Video Article

Genetic Manipulation of Cerebellar Granule Neurons *In Vitro* and *In Vivo* to Study Neuronal Morphology and Migration

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Abstract

Developmental events in the brain including neuronal morphogenesis and migration are highly orchestrated processes. *In vitro* and *in vivo* analyses allow for an in-depth characterization to identify pathways involved in these events. Cerebellar granule neurons (CGNs) that are derived from the developing cerebellum are an ideal model system that allows for morphological analyses. Here, we describe a method of how to genetically manipulate CGNs and how to study axono- and dendritogenesis of individual neurons. With this method the effects of RNA interference, overexpression or small molecules can be compared to control neurons. In addition, the rodent cerebellar cortex is an easily accessible *in vivo* system owing to its predominant postnatal development. We also present an *in vivo* electroporation technique to genetically manipulate the developing cerebella and describe subsequent cerebellar analyses to assess neuronal morphology and migration.

Video Link

The video component of this article can be found at http://www.jove.com/video/51070/

Introduction

The cerebellum is an excellent system to study mechanisms of axon growth and migration. The cerebellum has been the subject of anatomical studies since the dawn of neuroscience[1]. Modern microscopy and immunohistochemical techniques have significantly expanded and refined the initial discoveries by Santiago, Ramon, and Cajal[2-4]. Mouse genetics and molecular studies uncovered essential growth and transcription factors in the control of cerebellar development, which led to greater understanding of crucial events required for proper wiring of different types of neurons including cerebellar granule neurons (CGNs)[5-7].

The cerebellum is a derivative of rhombomere 1 of the developing hindbrain[8]. The rhombic lip, which is part of the roof of the 4th ventricle, gives rise to cerebellar granule neuron progenitors, which will constitute the most numerous neuronal population in the adult cerebellum[9]. Following rostral migration, they settle in the cerebellar anlage. Here, mitosis of granule neuron precursors leads to the dramatic expansion of the external granular layer (EGL), which takes place postnatally in rodents. From the EGL, neurons start migrating inward through the molecular layer (ML), past the Purkinje cell layer to ultimately take up residence in the internal granular layer (IGL[2]). During this migratory process, they acquire a bipolar shape with two axons extending into the ML. Upon further migration, the cell body migrates away from the axons and the two processes fuse to form one bifurcated, T-shaped axon[10]. Subsequently, these axons fasciculate and are referred to as parallel fibers. Having settled in the IGL, CGNs grow dendrites, which form dendritic claws to establish synapses with mossy fibers. To examine fundamental processes in the developing cerebellum, a combined *in vitro* and *in vivo* approach allows for reliable results and conclusions.

CGNs are not only the most numerous neurons of the cerebellum but of the entire brain and can be cultured to high purity[11-13]. In culture, this highly homogeneous neuronal population becomes rapidly postmitotic and acquires a polar morphology with easily identifiable axons and dendrites. Cultured CGNs have proven to be extremely useful to study various aspects of neuronal development including progenitor proliferation, differentiation, axonal and dendrite development, neuronal migration, apoptosis and electrophysiological properties ([14-19] and many others). The use of genetic manipulation has expanded the versatility of cultured CGNs and allowed for further mechanistic insight into the aforementioned events. Transfection of cultured neurons using low-efficiency calcium phosphate or lipophilic methods followed by immunocytochemistry with polarity markers or software-supported analysis facilitates the assessment of *e.g.* the morphology of individual neurons in a dense neuronal culture. With this approach, the role of proteins of interest in axon or dendrite growth can be studied[20-25,26-28]. This culture system however is less useful to analyze neuronal migration as migration is very limited in high-density cultures and would require cocultures. The *in vitro* analysis of axon and dendrite growth also allows for the examination of interconnected proteins of a signaling pathway using combinations of RNA interference (i), over-expression or small molecules.

To establish the relevance of the protein of interest in axon and dendrite growth regulation or neuronal migration, the *in vivo* electroporation (IVE) technique allows for the analysis in the developing cerebellar cortex. Owing to the fact that cerebellar development in rodents extends way into the first two postnatal weeks, the cerebellum represents an accessible brain structure for genetic manipulations to examine developing axons

and dendrites, neuronal migration, synaptogenesis and apoptosis[20-24,29,30,26,27,31-34]. In addition, this model system is also useful for other aspects of neuronal development that require the intact cerebellar cortex such as axon pathfinding, wiring and connectivity of neurons and neuron-glia interactions Taken together, this protocol provides *in vitro* and *in vivo* techniques to tackle a complementary approach regarding neuronal morphogenesis and migration.

Protocol

CGNs can be prepared either from postnatal day (P) 5 mouse pups or P6 rat pups. We follow a protocol, described by Bilimoria and colleagues, which uses a mitotic inhibitor to select for postmitotic CGNs[13].

Ethics statement:

All experiments involving live animals have been conducted according to the animal protocol approved by the "Verbraucherschutz und Lebensmittelsicherheit" of Lower Saxony, Germany.

***In vitro* assay:**

1. Preparation of DNA Plasmid, Media, and Buffers for the Calcium Phosphate Transfection Method

1. Dissolve plasmid DNA in sterile, endotoxin-free water; DMEM (high glucose); make 2.5 M $CaCl_2$; make 2x HBSS (dissolve 4 g NaCl, 0.1775 g KCl, 0.095 g $Na_2HPO_4 \cdot 7H_2O$, 0.675 g glucose and 2.5 g HEPES in 250 ml ultrapure water and adjust pH to 7.05, 7.08, and 7.11). Note: When preparing the 2x HBSS solution, test which pH gives best results regarding transfection efficiency of given combination of plasmids.

