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Novel strategy to study gene expression and function in developing cerebellar granule cells

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Abstract

The advent of techniques for global analyses of cell biology, such as genomics and proteomics, opens the way to rapid progress in understanding the molecular control of developing tissues. However, such studies in the CNS are hindered by the complexity of this tissue. In particular, few approaches allow cells to be isolated that are enriched for specific stages of their maturation. We describe a new strategy to study gene expression and function in cerebellar granule cells. In these experiments, we have used square pulse electroporation to introduce fluorescent dye or DNA constructs into immature granule cell precursors in situ. This method only labels granule cell precursors in the superficial part of the external granule layer. Combining this labelling with fluorescent activated cell sorting (FACS) allows the transfected cells to be isolated at any time during their subsequent development, thus providing a means of analysing granule cells as they undergo maturation. This transfection method can be used to study events in the normal maturation of granule cells or the effects of introduced transgenes. Such studies can be carried out on cells purified from primary cultures or cells in situ using cerebellar slice cultures. Our strategy provides a new route to detailed analysis of the role of genes in controlling many aspects of granule cell biology. These approaches will allow recent global analyses to be more readily applied to subpopulations of cells in complex tissues.
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Keywords: Cerebellum; Electroporation; FACS; Dextran conjugates; Slice culture

1. Introduction

Granule cells of the cerebellum are the most abundant neuronal cell type in the vertebrate brain, accessible to study, both in vivo and in vitro, and are therefore a favoured system in which to study neuronal maturation (Burgoyne and Cambray-Deakin, 1988). In particular, the external granule layer (EGL) on the outer surface of the cerebellum, from which granule cells arise, provides a population of precursors already specified to produce this one type of neuron, such that studies are not complicated by the presence of multiple cell types (Goldowitz and Hamre, 1998). Recent studies have been focused on this cell type as the progenitor of the most common paediatric brain tumour, medulloblastoma (Pomeroy et al., 2002).

Many studies of granule cells make use of primary cultures, which can be grown at a high level of purity. However, such cultures contain about 5% non-granule cells (Levi et al., 1984) and are heterogeneous with respect to the maturational state of the granule cells. This maturational heterogeneity precludes studies of the changes that granule cells undergo in the first hours after exit from cell cycle.

The advent of new electroporation methods for introducing DNA and other molecules into mammalian cells and tissues has allowed us to overcome the problems described above. Electroporation has already been shown to provide an efficient means to transfect cells of the cerebellum in slice cultures (Murphy and Messer, 2001). Here, by transfecting the EGL with the cerebellum intact we have increased both the efficiency and selectivity of transfection to facilitate analysis of granule cell differentiation. We have developed

Abbreviations: EGL, external germinal layer; FACS, fluorescence activated cell sorting; IRES, internal ribosome entry segment; GCPs, granule cell precursors

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a protocol to select cells of the EGL using fluorescence activated cell sorting (FACS) after transfection with fluorescent dye or pEGFP. Dividing granule cell precursors (GCPs) are initially located in the superficial EGL, migrating first into the deep EGL when they exit mitosis and then to their final destination, the inner granule layer (IGL) (Goldowitz and Hamre, 1998). Standard granule cell cultures contain cells from both the superficial EGL and deep EGL (Raetzman and Siegel, 1998). Due to a lack of appropriate surface antigens, no methods to isolate the cells of the superficial EGL have been described. We show that electroporation can be used to label cells of the superficial EGL, which can then be isolated by FACS either immediately or after any chosen period of maturation. We show that these cells exhibit a high degree of maturational purity and that molecular aspects of the cells' biology can be readily assayed following this purification procedure. This approach facilitates two main types of study. First, transient labelling of the cells of the EGL with fluorescent dye allows analysis of normal events over the first 24 h, whereas transfection of DNA constructs allows longer term studies due to the continued expression of the marker protein. Secondly, constructs carrying transgenes can be transfected into the most immature granule cells, such that the effects of those genes on the granule cells can be studied in primary cultures or in situ, in cerebellar slice cultures.

In conclusion, this new methodology provides a means to gain even greater insight into the mechanisms that control granule cell development. Furthermore, there appears to be no restriction on the cell types susceptible to this method of transfection, so it is also likely to be applicable to the study of other regions of the brain.

2. Materials and methods

2.1. Electroporation

Cerebella were prepared from postnatal day 6 (P6) Wistar rat pups. All procedures relating to the care and treatment of animals were carried out according to the European Communities Council Directive for the care and use of laboratory animals. After sacrifice, rat brains were rapidly removed and placed into chilled PBS. The cerebellum was isolated and the pial layer peeled away. Half a cerebellum was placed in the electroporation well (Fig. 1A and B) orientated such that the transverse fissures ran parallel to the electrodes fixed 7 mm apart.

pEGFP (Clontech, USA) and DsRed (Clontech, USA) plasmids were prepared to a final concentration of 1.5 μg/μl in PBS using a DNA Maxi-prep Kit (Qiagen, Germany). Dextran conjugates (Eugen, USA), were used at a final concentration of 1 μg/μl in PBS. For gene constructs, 15 μl pEGFP or DsRed was injected into the transverse fissures of a half cerebellum with a micropipette. One-tenth volume of Fast Green solution (0.1%) was added to the plasmid solution to monitor the treatment. For dextran conjugates, a fluorescent dissection microscope was used to ensure that most cerebellar fissures were loaded with dye.

Electroporation parameters (voltage, pulse length and numbers) were controlled by a ECM830 electroporator (BTX, Genetronics Inc., USA). The square pulse was delivered with an interval time set as 500 ms. After pulse delivery, the cerebellum was immersed in ice-cold PBS to avoid heat damage from the electroporation. Transfected cells on the surface of the cerebellum were visualised using a fluorescent microscope (Fig. 1C).

After electroporation, we assessed cell damage by a "cell survival index", which was calculated based on following formula:

$$\text{cell survival index} = (\text{Ne} - \text{Ce})/(\text{Nne} - \text{Cne})$$

Ne and Nne represent the total cell number resulting from disaggregating cerebellum either with electroporation or without electroporation, respectively. Ce and Cne refer to the dead cell number (after 6 h culture), from electroporated and non-electroporated cerebellum as assayed by Trypan Blue staining.

2.2. Granule cell cultures

All chemicals were obtained from Sigma unless otherwise stated. Cultures of dissociated granule neurons were prepared as previously described (Behringer et al., 1996). In brief, after electroporation, cerebella were enzymatically dissociated with 0.05% trypsin, and triturated in 4000 U DNase. Following dissociation, granule cells were resuspended in granule cell culture medium (DMEM containing 1× ITS (insulin, transferring, sodium selenite liquid media supplement, Sigma I-3146), 10 mM glutamate, 1 mM putrescine and 1/3 mM progesterone). The dissociated cells were then plated onto 22 mm coverslips coated with poly-D-lysine at a density of 8–10 × 10^5 cells per coverslip.

2.3. Fluorescence activated cell sorting (FACS)

A FACScan flow cytometer (Becton Dickinson Immunocytometry System, BDIS) equipped with a 15 mW air-cooled 488 argon-ion laser was used to analyse cell purity and transfection efficiency. Green fluorescent cells were collected using a 530/630 nm bandpass (BP) filter. Electronic compensation was applied to the fluorescence channels to remove residual spectral overlap. Photomultiplier tube voltage and spectral compensation were initially set using cells stained with either pEGFP or Dextran fluoroscein conjugate. Gates were set to exclude necrotic cells, cellular debris and cell aggregates and the fluorescence intensity of events within the gated region were quantified. For each sample, a minimum of 50,000 events were collected within the single gate for comparisons of transfection efficiency. Analysis of multivariant data was performed with CELLQuest software (BDIS). Two distinct populations of cells were visible on flow histograms.

