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An efficient method for the long-term and specific expression of exogenous cDNAs in cultured Purkinje neurons

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ABSTRACT

We present a simple and efficient method for expressing cDNAs in Purkinje neurons (PNs) present in heterogeneous mouse cerebellar cultures. The method combines the transfection of freshly dissociated cerebellar cells via nucleofection with the use of novel expression plasmids containing a fragment of the *L7* (*Pcp2*) gene that, within the cerebellum, drives PN-specific expression. The efficiency of PN transfection (determined 13 days post nucleofection) is approximately 70%. Double and triple transfections are routinely achieved at slightly lower efficiencies. Expression in PNs is obvious after one week in culture and still strong after three weeks, by which time these neurons are well-developed. Moreover, high-level expression is restricted almost exclusively to the PNs present in these mixed cultures, which greatly facilitates the characterization of PN-specific functions. As proof of principle, we used this method to visualize (1) the morphology of living PNs expressing mGFP, (2) the localization and dynamics of the dendritic spine proteins PSD-93 and Homer-3a tagged with mGFP and (3) the interaction of live PNs expressing mGFP with other cerebellar neurons expressing mCherry from a *β-Actin* promoter plasmid. Finally, we created a series of *L7*-plasmids containing different fluorescent protein cDNAs that are suited for the expression of cDNAs of interest as N- and C-terminally tagged fluorescent fusion proteins. In summary, this procedure allows for the highly efficient, long-term, and specific expression of multiple cDNAs in differentiated PNs, and provides a favorable alternative to two procedures (viral transduction, ballistic gene delivery) used previously to express genes in cultured PNs.

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1. Introduction

Purkinje neurons (PNs) are key players in the circuitry of the cerebellum in that they provide the sole output from the cerebellar cortex (Hansel et al., 2001). These neurons integrate excitatory input from the axons of cerebellar granule neurons (GNs) (parallel fibers) and the axons of neurons present in the inferior olive (climbing fibers). Methods for the preparation of dissociated cerebellar cultures containing PNs from the cerebellar primordium of embryonic or newborn mice/rats have been developed (Cohen-Cory et al., 1991; Furuya et al., 1998; Gruol and Franklin, 1987; Hockberger et al., 1989; Okubo et al., 2001; Schilling et al., 1991; Tabata et al., 2000; Weber and Schachner, 1984; Yuzaki and Mikoshiba, 1992). These primary cultures are very heterogeneous, containing GNs, inhibitory interneurons and glial cells as well as PNs. Notably, the survival and development of PNs in culture depends on the presence of GNs (Baptista et al., 1994; Hirai and Launey, 2000; Hisatsune et al., 2006; Morrison and Mason, 1998). Various aspects of PN biology, including their morphological development, electrophysiological properties, sub-cellular organization, and relationships with other cell types have been investigated using these dissociated cerebellar cultures (Cohen-Cory et al., 1991; Dunn et al., 1998a,b; Gruol and Franklin, 1987; Hirai and Launey, 2000; Hirano and Hagiwara, 1988; Hisatsune et al., 2006; Hockberger et al., 1989; Kuroyanagi et al., 2009; Linden, 1997; Mashimo et al., 2008; Matsuda et al., 2006, 2010; Nagata et al., 2006; Okubo et al., 2001; Schilling et al., 1991; Stepanova et al., 2003; Tanaka et al., 2006; Tu et al., 1998; Uemura et al., 2010). Moreover, the existence of extensive afferent synapses on the dendritic spines, dendritic shafts and cell bodies of the cultured PNs has been documented (Dunn et al., 1998a,b; Hirano and Kasono, 1993; Ito-Ishida et al., 2008), and the molecular mechanisms of synaptic plasticity at the GN–PN synapse

Abbreviations: DIV, days *in vitro*; GN, granule neuron; MCS, multiple cloning site; ORF, open reading frame; PLO, poly-L-ornithine; PN, Purkinje neuron; PSD, postsynaptic density.