2. Transfection of Cultured Neurons

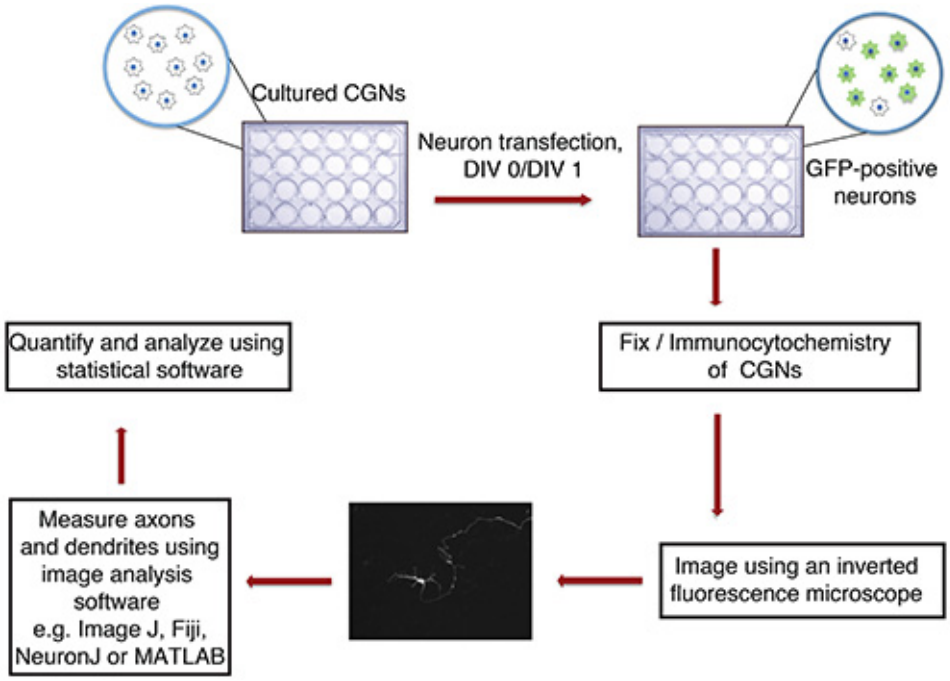


Figure 1. Flowchart of *in vitro* axon and dendrite growth assay. Cultured CGNs (24-well plate with glass coverslips), isolated from P6 rat pups, are transfected at DIV 0 or 1 with DNA precipitate containing a fluorescent transfection marker (*e.g.* GFP). After fixation and immunocytochemistry, neurons are imaged in a blinded manner. Images are imported into ImageJ and processes are measured. Measurements are then processed using a statistical program.

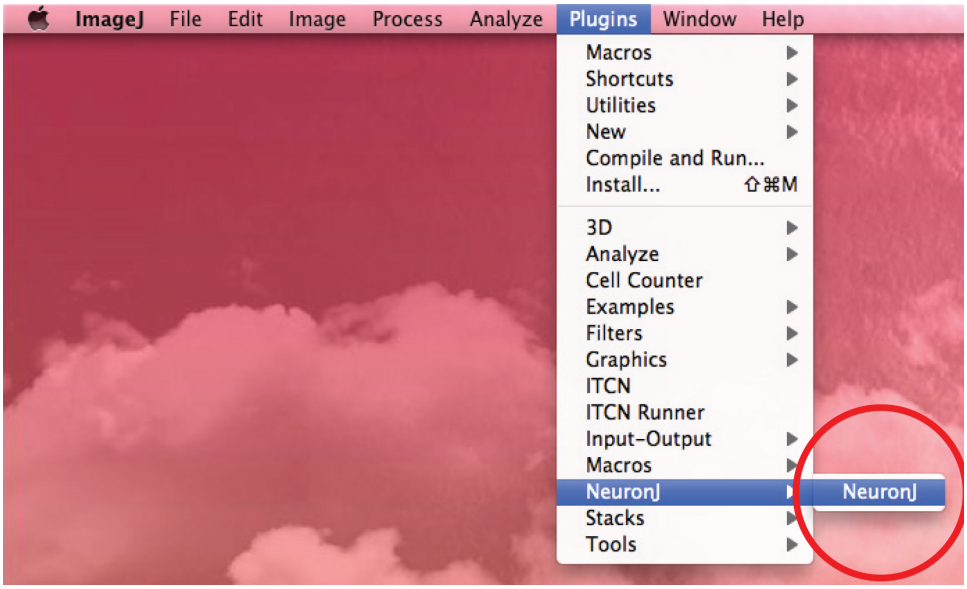
1. Seed CGNs (20 x 10^6 per 24-well plate; BME, 10% calf serum, 2 mM Penicillin-Streptomycin-Glutamine (PSG), 25 mM KCl) on nitric acid-washed, polyornithine-coated 12 mm glass coverslips in a 24-well plate with 500 µl of media per well.
2. On day *in vitro* (DIV) 0 (at least 8 hr after plating) or DIV 1, collect growth media and keep at 37 °C. Wash neurons twice with 500 µl of prewarmed DMEM and add 500 µl of DMEM.
3. Place neurons in incubator (37 °C, 5% CO_2) for 45 min.
4. Prepare 40 µl DNA precipitate for each well by mixing: DNA (2-2.5 µg/well, 10% of total DNA should be a transfection marker *e.g.* GFP to visualize transfected neurons), water (up to 18 µl), add 2 µl of 2.5 M $CaCl_2$, mix well and add 20 µl of 2x HBSS.
    1. Incubate DNA precipitate for 5 min at RT.

5. Add DNA precipitate to each well and incubate neurons for 18 min in incubator.
6. Remove DMEM/DNA mix and wash neurons twice with 500 µl of prewarmed DMEM.
7. Add collected media from step 2.2 back to neurons. If neurons will be in culture for more than 3 days, supplement media with 25 mM glucose at DIV 3 to replenish carbon source.
8. After 1-5 days, subject neurons to immunocytochemistry using GFP antibodies.
9. Image at least 30 individual neurons per condition in a blinded manner using a fluorescent microscope.

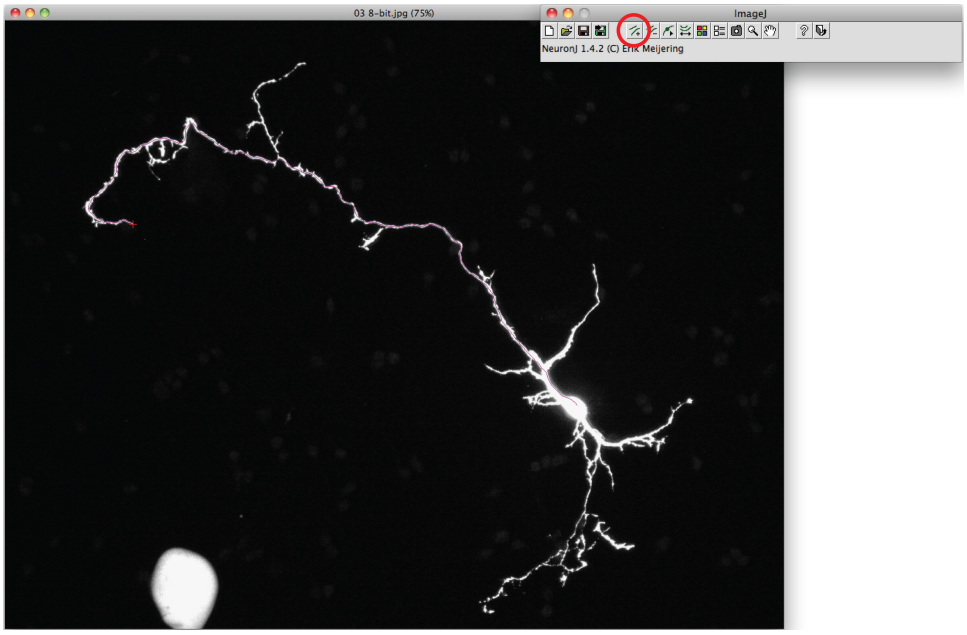
3. Measure Axons and Dendrites with NeuronJ, an NIH ImageJ Plugin

Important: Ensure that Images are scaled correctluy by using appropriate pixel:µm ratio depending on magnification and resolution of image.