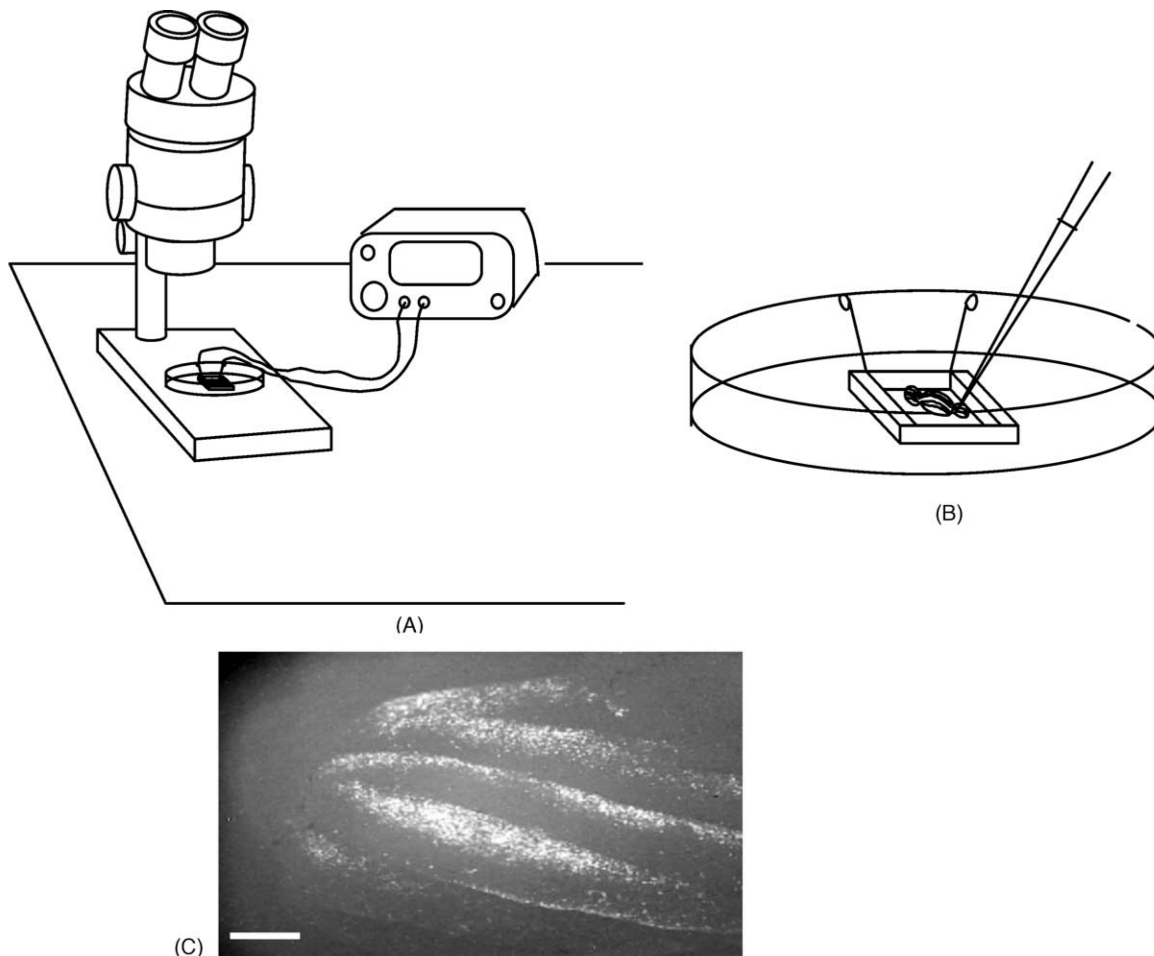

Fig. 1. Diagram of electroporation apparatus for use on cerebellum. (A) Electroporation simply requires a micropipette filled with DNA solution or other molecules to be delivered, a microscope, an electroporator and wire connections. (B) Electroporation of the cerebellum was carried out by inserting the micropipette with solution into fissures of cerebellum. The fissures were then aligned perpendicular to the direction of electrophoresis. Delivery of electrical pulses causes electrophoresis of the DNA towards the positive electrode and causes the cells to take up the DNA. (C) Cerebellum electroporated with GFP. Following electroporation with pEGFP, whole cerebellum was incubated in culture medium. Six hours later, fluorescence was seen over most of the surface of the cerebellum. Scale bar approximately 150 μm.

Only cells exhibiting high levels of fluorescence were included for determination of the percentage of transfected cells and cells adjacent to auto-fluorescent non-transfected cells were excluded. Sorted cells were collected into vials containing complete medium at a rate of 3000 cells/s.

2.4. Slice culture

Cerebellum slice cultures were prepared and cultured as previously described (Haydar et al., 1999). In brief, after electroporation, cerebella were transferred into 3% agarose (tissue culture grade) at 50 °C. After the agarose gel had set, 300 μm coronal slices were cut using a VT100S vibrating microtome (Leica, German). Slices were transferred to a 0.4 μm Nuclepore membrane (Millicell, USA) at the interface between air and culture medium (DMEM containing 10% horse serum, 0.3 M glucose, 10 mM ITS, 0.3 mM progesterone, 100 IU/ml penicillin) in a 6-well culture plate. The culture plates were incubated at 37 °C in 5% CO_2.

2.5. Immunohistochemistry

After various periods of incubation, cerebellum slices were fixed for 3 h with 4% paraformaldehyde (PFA) at 4 °C and then cryoprotected in 30% sucrose for 4 h. Slices were embedded with OCT compound (BDH, UK) and frozen in liquid nitrogen. Fourteen micrometers coronal sections were cut, dried at room temperature for 1 h, and stored at −20 °C until needed.

For Tag1 (1:20, gift from Dr. Andrew JW Furley, Centre for Developmental Genetics, Biomedical Sciences, University of Sheffield, UK) and Tuj1 (Babco, USA, 1:300) immunostaining, sections were processed as follows. Sections were rehydrated in PBS for 10 min and incubated in primary antibody overnight after 1 h blocking in 1% BSA. After three washes of 5 min in PBS sections were incubated with secondary antibody (Vector, USA, 1:100) in 1.5% goat serum/PBS for 1 h at room temperature and washed three times for 5 min in PBS. For BrdU staining, sections were

treated with 2N HCl and 0.3% Triton X-100 for 20 min, blocked for 30 min with 10% normal goat serum and incubated firstly in mouse anti-BrdU antibody (Becton Dickinson, USA) diluted 1:100 in PBS overnight, and then 1 h in fluorescent secondary antibody (Vector, CA) diluted 1:150 in PBS, followed by washing three times for 5 min in PBS. Before visualisation, the sections were stained with DAPI (Vector, CA) to distinguish different layers of cerebellum. For immunostaining on primary culture, cells were firstly fixed with 4% PFA for 1 min at 4 °C, then incubated in primary antibody for 1 h, then second antibody for another 1 h (the antibodies concentrations are similar to those used for sections staining).