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has been elucidated with the help of these mixed cerebellar cultures (Chung et al., 2003; Eto et al., 2002; Hirai et al., 2003; Kawaguchi and Hirano, 2007; Launey et al., 2004; Leitges et al., 2004; Linden, 1996; Linden and Ahn, 1999; Linden and Connor, 1991; Linden et al., 1991; Lonart et al., 2003; Matsuda et al., 2000; Simsek-Duran et al., 2004; Smith-Hicks et al., 2010; Steinberg et al., 2006; Takamiya et al., 2008; Wang and Linden, 2000; Yawata et al., 2006).

The ability to transfect and express exogenous cDNAs in cultured cells is a central tool in cell biological research. In the case of PNs, the procedures that have mainly been used to deliver exogenous DNAs are ballistic gene delivery via the 'gene gun' (Chung et al., 2003; Eto et al., 2002; Leitges et al., 2004; Linden and Ahn, 1999; Lonart et al., 2003; Simsek-Duran et al., 2004; Smith-Hicks et al., 2010; Steinberg et al., 2006; Tu et al., 1998) and viral transduction (Gimenez-Cassina et al., 2007; Mashimo et al., 2008; Matsuda et al., 2006; Okubo et al., 2001; Stepanova et al., 2003). Both of these techniques possess significant disadvantages, however, as gene gun-mediated DNA delivery suffers from low transfection efficiency (Biewenga et al., 1997) and physical damage to the cells, while viral transduction is hampered by the time-consuming preparation of viral stocks, possible adverse effects on cellular physiology (Warren et al., 2006; Yedowitz et al., 2005), limitations in DNA size in the case of some viral vectors (Gimenez-Cassina et al., 2007; Lundstrom et al., 2001; Takayama et al., 2008), and safety concerns.

Recently, a highly efficient electroporation technique called nucleofection has been developed that circumvents many of the disadvantages of gene gun- and virus-mediated gene transfer. Nucleofection has been applied successfully to the transfection of several types of primary neurons, including hippocampal neurons and cerebellar GNs (Dityateva et al., 2003; Gartner et al., 2006; Zeitelhofer et al., 2007), but not to PNs. Since nucleofection is performed with cells in suspension, it is applied in the case of primary neurons to freshly dissociated cells before they are plated. Primary neurons, including PNs, take weeks in culture to mature, i.e. to develop axons, dendrites, and synapses. Therefore, the successful application of nucleofection to achieve expression of exogenous cDNAs in differentiated PNs requires the use of a promoter element that is not only sufficiently active to generate detectable amounts of protein in cells, but also active long enough (i.e. weeks) for the PNs to fully develop.

The heterogeneous nature of dissociated cerebellar cultures represents an additional challenge for the characterization of expressed cDNAs in PNs. For example, the uniform expression of a GFP-tagged protein in all of the cell types present in these densely populated, heterogeneous cultures would make it difficult to characterize the distribution of the protein within just PNs, especially since they are sparsely distributed. Furthermore, certain experimental questions require the ability to express cDNAs in PNs, but not, for example, in GNs.

Here, we provide an in-depth description and characterization of a simple and highly efficient method that allows for the high-level, long-term expression of exogenous cDNAs specifically in PNs present in heterogeneous dissociated cerebellar cultures. This method was key to our recent efforts to define the role of myosin-Va in the targeting of endoplasmic reticulum to the dendritic spines of PNs (Wagner et al., 2011).