1. Convert images to 8-bit with ImageJ: Open image, choose 'Image' -> 'Type' -> '8-bit' -> 'Save' image.
2. Run NeuronJ plugin and open the 8-bit image.

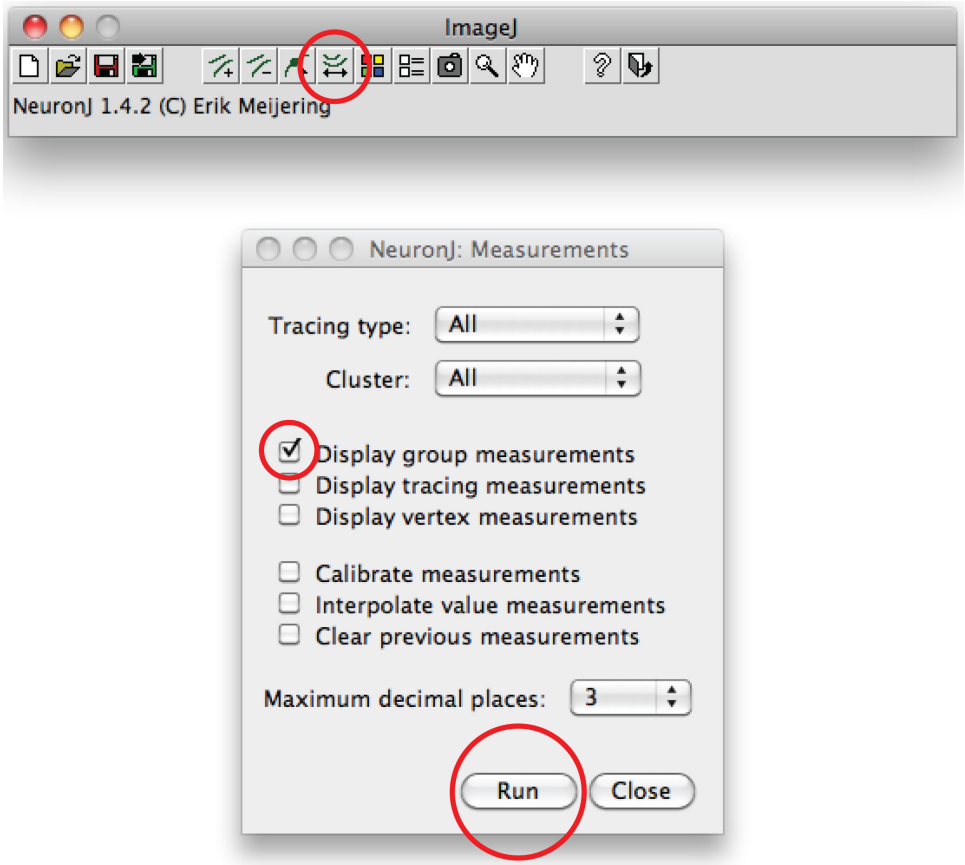


3. Use the 'Add tracings' option to track the axon: click the left mouse button once at the beginning of the axon and move the mouse along the process. Double click on tip of axon if trace matches axonal shape.



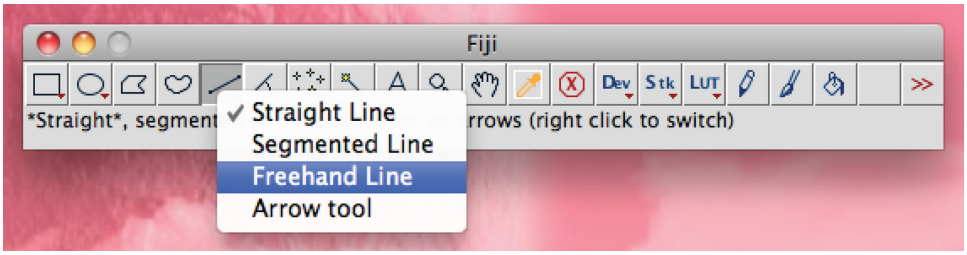
Note: Should the suggested trace differ from axonal shape, click once on the axonal process to anchor trace, then double click on tip of axon.

4. Click on 'Measure tracings', choose the 'Display tracing measurements' option and press 'Run'. Axon measurements are all displayed in a new window. For dendrites, choose the 'Display group measurements option' and press 'Run'.



Total dendrite measurements are all displayed in a new window. Save them as a separate file that can be opened in any spreadsheet program.

5. Alternatively for manual tracing, use Fiji software: Right click on the 'Straight line' option, choose 'Freehand line',



keep the left mouse button pressed and manually trace the process, press 'Ctrl+M' to measure.

6. Calculate average axonal/dendritic length per condition and use appropriate statistical test.

***In vivo* electroporation:**

1. Equipment and Preparation of Reagents

1. You need 30 G needles, spacer (1-2 mm), syringe, dead volume reducer (DVR), electroporator, and tweezertrodes, heating pad or infrared heat lamp, gooseneck lamp and isoflurane.
2. Put DVR into needle, then attach needle to syringe, and finally put spacer onto needle (**Figure 2**).