2.6. RT-PCR

RT-PCR was essentially as described (Raetzman and Siegel, 1998). Briefly, total RNA was extracted from cells using Trizol reagent (Invitrogen, CA) and cDNA was prepared from 1 μg of RNA using AB gene RT-PCR system (Abgene, UK). Specific genes were amplified, after an initial 3 min denaturation at 94 °C, for 25–30 cycles of 94 °C for 30 s, 55–60 °C for 30 s, 72 °C for 30 s and a final 5 min extension at 72 °C. PCR products were separated by electrophoresis in a 2% agarose gel and visualised by ethidium bromide staining. The following primers were used (written 5′ to 3′), cyclin D1: 5′ GGGGATGTGTTGTTACCAGAAGG; 3′ ACCCACGACCCGCTAGAG; BM28: 5′ GAAGCGAATGAAGGCACTGG; 3′ GCAGACACAGCCCACTTCCAC; Tag1: 5′ TAGACACCTACTCCCAACAG; 3′ TGCCCAGTGCCTCTTGACTT. Math1: 5′ ATGGCACAGAAGGACCTGTC; 3′ CGTCACTTCTGTGGGATATG. β-tubulin 5′ CCGCCTGCCTCTTCGTCTCTA, 3′ TGCAGGCAGTCACAATTCTCAC. Primers specific for GAPDH (5′ TGGTCTACATGTTCCAGTATG; 3′ TCCACCACCCTGTTGCTGTA) were used as a control for equal loading of first-strand cDNA.

3. Results

3.1. Determining key parameters for electroporation

Previous studies have demonstrated that different tissues and cells have variable responses to electrical pulses, so that each tissue requires specific electroporation conditions for optimal transfection efficiency (Lurquin, 1997). Therefore, we first had to determine optimal conditions for electroporation of the superficial EGL at the surface of the cerebellum.

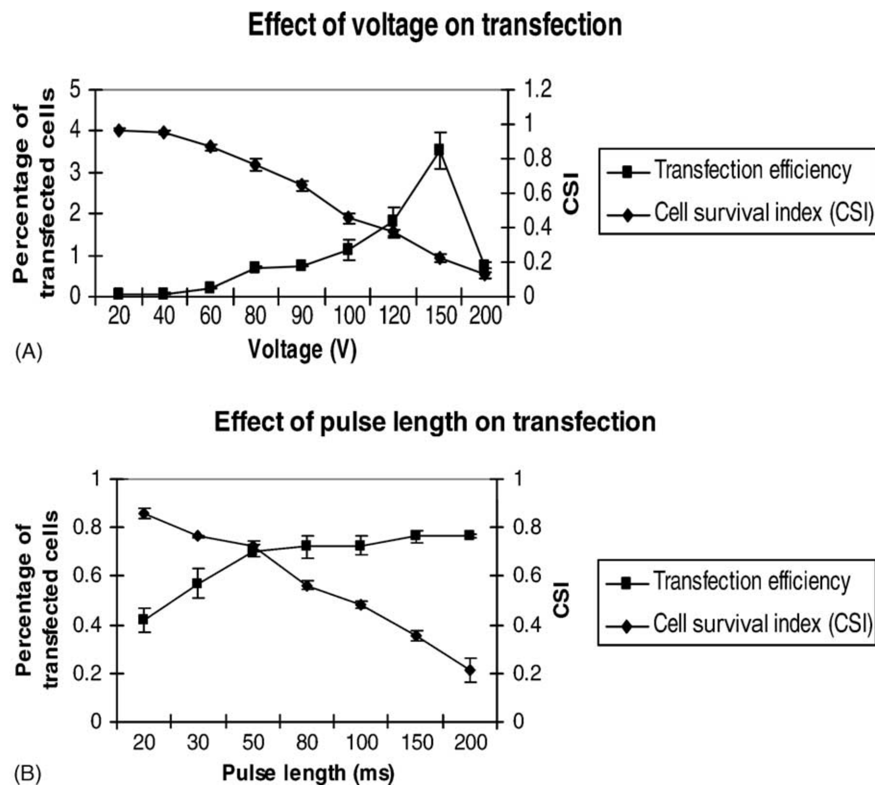

Fig. 2. Optimisation of conditions for cerebellum electroporation using the plasmid pEGFP. The effects of varying the voltage, pulse length and pulse number were determined. Transfection efficiency (assessed by FACS analysis) and cell survival (assessed by trypan blue staining) were analysed 6 h after electroporation. All data were obtained from three independent experiments. (A) The effect of varying pulse voltage was assessed with the pulse length and pulse number fixed as 50 ms and five pulses, respectively. (B) The effect of varying pulses length was assessed with the pulse voltage and pulse number fixed at 80 V and five pulses, respectively. (C) The effect of varying pulse number was assessed at 40, 80 and 120 V with pulse length fixed at 50 ms.

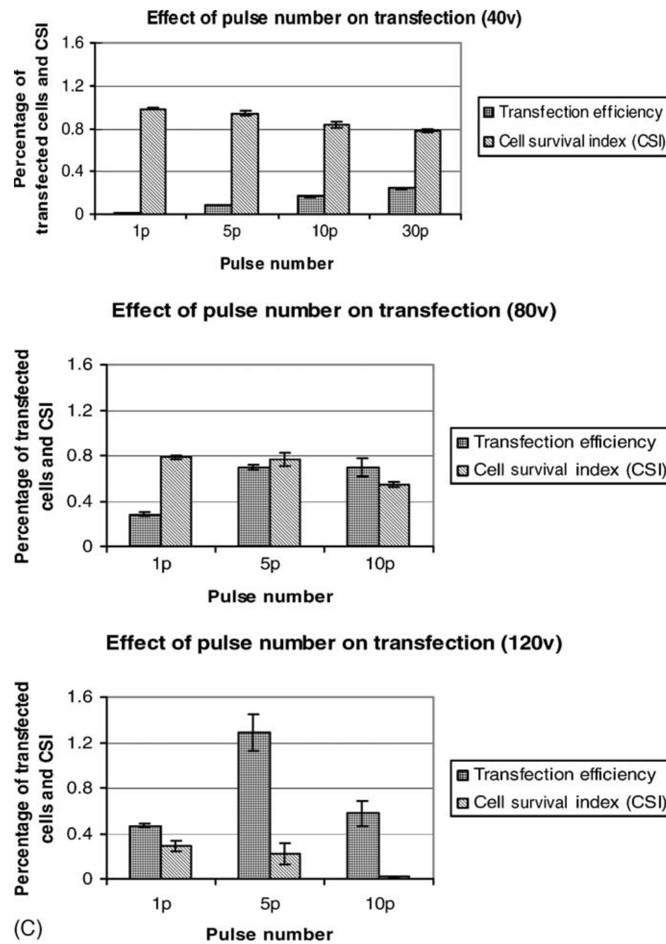

Fig. 2. (*Continued*).

Transfection efficiency was analysed using a plasmid carrying the EGFP gene downstream of a CMV promoter. This plasmid was injected into the cerebellar fissures and the tissue was subjected to electrical pulses perpendicular to the fissures while varying a range of parameters (Fig. 1B). After electroporation, cerebella were disaggregated, and granule cells cultured for 6 h to allow time for EGFP expression. Effective electroporation parameters require a balance between the requirement for temporary pore formation and DNA electrophoresis and the damaging effects of strong electric fields (Neumann et al., 1998). Cell damage was assessed by a "cell survival index" (CSI, described in Section 2) and transfection efficiency was evaluated by FACS analysis.

