019). Quantification of viral cell infection in organotypic hippocampal cultures from other groups via microinjection has been limited to around 100 cells per slice (Ehrengruber, Hennou et al. 2001). Adenovirus has been shown to successfully infect primary neuron cultures in vitro (Ehrengruber, Doupnik et al. 1997, Robert, Bouilleret et al. 1997). However, in injected organotypic hippocampal slice cultures, adenovirus has previously been observed to infect primarily glial cells (Robert, Bouilleret et al. 1997, Ehrengruber, Hennou et al. 2001). Inclusion of the glial cell growth inhibitor cytosine beta D arabinofuranoside hydrochloride (AraC) in the culture media, alongside utilization of the droplet method with addition of collagen, resulted in infection of both neurons and glial cells in mature slice cultures. This technique also resulted in visibly good infection efficiency, though this was not quantified by cell number, and infected cells were only reported on the slice surface (Miyaguchi, Maeda et al. 1999). Glover and colleagues observed infection of both neurons and glia in mature slices, but infection method was not detailed (Glover, Bienemann et al. 2002). Our results show that infecting organotypic hippocampal slice cultures within a few hours after plating results in good infection efficiency quantified as up to 41% infection of all cells, including reliable infection of neurons. We observed no colocalization between GFP expression and the GFAP staining of the glial scar on the slice surface. Notably, the GFAP staining of the slice culture was not observed below the surface layer, where supporting astrocytes would be expected to be found. It is possible that some of the GFP-positive cells deep in the slice culture are astrocytes and were just not stained by the GFAP antibody due to poor antibody penetration, so we cannot rule out astrocyte infection in addition to neuronal infection from adenovirus. Slices were infected soon after plating to ensure that reactive gliosis had not had time to form a glial scar over the surface which could prevent viral infection. The glial scar has been previously reported to form between 0 and 3 days in culture, seen as a large increase in GFAP immunoreactivity (Gerlach, Donkels et al. 2016) in response to the slicing. Reactive gliosis occurs following CNS insult such as trauma, and commonly leads to a glial scar, as well as other detrimental and beneficial effects (Sofroniew 2009). We propose that by infecting the slice in the acute stage before reactive gliosis creates a glial scar to prevent viral dispersion, the droplet method is highly

efficient, and avoids potential mechanical disruption by a micropipette. Furthermore, unlike Miyaguchi and colleagues (Miyaguchi, Maeda et al. 1999), we found that infecting at day 0 in vitro did not require collagen addition to the droplet and resulted in reliable neuronal infection without use of glial cell growth inhibitors in the media.

The GFP marker is expressed throughout the entire cell, allowing clear visualization of neuron soma and neurite projections within the hippocampal network. This makes it possible to focus on adenoviral infected cells expressing a transgene of interest within a hippocampal culture and compare them to nearby non-infected cells of the same type through techniques such as microscopy or patch clamp electrophysiology. Various virus forms have previously been used for transgene delivery. While adenovirus can cause immune reactions when used in high titres in vivo, it is particularly appropriate for in vitro work such as infection of organotypic cultures due to its rapid amplification process and efficient infection (Teschemacher, Wang et al. 2005). Furthermore, we did not observe any increase in cell death in adenoviral infected cultures from ethidium homodimer staining, suggesting the titres used here do not have significant cytotoxic effects. This is further supported by continued visualization of GFP expression in infected cultures up to 5 weeks in vitro, mirroring results found previously (Miyaguchi, Maeda et al. 1999). Altogether this supports the reliable use of adenovirus for transfections in organotypic hippocampal slice cultures for future studies.

Infection of the organotypic cultures with adenovirus carrying the transgene REST resulted in overexpression of REST in cell nuclei, demonstrating the potential of the infection technique for gene modulation studies. The localization of REST has not been previously examined in organotypic hippocampal slice cultures, but the similarities of organotypic cultures to in vivo brain make them particularly relevant. In support of this, REST was observed in the nucleus of cortical neurons from postmortem human brain sample (Kawamura, Sato et al. 2019). However, endogenous REST localization is likely to vary under different conditions, with greater nuclear REST occurring during times of REST repression of its many target genes. Nuclear REST levels have been found to increase naturally in ageing in healthy brains to repress genes involved in neurodegeneration, and disturbance of this mechanism can contribute to the pathology of Alzheimer's disease, frontotemporal dementia, dementia with Lewy bodies, and Parkinson's disease. In contrast, Huntington's disease is associated with excessive nuclear REST (Zuccato, Tartari et al. 2003). REST nuclear localization from the cytoplasm can be induced by ischemia-reperfusion in rat cortex and hippocampus (Luo, Fu et al. 2020), or kainite or hydrogen peroxide exposure in cultured hippocampal neurons (Spencer, Chandler et al. 2006), suggesting potential involvement in stroke and epilepsy pathogenesis. Therefore, the complete REST nuclear localization observed here in organotypic hippocampal slice cultures highlights their potential as a useful model for investigations into the molecular aspects of Huntington's disease, stroke, epilepsy or ageing.

In conclusion, we have demonstrated that infection of up to 41% of cells in organotypic cultures is possible by exposing them to virus in media at 1-2 hours after plating and does not require invasive microinjection. Adenovirus was observed to infect neurons and microglia, with weaker adenoviral-mediated transgene expression observed in oligodendrocytes. No signs of toxicity were observed and only a gradual diminishment of GFP reporter fluorescence was observed up to 5 weeks. The transgene REST was overexpressed in cells infected with AdGFP-REST compared to AdGFP, demonstrating the effective use of this method for modulating gene expression in organotypic cultures.

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