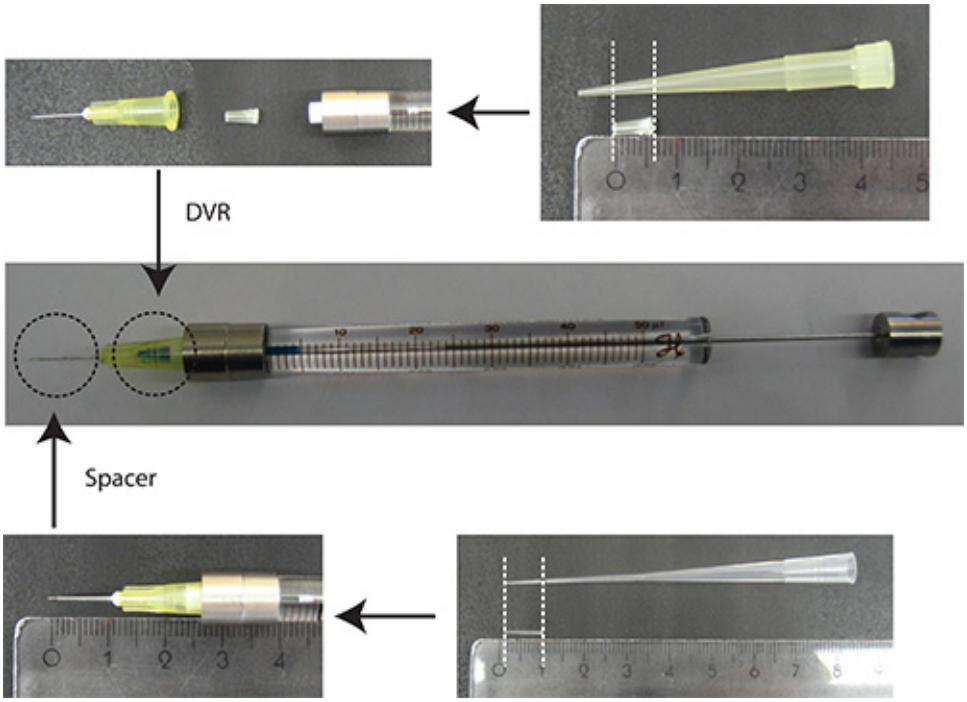


Figure 2. Preparation of needle. DVR is cut off a 200 µl pipette tip and placed into the needle to reduce the dead volume. Spacer is derived from a 200 µl loading tip and is placed on the end of the needle to regulate the depth of penetration into the cerebellum to approximately 2 mm. Ruler units: cm

3. Dissolve DNA in PBS/0.03% Fast Green. Note: As a transfection marker, it is advantageous to use a fluorescent protein that is under a neuron-specific promoter (*e.g.* Synapsin) to visualize neurons only. 25% of the total plasmid amount should be the plasmid encoding the transfection marker.
4. Make 70% ethanol.
5. Mix equal volumes of OCT and 30% sucrose dissolved in PBS.
6. Fill syringe with 4 µl of DNA (4 µg/µl of plasmid DNA in PBS/0.03% Fast Green).

2. IVE of Rat Pups

Flowchart of IVE: see **Figure 3**

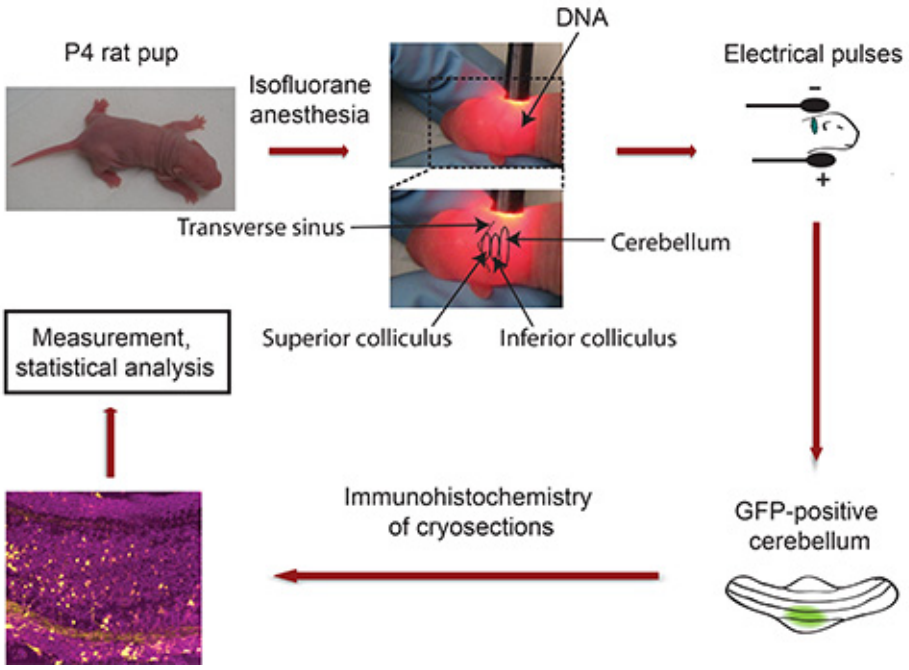


Figure 3. Flowchart of *in vivo* electroporation. P4 rat pups are anaesthetized with Isoflurane and plasmid DNA encoding a fluorescent transfection marker (*e.g.* GFP) is injected into the cerebellum, followed by exposure to 5 electrical pulses. Five days later, isolated GFP-positive cerebella are sectioned and subjected to immunohistochemistry. Images are captured using a confocal microscope and analyzed using Imaris software. Data are processed with a statistical program.

1. Use P4 rat pups from albino strain (Wistar or Long Evans).
2. Anesthetize pups (one after another) with isoflurane in small box (*e.g.* P1000 pipette tip box) with 200 µl of isoflurane (soaked in tissue) for 1-2 min until pup is no longer moving. Take care that pups do not get into contact with liquid isoflurane. Monitor time closely as individual pups respond differently to anesthesia.
3. Sterilize back of pup's head with 70% ethanol.
4. Fix head of pup between thumb and index finger and use gooseneck lamp to locate cerebellum of albino pup. The transverse sinus sharply demarcates the midbrain (superior and inferior colliculus) from the cortical hemispheres (**Figure 3**). The cerebellum is located adjacent to midbrain and appears in a darker shade. Use a permanent marker to indicate the cerebellum with a dot. Important: Keep pup in fixed position! Note: Should anesthesia wear off during this procedure, expose pup to isoflurane prior to injecting the DNA.
5. Insert needle (**Figure 3**) and slowly inject 3 µl of DNA into the cerebellum.
6. Let the DNA solution diffuse for 30-60 sec.
    1. Place head of pup between tweezertrodes so that the minus pole makes contact with the back of the head (cerebellar region) and that the plus pole contacts the opposite side of the head (**Figure 3**).
    2. Subject pup to 5 electrical pulses. Adjust voltage to weight of pups to ensure good electroporation efficiency without compromising their survival (**Table 1**).

Weight	Voltage	Pulse	Interval
8-9 g	160 V	50 msec	950 msec
9-10 g	165 V	50 msec	950 msec
> 10 g	170 V	50 msec	950 msec

Table 1. Electroporation of P4 rat pups.