Analysis of cultures 6 h after electroporation revealed a low number of dead cells (Ce) similar to that in non-electroporated cultures (Cne), no matter what electric parameters we used, which is consistent with previous reports that most cell death associated with electroporation occurs immediately after the shock (Espinos et al., 2001). Thus, although the number of cells surviving the treatment varied, those cells that did not survive were no longer in evidence after 6 h in culture. Electroporation efficiency depends on a number of different parameters including voltage, pulse length and the number of pulses delivered (Kirchmaier, 2001). We first assessed the effect of voltage on the level of transfection and cell survival. It should be noted that, since only cells at the surface of the cerebellum are transfected, these make up only a small proportion of the total number of cells in the culture.

As shown in Fig. 2A, pulse voltage can dramatically affect transfection efficiency and cell survival. Selecting five pulses and varying the voltage, we found that the transfection efficiency initially rose with increasing voltage. The highest level of transfection (3.53% of the total cell population) was obtained using 150 V, above which the number of transfected cells decreased. In contrast, the cell survival index always decreased with increasing voltage, indicating that higher voltages cause more cell damage. Only about 10% of cells (CSI = 0.12) survived electroporation at 200 V, which probably explains the reduced number of cells exhibiting transfection. Since a low level of cell damage is a high priority for most experimental purposes, we chose 80 V as our optimal pulse voltage, which still produced a satisfactory transfection efficiency (0.7%) and 76% survival cells. However, even lower voltages might be used for studies where a large number of transfected cells are not required.

Varying pulse length had a less dramatic effect on transfection efficiency (Fig. 2B), although longer pulses did result in more cell death. A significant increase in transfection efficiency was achieved by increasing the pulse length from 20 to 50 ms. However, increasing the pulse length from 50 to 200 ms achieved only a 0.06% (0.70 → 0.76%) improvement in transfection efficiency. Conversely, the cell survival index decreased from 76 to 21% between 50 and 200 ms. Therefore, we chose 50 ms as our routine pulse length, since this gave an optimal balance between transfection efficiency and survival.

We next analysed the effect of pulse number on transfection, as shown in Fig. 2C. We chose to analyse the effect of pulse number at three different voltages (40, 80 and 120 V). Forty volts appeared too low a voltage to permeabilize cell membranes efficiently since even 30 pulses with this voltage only resulted in a transfection rate of 0.2%; although most cells survived this voltage. On the other hand, 120 V was considered unsuitable since even one pulse killed the majority of cells. Eighty volts was considered as relatively safe voltage, because 57% of granule cells still survived 10, 80 V pulses. Moreover, with five pulses, 80 V electroporation could result in 0.7% transfected cells. Overall, we selected a treatment of five 50 ms pulses of 80 V as the best compromise between transfection efficiency and cell survival. These results represent a significant increase in efficiency of transfection over those described by Murphy and Messer (2001) who transfected all cell types in cerebellar slice cultures. There experiments described a maximum efficiency of about 30 cells per slice (each slice representing a separate transfection experiment), as compared here to numbers in excess of 100 per slice with up to 20 slices per transfection experiment (thus, as many as 1000 or more transfected cells per transfection, see Fig. 1C).

DNA concentration is another factor that can affect transfection efficiency (Kovala et al., 2000). Optimal transfection efficiency was obtained by injection of 20 μl DNA (1–3 μg/μl) per cerebellum (data not shown). In order to diminish variance due DNA loading, in this study we always electroporated each cerebellum with 30 μl DNA (1.5 μg/μl), ensuring an excess of DNA.

3.2. Localised gene expression

The experimental strategy described herein, relies on transfection of only the most superficial cells of the EGL. It is also critical that the transfection procedure does not interfere with the normal migration and differentiation of GCPs. As seen in Fig. 1C, 5–6 h after transfection pEGFP fluorescence was obtained over most of the cerebellum surface.

In order to determine the location and development of transfected cells, cerebella were sliced after electroporation and grown in vitro. Such slices can be maintained in culture for a period of weeks. After 6 h of culture, cerebellar slices were frozen and 14 μM sections cut for immunostaining to determine whether labelled cells were dividing GCPs located in the superficial EGL or postmitotic cells located in the deep EGL. Tag1, which is considered as an early differentiation marker of granule cells (Rivas et al., 1998), was used to stain the deep EGL. Fig. 3A–D showed that after 6 h in culture EGFP-positive cells were confined to the superficial EGL, where GCPs normally reside. We did not observe any EGFP fluorescence in the deep EGL, where post-mitotic granule cells expressed Tag1. However, after 18 h in culture, many transfected cells had extended processes, and some EGFP-positive cells had already migrated into the deep EGL, where they became post-mitotic cells and expressed Tag1 (Fig. 3E and F). Finally, GFP expressing cells can be seen to migrate to the IGL and differentiate over a period of many days in slice cultures (Fig 3I and J). These results demonstrate that the cells initially transfected are restricted to the superficial EGL and the transfected cells could still migrate and differentiate normally.

3.3. Granule cell development in vitro

Analysis of granule cell primary cultures after transfection allowed us to study their development and morphology in greater detail. The cell population in primary cultures from cerebellum is very heterogeneous in terms of the maturational stage of the granule cells. We found that about 30% (31.4 ± 3.4%) of plated cells expressed Tag1 (Fig. 4A–C). These results are in agreement with a previous report, which demonstrated that cells from both the superficial EGL and deep EGL could attach to the substratum in granule cell cultures (Raetzman and Siegel, 1998). In order to examine the maturational stage of transfected cells, we electroporated cerebella with the pEGFP plasmid and immediately dissociated the cells into primary culture. After 6 h, staining of these cell cultures for TAG1 protein demonstrated that the EGFP expressing cells were uniformly TAG1 negative ($n = 50$, Fig. 4D–G). We next used BrdU incorporation to examine whether transfected cells were actively proliferating, as would be expected if they all derived from the superficial EGL. Following an 18 h BrdU pulse, almost all the transfected cells (96 ± 2%) incorporated BrdU ($n = 64$, Fig. 4H–K).

To determine whether the development of granule cells was disrupted by electroporation, we observed the morphological changes the transfected cells underwent in vitro. Just after plating, granule cells initially exhibited a round morphology (Fig. 4L), after which lamellopodia grew around the cell body after attaching the substratum (Fig. 4M). After 20 h, granule cells initiated neurite outgrowth at a single site, forming a unipolar morphology (Fig. 4N). Thereafter, a second process developed opposite the first outgrowth to give a bi-polar morphology (Fig. 4O). Finally, shorter dendritic processes were found to extend around the cell body (Fig. 4P). Such morphological changes of transfected cells accorded with those seen during normal development of granule cells in vitro (Powell et al., 1997) again confirming