2. Materials and methods

2.1. Preparation and transfection of dissociated cerebellar cultures from C57BL/6 mice

Dissociated cerebellar cultures were prepared from mice essentially as described (Linden, 1996; Linden et al., 1991; Schilling et al., 1991; Tabata et al., 2000), except that the cells were transfected by nucleofection just before plating (see below). Every embryo was treated separately, resulting in one culture from one cerebellum. This allows the simultaneous processing and transfection of cerebellar cells from embryos with different genotypes (followed by genotyping after the fact). Briefly, day E17 or E18 mouse embryos were obtained from timed pregnant C57BL/6 females that had been anesthetized using isoflurane (Forane, Baxter; 1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) and euthanized via cervical dislocation. Routinely, two pregnant females were processed in parallel. Sterile scissors and forceps were used. Immediately after euthanasia, the uteri with the embryos were removed and transferred into a sterile 140 mm tissue culture dish (Nunc #168381) that was kept on ice and filled with ~50 ml ice-cold, modified Hank's Balanced Salt Solution (MHS; 5.333 mM KCl, 0.441 KH_2PO_4, 137.931 mM NaCl, 0.336 mM $Na_2HPO_4{\cdot}7H_2O$, and 5.556 mM D-glucose (HBSS, Invitrogen 14185-052), 20 μg/ml gentamycin (Invitrogen 15710-064), pH was adjusted to 7.2 with NaOH, sterile-filtered). All reagents for cell culture, including MHS and culture medium (DFM; see below), were prepared using sterile-filtered, deionized and UV-treated water (KD Medical RGF-3410). Single embryos were isolated from the uteri and placed separately into the wells of a sterile six-well plate (BD Falcon #353046) containing 3 ml ice-cold MHS per well and kept on ice. Each embryo was then decapitated and the body was removed from the well. Next, each head was dissected and the cerebellar primordium was transferred into a sterile 1.5 ml tube containing 250 μl ice-cold MHS (one cerebellum per tube) and kept on ice. After all heads were processed, the isolated cerebellar primordia were minced in the tubes using a scalpel (handle #3 with blade #11) to obtain chunks of ~1 mm size. Subsequently, the cerebellar tissues were digested by adding 250 μl of freshly prepared, ice-cold papain solution (MHS containing 20 U/ml papain, Sigma P-5306) to each tube, followed by incubation in a 33 °C water bath for 30 min.

To stop the digestion, 1 ml MHS/FBS (84% (v/v) MHS, 16% (v/v) fetal bovine serum [FBS; Invitrogen 10082-139]) prewarmed to room temperature (RT) was added to each tube. After gentle mixing by inverting the tube, the cells were harvested by centrifugation at RT for 4 min at 0.6 × *g*. All subsequent steps were carried out at RT under the hood. After all supernatants were removed, 300 μl of freshly prepared DNase solution (MHS containing 11.86 mM $MgSO_4$ [Sigma M-2643] and 5 U/ml DNase I, RNase-free [Roche 10776785001]) was added to each of the harvested cerebellar cell pellets. Each cell pellet was then triturated carefully by pipetting up and down ~40 times using a Gilson Pipetman P1000 equipped with a sterile 1000 μl tip (Rainin RT-200S). The triturated cells were then passed through a 210 μm nylon mesh (Small Parts, Inc. CMN-0210-A; sterilized by submerging in 100% ethanol, followed by air-drying under the hood), collected in a fresh 1.5 ml tube, and harvested by centrifugation (RT for 4 min at 0.6 × *g*). The cells were then washed twice by resuspending them (via inverting the tube several times) in 1 ml of RT MHS and harvesting them by centrifugation (RT for 4 min at 0.6 × *g*).

The transfection of the cells was performed using the Amaxa Mouse Neuron Nucleofector Kit (Lonza VPG-1001) according to the manufacturer's instructions. The next steps (nucleofection, plating) were followed through the end for each pellet before proceeding to the next pellet. Immediately after removing the supernatant of the second wash, 100 μl of the nucleofection solution was mixed with the plasmid DNA to be transfected. This mix was then used to resuspend the cerebellar cell pellet (~1.5×10^6 cells). The resulting cell suspension was transferred into one of the cuvettes provided in the kit and subjected without delay to nucleofector program O-03 (or another program, if indicated). Immediately after nucleofection, one of the pipettes provided in the Amaxa kit was used to dilute the cell suspension with 300 μl of a mix containing 90% (v/v) DFM (see below) and 10% (v/v) FBS and to transfer the mixture