    3. Let pups recover on heated pad or underneath an infrared lamp. Return pups to dam. Important: Make sure that heating source does not inflict any burns.

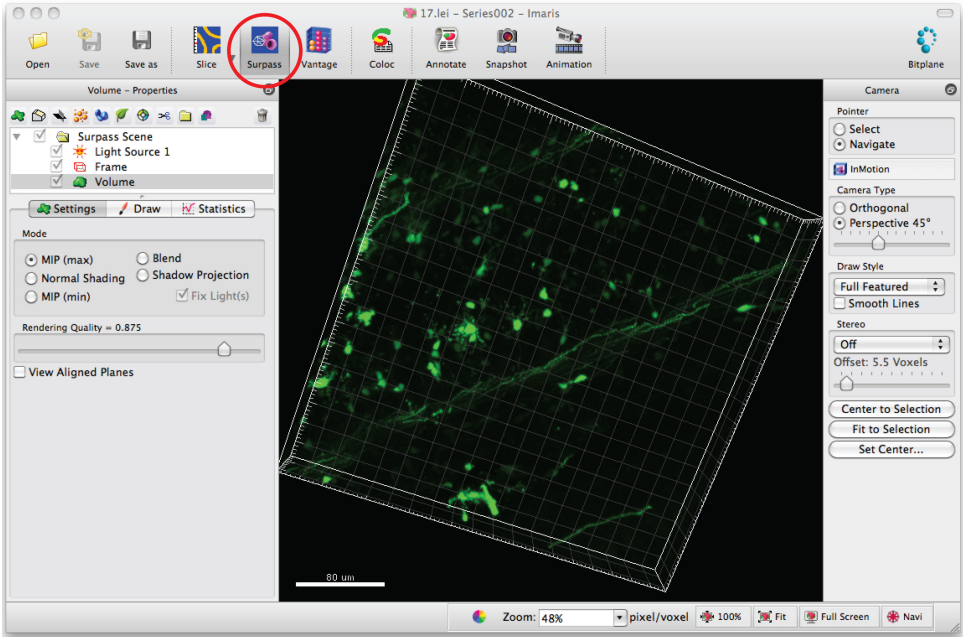
7. Sacrifice pups 5 days after electroporation by placing them in CO_2 followed by decapitation.
    1. Isolate the cerebella and screen for GFP-positive ones cerebella using a fluorescent microscope.

8. Fix cerebella in 4% PFA O/N at 4 °C, then incubate in 30% sucrose at 4 °C until cerebella sink to bottom of tube.
9. Embed cerebella in OCT/30% sucrose and cut 40 µm coronal sections using a cryostat. Note: Section each cerebellum in a blinded manner.
10. Subject sections to immunohistochemistry using the GFP antibody. Counterstain with nuclear dye (DAPI or Hoechst 33258) and determine localization of at least 200 transfected neurons per animal.
11. For an in-depth analysis, subdivide the IGL into halves, resulting in an upper IGL facing the ML and a lower IGL facing the white matter and count GFP-positive neurons residing in each half. Note: Count GFP-positive neurons of each section in a blinded manner.

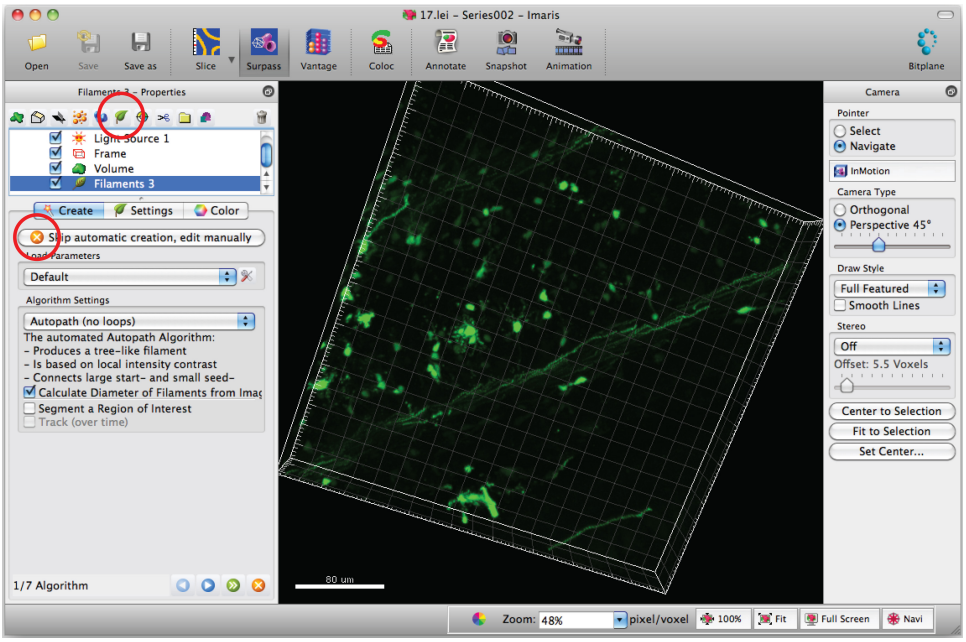
3. Measuring Dendrite Length, Acquire the Images of the Section in x, y, z Plane Using a Confocal Microscope

Note: for example, use 40 images for a 40 µm section with a z-stwp of 1 µm.

1. Open image series in the software, Imaris, to generate a 3D image of the dendrites.
2. Click on 'Surpass' mode to view the neuron in 3D.