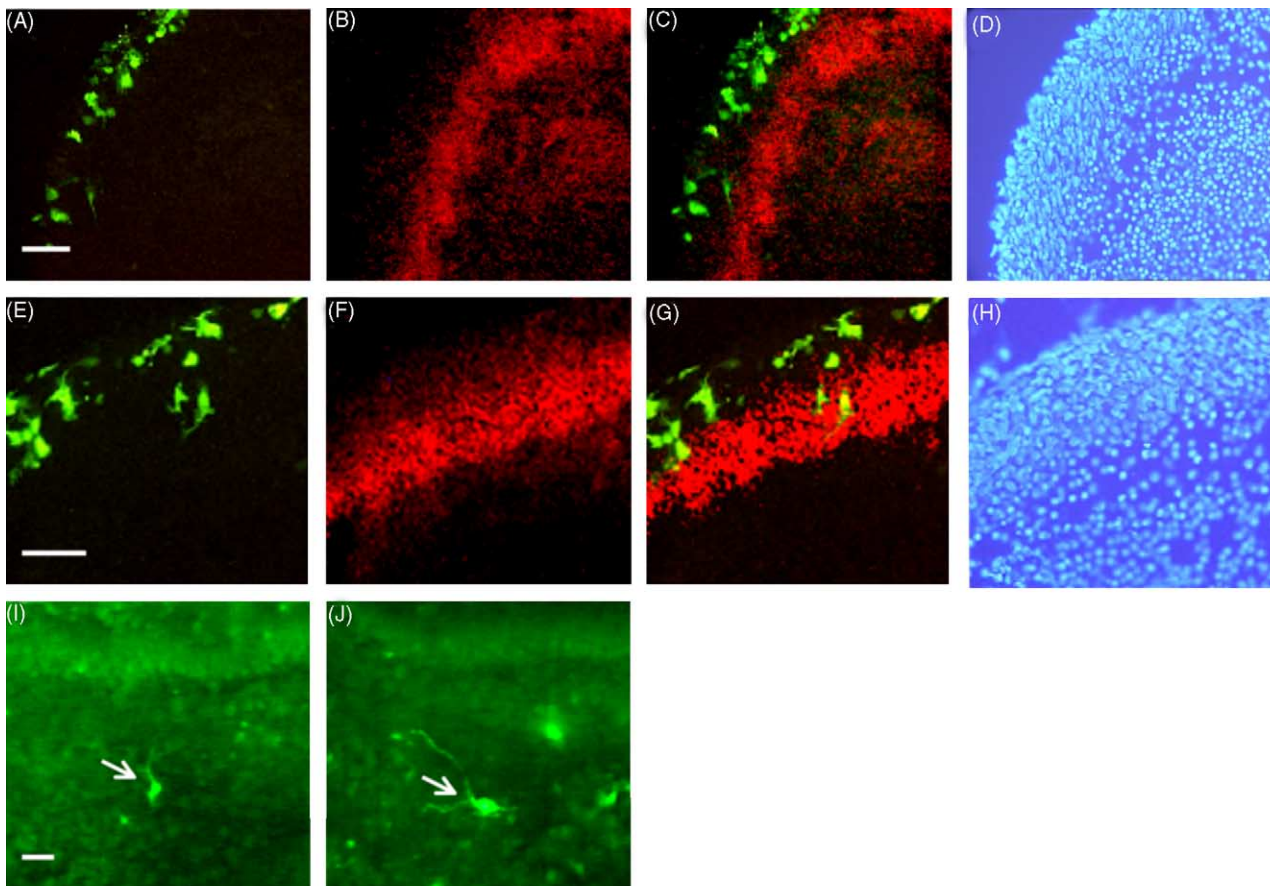

Fig. 3. Localization of EGFP expressing cells in transfected cerebellum sections were analysed 6 h (A–D) and 18 h (E–H) after electroporation with pEGFP. After 6 h, EGFP expressing cells were restricted to the superficial EGL and most transfected cells displayed round morphology (A). (B) Demonstrates that Tag1 expression was predominantly in the deep EGL (as shown by red fluorescence). A merged image (C) shows that almost no transfected cells were Tag1 positive. (D) DAPI staining identifies all cells of the cerebellum. After 18 h of slice culture following electroporation some EGFP-expressing cells changed their round morphology and extended processes (E). (F) The merged image in (G) shows that some transfected cells ($12 \pm 2.5\%$) had migrated into the deep EGL and now co-expressed EGFP and Tag1. (H) DAPI staining identifies all cells of the cerebellum. (I and J) GFP expressing cells (arrows) in the IGL 5 days after electroporation. Scale bar approximately 50 μm.

that the electroporation treatment does not disrupt normal development.

3.4. Electroporation of fluorescent dextrans

Having established conditions for electroporation of a DNA construct, we next determined if a similar approach would be effective for introducing molecules other than DNA into cells. Fluorescent dextran is a polymer of glucose that is non-toxic to cells and has a relatively lower molecular weight, making it easier for it to enter cells (Craziadei et al., 1991), thus allowing a much higher level of transfection to be achieved (greater than 20% of cells) than was obtained for the DNA transfection described above. The most important advantage, however, is that dextrans can emit florescence rapidly, allowing the labelled cells to be identified immediately after electroporation. Thus, this method is particularly useful for applications in which biochemical analysis of the proliferating superficial EGL is needed.

Cerebella were isolated and fluorescent dextrans were electroporated in a manner similar to that described above for pEGFP constructs. In order to examine which cells were labelled, cerebella were processed for frozen sectioning immediately after transfection. Sections were stained for TAG1 or β-tubulin (using Tuj1 antibody), a marker of post-mitotic neurons (Alexander et al., 1991). As was seen with DNA transfection, dye-positive cells were located in outer EGL, with almost no dye-positive cells found in the Tag1 or Tuj1-positive regions (Fig. 5). It was striking that the number of cells labelled with fluorescent dextran was far greater than was obtained using EGFP-coding DNA constructs.

3.5. FACS

Although the above methods are useful for introduction of genes into immature granule cells, and for cellular and molecular studies as those cells mature, they do not allow for

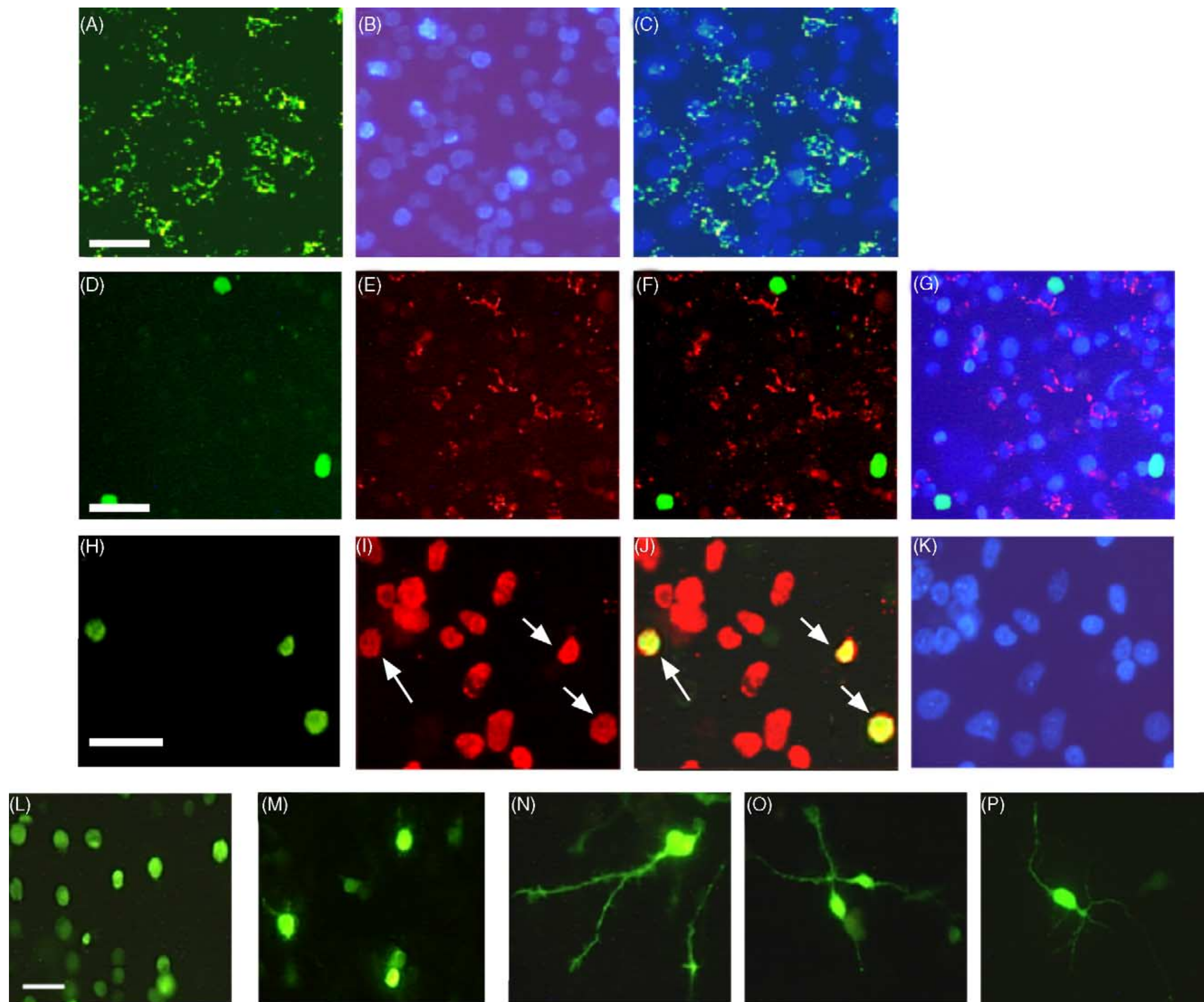

Fig. 4. Granule cell development in vitro (A–C), granule cells from 6 days postnatal cerebella were plated on coverslips and, after 30 min, stained with the Tag1 antibody. (A) Green staining represents immunoreactivity for Tag1. (B) DAPI staining of the same field. (C) Merged image of (A) and (B) shows that about 30% cells already expressed Tag1 after only 30 min in culture. (D–G) Granule cells from 6 days rat cerebella were cultured for 6 h after electroporation with pEGFP. (D) GFP-expressing cells (green). (E) Same field as (C) stained with the Tag1 antibody. (F) Merged image of (C) and (D). (G) Merged image of (F) with DAPI staining. Note that all GFP-expressing cells were Tag1-negative. (H–K) Granule cells from 6 days rat cerebella cultured for 18 h in the presence of BrdU after electroporation with pEGFP. (H) EGFP expressing cells, (I) BrdU labelled cells (red), arrows indicate GFP-positive cells. (J) Merged image of (H) and (I) with DAPI staining. (K) DAPI staining. Note that most EGFP-expressing cells also incorporated BrdU ($96 \pm 2\%$, three counts from two independent experiments, $n = 100$), although only about $50 \pm 3\%$ of the total cell population had incorporated BrdU. Thus, almost all transfected cells were still proliferating during the early period of culture and it is therefore the proliferating cells of the superficial EGL that were labelled by transfection. (L–P) Morphological changes of granule cells after electroporation. After electroporation with fluorescent dextran (L) or pEGFP (M–P), granule cells were plated on coverslips and analysed after various periods of culture. (L) 30 min culture. (M) 10 h culture. (N) 20 h culture. (O) 40 h culture. (P) 60 h culture. Scale bar approximately 25 μm.

more biochemical analyses where material must be extracted from such cells. Such applications would include RT-PCR, microarrays, and a range of protein studies. FACS provides a means to isolate the transfected cells at a high level of purity for such analyses. Moreover, because dead cells can be excluded from the desired population during sorting, the quality of protein and RNA in the separated cells will not be compromised by such contamination (Harvey et al., 1996). In our studies, we used FACS to isolate EGFP-positive or dextran-positive cells.

In order to define appropriate parameters for FACS, we designed controls to determine the background level of fluorescence. To this end, we injected pEGFP or dextran dye into the transverse fissures of cerebellum, but did not electroporate the cerebella. From Fig. 6B, it can be seen that pEGFP electroporated cerebella produce two distinctive cell populations: EGFP-positive cells and EGFP-negative cells. After 6 h culture, about 0.7% of cells are EGFP-positive based on the FACS data (Fig. 6B), while almost no cells were obtained from control cerebella within the same

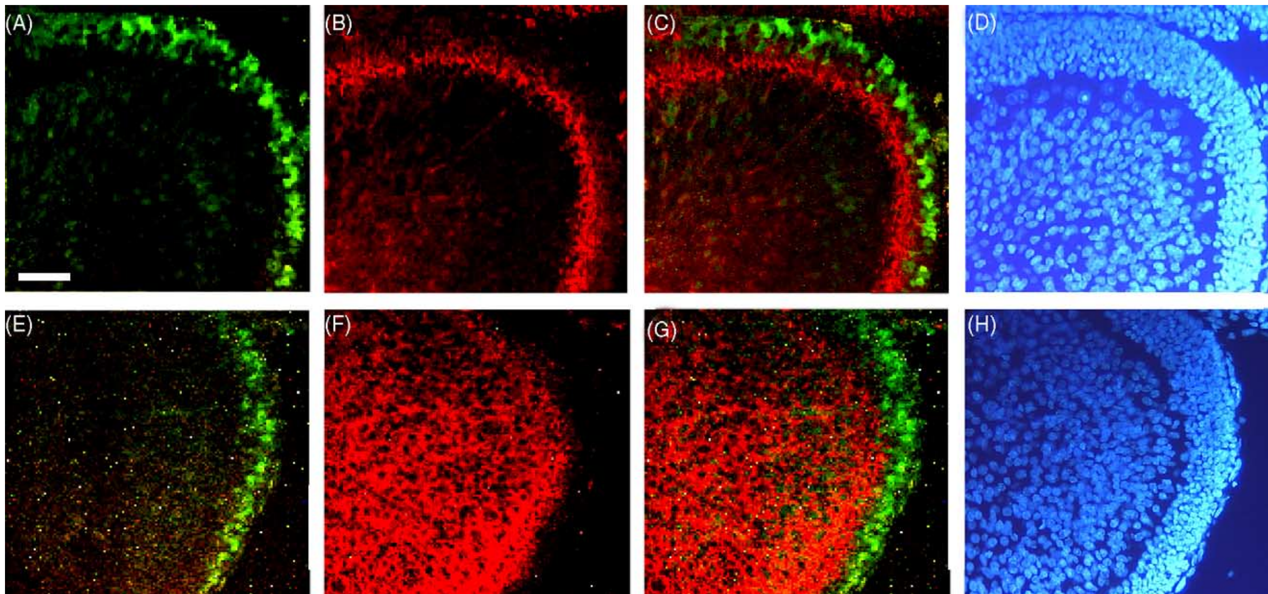

Fig. 5. Cerebellum sections immediately after electroporation with fluorescent dextran. (A–D) Same section showing green fluorescent dextran labelling evenly distributed throughout the superficial EGL (A) and Tag1-positive cells restricted to the deep EGL (B). (C) Merged image of (A) and (B) shows virtually no overlap between fluorescent dextran and Tag1 labelled populations. (D) DAPI staining. (E–H) Same section showing green fluorescent dextran labelling (A) and β-tubulin-positive cells (using Tuj1 antibody) extended through all the cerebellar layers except the superficial EGL (F). (G) Merged image of (E) and (F) shows no overlap between fluorescent dextran and β-tubulin labelled populations. (H) DAPI staining. Scale bar approximately 50 μm.

sorting region (Fig. 6A). Compared with pEGFP electroporation, electroporation of dextran dye resulted in a more variable fluorescence signal (Fig. 6D). For the control with dye injection but no electroporation, the fluorescent background was higher than pEGFP controls (Fig. 6C), possibly because the small size of dextran molecules allows them to enter a few cells without electroporation. However, this seems unlikely since cells dissociated immediately after treatment with dye, but without electroporation, exhibited no signs of dye uptake when observed under a fluorescence microscope. Another possibility is that cerebellum disaggregation resulted in death of some cells, which can emit auto-fluorescence (Craziadei et al., 1991). Similar damaged cells would be absent from the pEGFP controls because these are analysed 6 h after transfection, by which time dying cells had already floated into the culture medium and were removed prior to FACS analysis. Thus, when carrying out dye transfection it was necessary to reduce the size of the sorting region to avoid false-positive cells. Using the R2 region, about 15% of the total population were dye positive cells, compared with 0.03% from control cerebella.