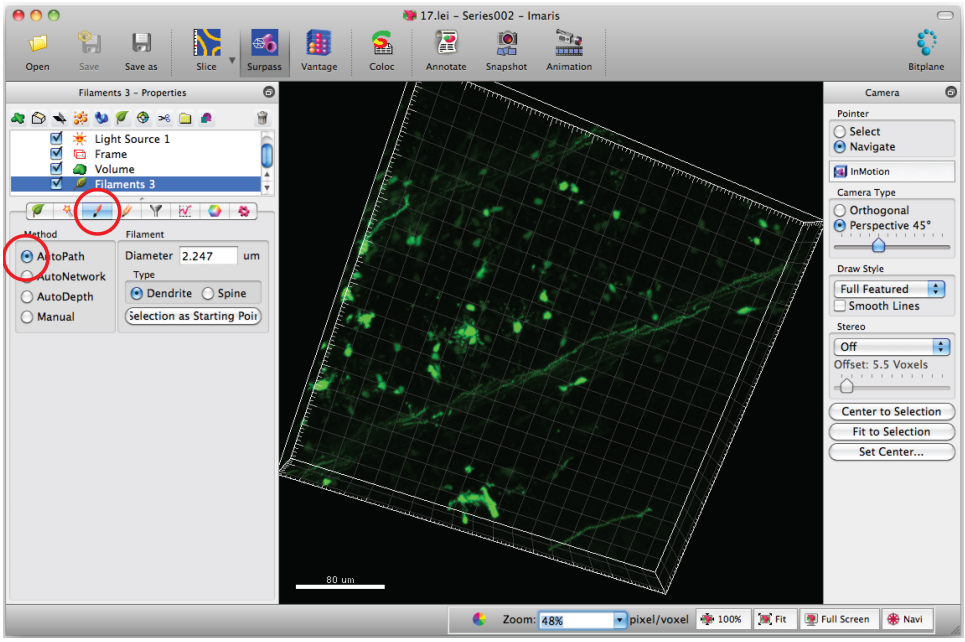


3. Select 'Add new filament' and click 'Skip automatic creation' to start semiautomatic tracing.

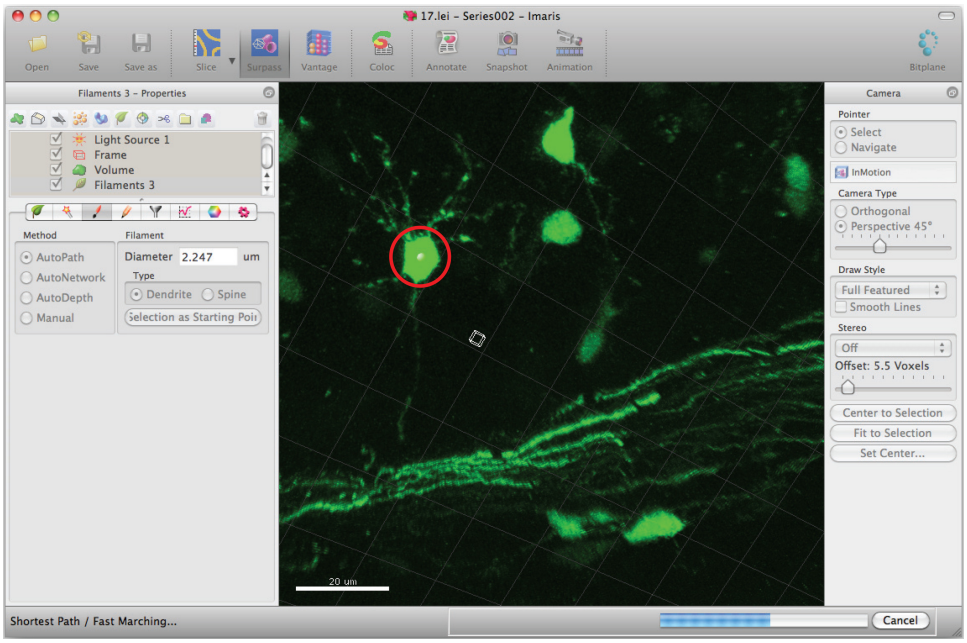


Note: Analyze each 3D image in a blinded manner

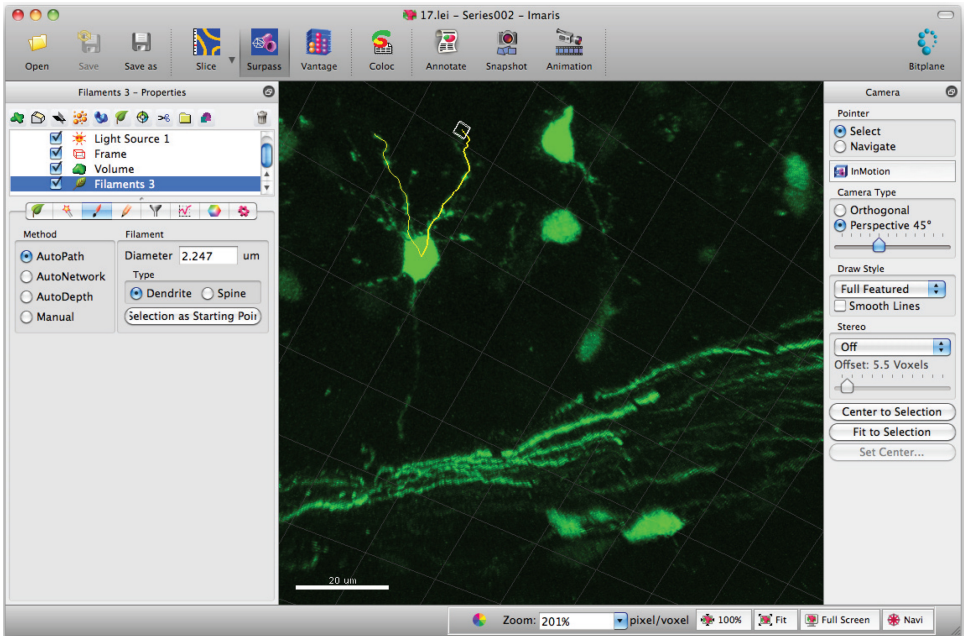
4. Select 'Draw' tab and 'AutoPath'.



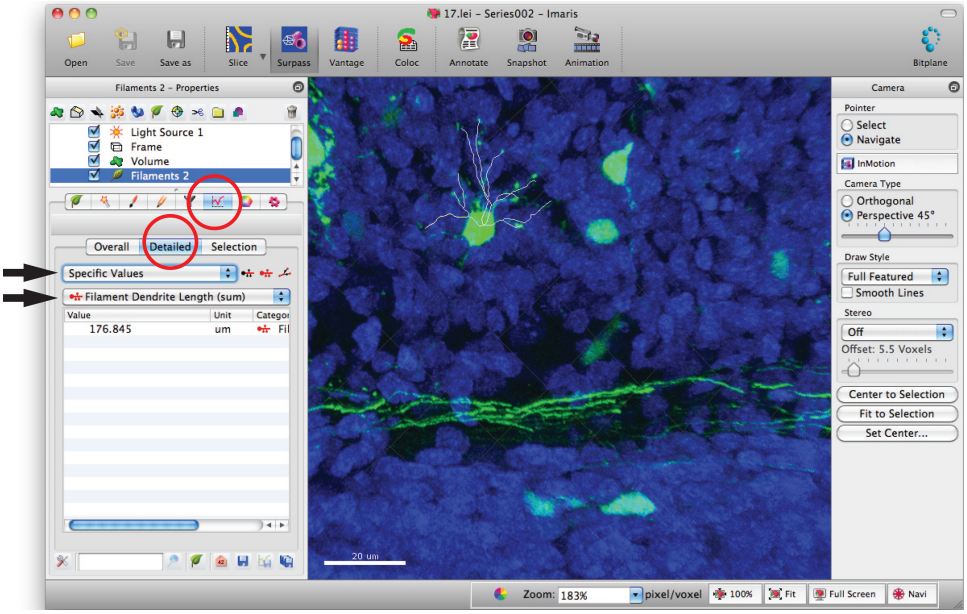
5. Move the mouse cursor on the cell body and Shift+mouse right click to select the cell body. Note: Autocalculation by software may require a few minutes.