To demonstrate the purity of sorted cells at the molecular level, we analysed several genes known to exhibit stage-specific patterns of expression by RT-PCR in sorted and unsorted cells (Fig. 6E). Math1 is specifically expressed in immature granule neurons in the rhombic lip and in the EGL prior to process outgrowth (Ben-Arie et al., 1997). Cdc1 and BM28 have recently been demonstrated to be expressed only in the superficial EGL (Zhao et al., 2002). When granule neurons in the EGL cease mitosis and begin to extend parallel process, the axonal glycoprotein Tag1, is transiently expressed in the deep EGL (Pickford et al., 1989). Granule cells go on to express β-tubulin as they migrate through the molecular layer and Purkinje cell layer (Wang and Zoghbi, 2001). As expected, expression of Math1, Cdc1 and BM28, markers of the proliferating superficial EGL, is much higher in sorted cells than in un-sorted cells (which would also include cells of the deep EGL). Sorted cells might also be expected to be negative for Tag1 staining. However, some Tag1 expression was detected in sorted cells, which might be due to some GCPs beginning to differentiate while still in the superficial EGL (Wolf et al., 1997). In fact, TAG1 does appear to be expressed in some proliferating cells, probably those in their final cell cycle (Furley, personal communication). Unlike unsorted cells, β-tubulin mRNA was not detectable in sorted cells, demonstrating the maturational purity of the immature GCPs obtained. Thus, although the population of transfected and sorted cells does exhibit some variation in maturational state (Tag1-negative and -positive cells) they are of restricted maturity (β-tubulin negative) and hence allow subsequent changes in maturation to be studied.

3.6. *Co-transfection of pEGFP and DsRed*

Co-expression of multiple genes is often required to study the interaction of proteins, but traditional transfection techniques are insufficient for controlled spatial and temporal expression of multiple genes in intact tissues (Haas et al., 2001). Hence, we have assessed the efficiency of co-transfection using our electroporation technique.

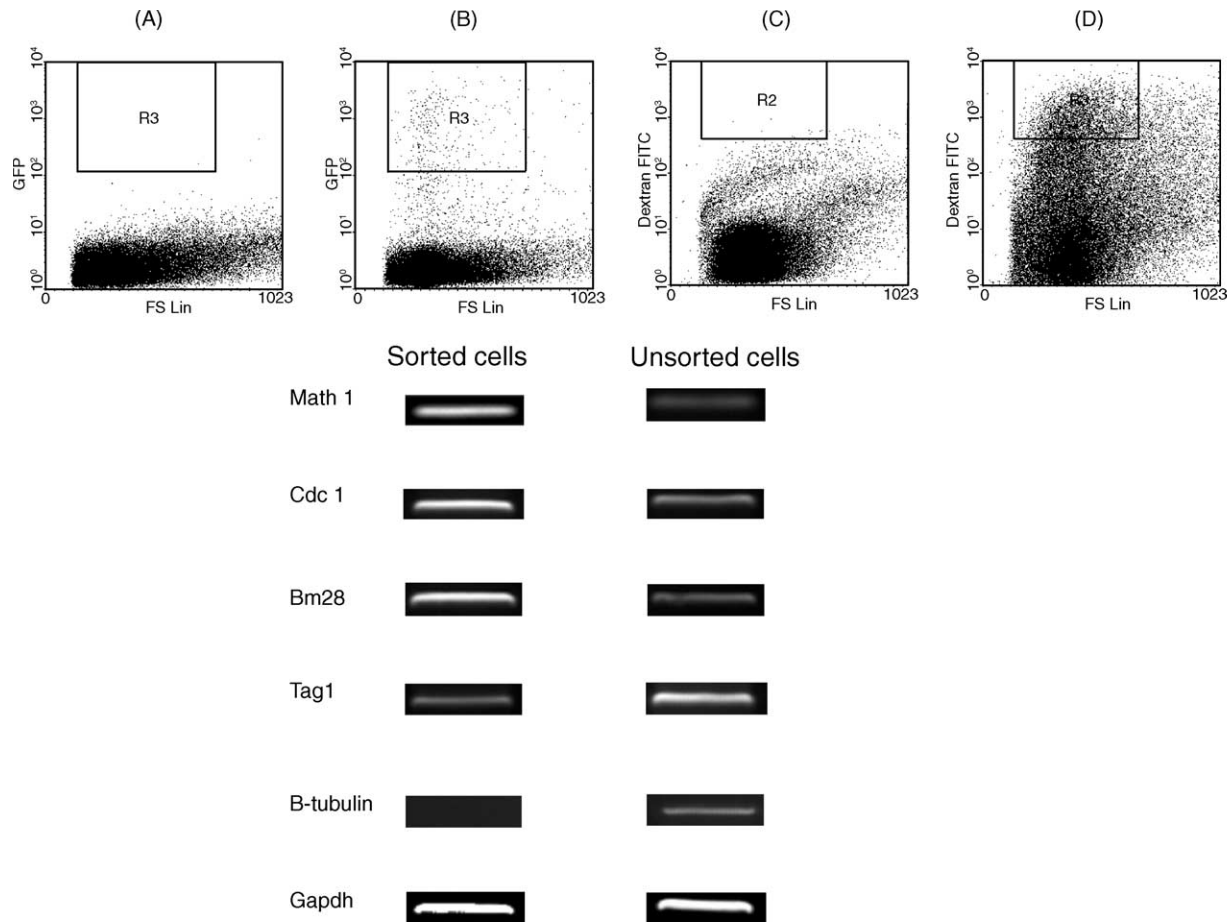

Fig. 6. FACS sorting transfected cells. (A–D) Bioparameter histograms showing log fluorescence versus forward angle light scatter (FLS) following treatments with either pEGFP or fluorescent dextran. On this scatter plot, the number of events was represented by point density. Windows R2 and R3, sorting region, represented identical FLS in each figure. (A) Granule cells cultured for 6 h after treatment with pEGFP but without electroporation. Almost no cells were obtained from windows R3. (B) Granule cells cultured for 6 h after electroporation with pEGFP 0.8% of the cell population were identified as GFP-positive located in the R3 window. (C) Granule cells analysed immediately after treatment with fluorescent dextran but without electroporation. Only 0.02% of cells were in window R2. (D) Granule cells analysed immediately after electroporation with fluorescent dextran. More than 15% of the cell population was identified as dextran-positive located in the R2 window. (E) RT-PCR analysis of FACS sorted granule cells. RNA was extracted from granule cell precursors before or after FACS sorting after electroporation with fluorescent dextran. GAPDH mRNA levels were shown as a control. Math1, Cdc1 and BM28, markers of undifferentiated granule cells, were present at higher levels in sorted cells than unsorted populations. However, Tag1 transcripts, expressed by differentiating neurons, were much less abundant in sorted cells compared with unsorted cells. β-tubulin, which is expressed in granule cells just after Tag1 during development, was only detected in unsorted cells. Thus, sorted cells are clearly enriched for the cells of the superficial EGL.