6. Add paths to the filament (dendrite) using Shift+mouse left click. Note: Paths can be visualized in real time.



7. Go to filament statistics window and click on 'Detailed', 'Specific values' and 'Filament dendrite length (sum)' for sum of total dendrite length.



8. Use appropriate statistical tests to analyze data.

Representative Results

To analyze the morphology of CGNs in response to different culturing conditions, we transfected the neurons on DIV 0 as described above. After transfection, we placed one set of neurons into full medium (BME, 10% calf serum, 2 mM PSG, 25 mM KCl) and another set into minimal medium containing insulin (BME, 25 mM glucose, 2 mM PSG, 10 µg/ml insulin). We subjected the neurons to immunocytochemistry using the GFP antibody at DIV 1, 2, and 3, followed by measuring axons and dendrites for set 1 and axons only for set 2. Owing to serum and KCl, which provide growth factors and mimic neuronal activity, respectively, axons and dendrites developed and grew rapidly over the time window indicated (**Figure 4A**). Axonal growth of set 2 was mainly a consequence of intrinsic stimulation and thus much reduced. Dendrites however failed to develop properly owing to the lack serum and KCl, which stimulate dendrite growth (**Figure 4B**).

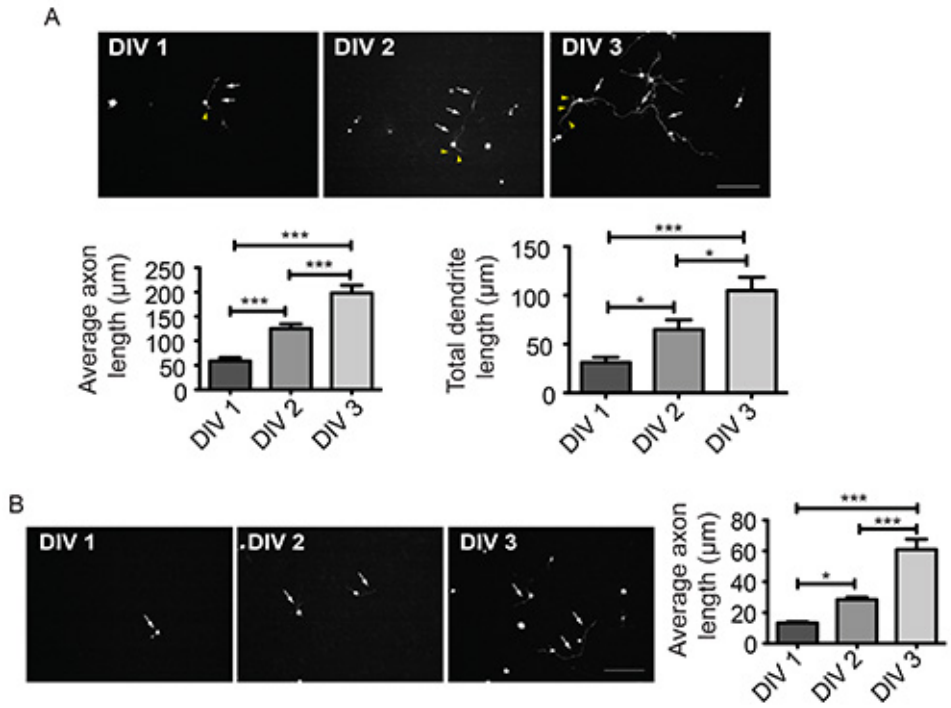


Figure 4. Analysis of axon and dendrite growth in CGNs (**A, B**) CGNs, transfected with a plasmid encoding GFP at DIV 0, were cultured for 1, 2, or 3 days in either full medium (**A**) or BME supplemented with insulin (**B**). After fixation, neurons were subjected to immunocytochemistry using the GFP antibody and axon and dendrite lengths were measured. A total of 82 (**A**) and 65 (**B**) neurons were measured (ANOVA, *p<0.05, ***p<0.001, mean + s.e.m.). White arrows and yellow arrowheads indicate axons and dendrites, respectively. Scale bar equals 100 µm. Please click here to view a larger version of this figure.

To perform morphological analysis of the rat cerebella, we subjected P4 pups to IVE as described above and isolated cerebella 5 days later. Immunohistochemistry of 40 µm coronal cryosections revealed that 86% GFP-positive neurons descended from the EGL into the IGL (**Figure 5A**). Among those, 50% were observed in the upper part of the EGL and 36% migrated farther into the lower part of the IGL. We also determined dendrite growth of three independent electroporated cerebella and compared average lengths (**Figure 5B**).

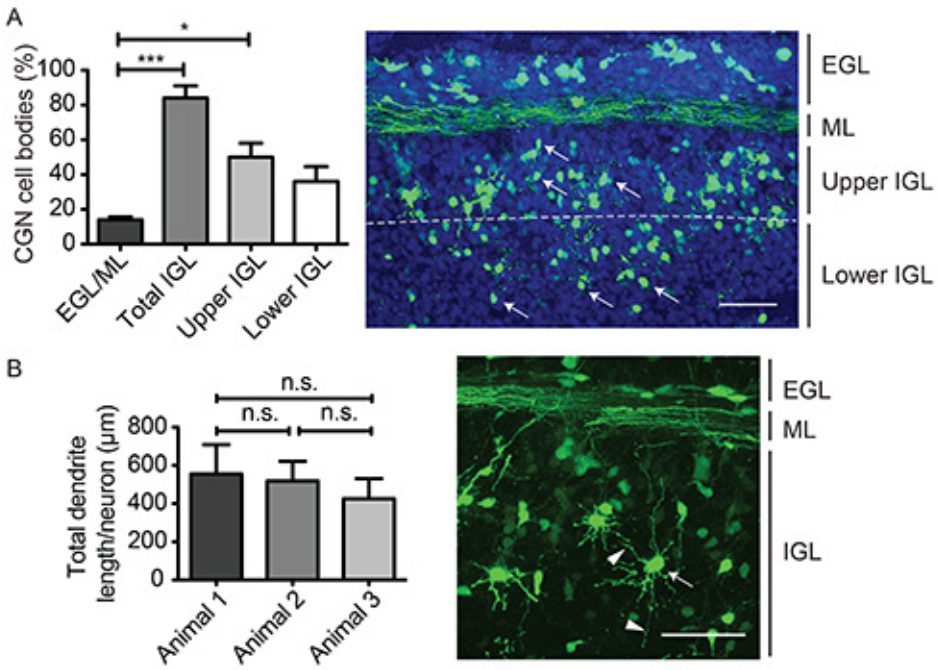


Figure 5. Analysis of neuronal migration and dendrite length in *in vivo* electroporated cerebella. Cerebella of P4 rat pups were electroporated with the pSyn-GFP plasmid and isolated 5 days later. 40 µm coronal sections were subjected to immunohistochemistry using the GFP antibody. (**A**) Localization of CGNs in cerebellum was assessed. (Kruskal Wallis, Mann Whitney correction, *p<0.05, ***p<0.001) (**B**) Total dendrite length was measured using Imaris software (ANOVA, Bonferroni correction, n.s. = nonsignificant, mean + s.e.m.). Arrows indicate CGN cell bodies. Arrowheads indicate dendrites. Scale bar equals 50 µm. Please click here to view a larger version of this figure.

Discussion

Advantages and limitations of the described *in vitro* and *in vivo* methods:

Cultured CGNs from mouse and rats are equally well suited for morphological analyses. Owing to the bigger size of a rat cerebellum, the yield of CNGs from rat pups exceeds that of mouse pups 3-4x. Aside from CGNs, cortical and hippocampal neurons can be used as culture system as well. The calcium phosphate method results in a low (0.01-5%) transfection efficiency, which is desired to analyze the morphology of individual neurons. Alternative lipophilic transfection methods can be used as well, but are excessively expensive without further gain. Viral transfection methods of high-density cultures such as CGNs should be avoided as high efficiencies will make it very difficult to distinguish individual processes. In CGNs, we typically find a correlation of transfection efficiency and days *in vitro*. The longer the neurons have been cultured, the less affected they will be by transfection-induced stress. As a consequence the transfection efficiency will go up. Also, to focus on intrinsic mechanisms of axon growth and exclude the influence of growth factors derived from serum present in the media, CGNs can be cultured in survival media supplemented with insulin, a cheap surrogate for insulin-like growth factor 1 (IGF-1), which promotes neuronal survival[35]. A media change from full media to insulin-containing media must occur either at DIV 0 or DIV 1 to prevent serum/KCl-withdrawal-induced apoptosis. The analysis of dendrite growth can be performed analogous to the axon growth assay. It is however important to keep CGNs in media supplemented with KCl to simulate neuronal activity, which promotes dendritic development. Analysis of neuronal migration should be carried out using the *in vivo* electroporation technique.

The transfection of cultured neurons will typically entail the cotransfection of at least two plasmids: a plasmid encoding the transfection marker, which can be a plasmid coding for a fluorescent protein (*e.g.* GFP) or b-Galactosidase, and either RNAi or overexpression plasmids. To ensure the successful coexpression of plasmids, the recommended amount of transfection marker should be 10% of the total amount of DNA (2-2.5 µg/well of a 24-well plate). We have previously established that the transfection of equal amounts of DNA (GFP together with DsRed) results in more than 85% of neurons coexpressing the two plasmids[22,24] . Should knockdown, overexpression or exposure to small molecules induce neuronal cell death, a plasmid encoding Bcl-XL can be cotransfected to ensure neuronal survival without effects on morphology[20]. Cotransfection of up to three or four plasmids is also unproblematic, which is useful to carry out epistasis analyses to establish a linear pathway or synergy of two proteins in axon or dendrite growth. Here, two independent RNAi or overexpression plasmids or a combination of both can be cotransfected together with a transfection marker.

The *in vivo* electroporation technique is an ideal method for the analysis of neuronal migration in developing cerebellar cortex. It is of advantage to use pups from an albino strain as compared to a strain with dark complexion. Also, it is easier to work with rat pups owing to their larger sized cerebella. In our hands, almost all cerebella are GFP-positive but to different degrees. A well-electroporated cerebellum has many hundred GFP-positive neurons, a badly-transfected cerebellum less than 100. With a little practice, it is also possible to use mice if transgenic mice are required for the study. This method is significantly faster than the generation of transgenic mice and it allows for the analysis of different conditions (loss-of-function, gain-of-function and structure-function-analyses). IVE does not require stereotaxis, a gooseneck lamp is sufficient to

detect the cerebellum of a fairly translucent albino pup. This will also shorten the procedure time per pup and a brief anesthesia with isoflurane suffices. It does however require a little practice to target the correct region and thus to master the technique.

Developmental problems caused by knockdown or overexpression are insignificant owing to the regionalized genetic manipulation of the cerebellum. This allows for the analysis of intrinsic mechanism as the electroporation results in a mosaic pattern of genetically-modified neurons embedded in a wild type environment. The downside is that the electroporated rats or mice cannot be subjected to behavioral test due to the low amount of transfected neurons. To ensure the coexpression of plasmids in neurons, we recommend the cotransfection with a plasmid encoding GFP (or any other fluorescent protein) under a neuron-specific promoter to avoid the visualization of transfected glial cells. Effects that result in a reduction of axonal length can be easily detected and measured[24]. In contrast, it is technically impossible to quantify stimulating effects on axons as their entire lengths cannot be traced. Defasciculation of parallel fibers however is measureable[20]. The same holds true for the assessment of dendrite length and neuronal migration[23,24,30]. We typically carry out our analyses 5 days after the electroporation. It is of course possible to perform the analysis sooner and later. A later time point is recommended to examine the formation of dendritic claws[29], which represent the synaptic connection between CGNs and mossy fibers.

To finalize the analyses, it is important to choose the appropriate statistical test. For this, one has to take into account if the values of the groups follow a normal distribution (*e.g.* axonal or dendrites lengths) and if 2 or more than 2 groups are included in the analysis. For 2 groups, we use Student's test, for more than 2 groups ANOVA. Should the values follow a non-normal distribution (*e.g.* migration distance), Mann Whitney U test and Kruskal Wallis test have to be used for 2 or more than 2 groups, respectively.

Disclosures

The authors declare no competing financial interests.

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