Transfection was carried out as described above, but with a mixture of two plasmids (pEGFP and DsRed). Since the green fluorescence of EGFP is brighter than the DsRed fluorescence we used the two plasmids at a ratio of 1:4 (pEGFP/DsRed). After 12 h, we observed the degree of co-transfection. As shown in Fig. 7A–D, the majority of transfected cells were seen in the superficial EGL. In order to see the level of co-transfection in individual cells more clearly we repeated the experiment but dissociated the cells into primary culture immediately after electroporation. After 12 h of culture we found 92 ± 2% of cells expressing pEGFP also expressed DsRed (Fig. 7E and F, three counts from two independent experiments, $n = 100$), and no cells were found to only express DsRed without pEGFP. Thus, this approach would be very effective for experiments where co-transfection is necessary.

4. Discussion

Our results demonstrate that electroporation is a powerful technique for the introduction of fluorescent dye or DNA into cerebellar GCPs. The procedure is simple and can be performed more easily and quickly than other transfection methods. Our results show that pulse voltage appears to be the most critical variable parameter, with pronounced effects on both transfection efficiency and cell survival. For the applications described herein, five 50 ms pulses of 80 V was

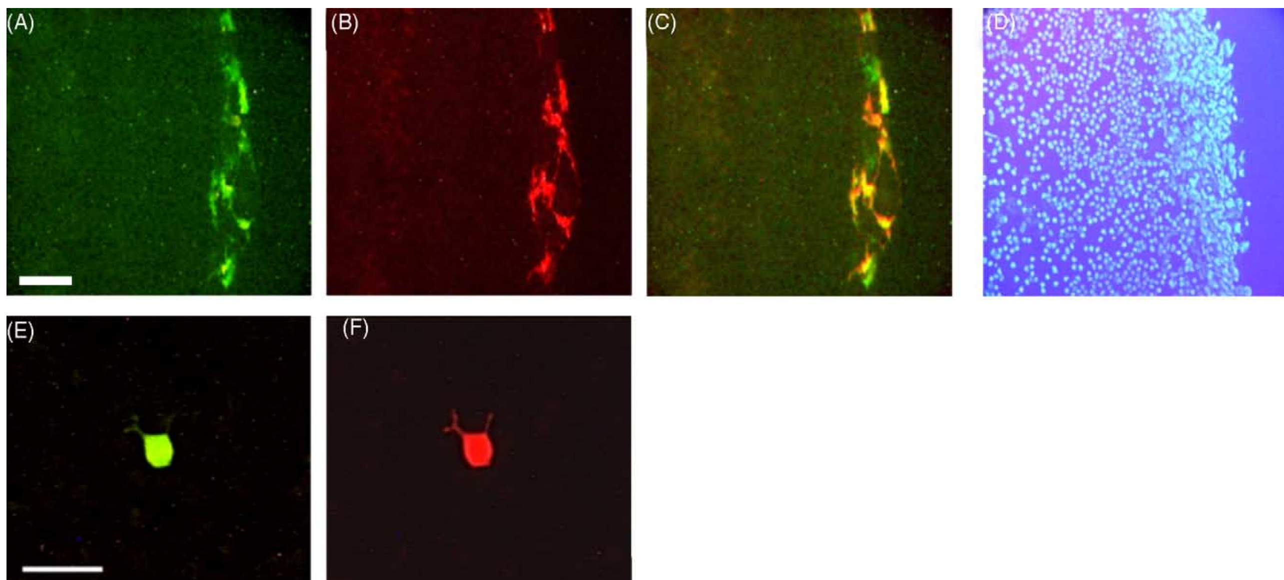

Fig. 7. Co-transfection of cerebellum with pEGFP and pDs-Red. (A–D) Section from cerebellum slice cultured for 12 h following electroporation with pEGFP and pDS-Red in a ratio of 1:4. (A) GFP-expressing cells restricted to the superficial EGL. (B) pDS-Red transfected cells also located in superficial EGL. (C) Merged image of (A) and (B), showing that most cells were co-transfected with the two plasmids. (D) DAPI staining. (E and F) illustrate the significant level of expression in single cells transfected with the two plasmids. Scale bar approximately 50 μm (A–D), 25 μm (E and F).

identified as optimal for transfection of DNA constructs, resulting in approximately 0.7% of cultured GCPs being transfected with approximately 80% cell survival.

We have demonstrated the versatility of this technique for the delivery of molecules other than DNA into cells. Electroporation of fluorescent dextran allowed rapid sorting of granule cells minutes after transfection, in contrast to the longer times required for expression of proteins following pEGFP transfection. Due to its low molecular weight and size, fluorescent dextran also yielded more than 15% labelled cells after electroporation, proving it to be an efficient method for isolating cells for molecular studies. Unfortunately, the fluorescent dye fades more quickly than EGFP, especially in dividing cells, making it unsuitable for long-term studies. Other than DNA and dextran, intracellular deposition of proteins has also been achieved by electroporation (Boron et al., 2000) suggesting that proteins could be similarly introduced into GCPs followed by analysis such as those described herein.

The greatest advantage of this method is that only GCPs in the superficial EGL are transfected and labelled. In vitro, such transfected or labelled cells were BrdU-positive and Tag1-negative, confirming that these cells came from superficial EGL.

We assessed the health of electroporated cells based on cell migration and morphological changes in vitro. After electroporation, cerebellar slices can survive several weeks on semiporous membranes. In our experiments, transfected cells underwent normal migration and differentiation. In vitro, the morphological changes the electroporated cells underwent followed: round → lamellopodium → unipolar → bipolar → T shapes, a pattern that has been described as reflecting the normal development of granule cells (Powell et al., 1997).

We also demonstrated a high efficiency of co-electroporation of individual cells with two plasmids (pEGFP and DsRed). We detected co-expression of these two proteins in over 90% of transfected cells. These experiments indicate that electroporation of a reporter plasmid (pEGFP/DsRed) can be used to identify cells co-transfected with a second plasmid expressing a gene of interest. This represents a significant improvement over current methods requiring the use of bicistronic IRES constructs, where expression levels of the gene downstream of the IRES can be quite variable (Haas et al., 2001).

In order to use this method to carry out a wide range of analyses we have shown that transfection can be combined with FACS to isolate the transfected cells. If done immediately after transfection with dye, this provides a means to study the proliferating cells of the superficial EGL. However, sorting can similarly be carried out any time after transfection allowing the cells to be analysed after any chosen period of maturation/differentiation.

It has become increasingly evident that the in vivo environment is crucial for granule cell development, but it is almost impossible to mimic such an environment in vitro. For this reason slice cultures, where the tissue architecture is maintained intact, are being used ever more widely (Tanaka et al., 1994). By culturing cerebella slices after electroporation, we have demonstrated not only that the cytoarchitecture was not damaged by electroporation, but also that the pEGFP-transfected cells can still migrate inwards. Thus, future studies will be able to use the system described here to analyse gene function in GCPs in slice culture.

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