Video Article

Ex Utero Electroporation and Organotypic Slice Culture of Mouse Hippocampal Tissue

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Abstract

Mouse genetics offers a powerful tool determining the role of specific genes during development. Analyzing the resulting phenotypes by immunohistochemical and molecular methods provides information of potential target genes and signaling pathways. To further elucidate specific regulatory mechanisms requires a system allowing the manipulation of only a small number of cells of a specific tissue by either overexpression, ablation or re-introduction of specific genes and follow their fate during development. To achieve this *ex utero* electroporation of hippocampal structures, especially the dentate gyrus, followed by organotypic slice culture provides such a tool. Using this system to generate mosaic deletions allows determining whether the gene of interest regulates cell-autonomously developmental processes like progenitor cell proliferation or neuronal differentiation. Furthermore it facilitates the rescue of phenotypes by re-introducing the deleted gene or its target genes. In contrast to *in utero* electroporation the *ex utero* approach improves the rate of successfully targeting deeper layers of the brain like the dentate gyrus. Overall *ex utero* electroporation and organotypic slice culture provide a potent tool to study regulatory mechanisms in a semi-native environment mirroring endogenous conditions.

Video Link

The video component of this article can be found at http://www.jove.com/video/52550/

Introduction

The hippocampus plays an important role in memory and learning as well as emotional behavior. One main function consists of the consolidation of short-term memory into long-term memory, which requires high plasticity of the nervous system. The dentate gyrus of the hippocampus acts as the primary gateway for input information and is also one of two brain regions with ongoing neurogenesis throughout adulthood[1,2]. The development of the hippocampal structure occurs during late embryogenesis and particularly during the first 3 to 4 weeks postnatal[3]. During early development of the dentate gyrus a stem cell pool is established required for postnatal as well as adult neurogenesis[4]. Developing neurons pass through various stages, from the stem cell through several stages of progenitor cells to the immature and finally the mature neuron during postnatal as well as adult neurogenesis. At different stages of neurogenesis the expression of specific genes is required to allow the maturation and integration of new neurons into the hippocampal circuitry[5,6].

Using mouse genetics and phenotype analysis by immunohistochemistry as well as molecular methods allowed defining the expression pattern and function of many of these genes. In addition microarray analysis as well as chromatin immunoprecipitation (ChIP) provided information about potential direct and indirect target genes[7,8]. However, there are still many open questions concerning the regulatory mechanisms of hippocampal development, in particular the development of the dentate gyrus. To gain further insight how specific genes are regulated a system is required allowing the manipulation of a small number of cells by down- or up-regulation of the gene of interest and/or its target genes and follow their fate during development. *In utero* electroporation of shRNAs, cDNA of genes of interest or *Cre* recombinase provides such a tool. To ensure the presence of the desired DNA or small RNAs expression plasmids should be used for electroporation. This approach is very successfully implemented in studying cortical development[9,10], but is a more challenging approach examining the development of the dentate gyrus due to the position of the hippocampal structures in deeper brain layers.

Ex utero electroporation followed by organotypic slice culture is one approach to circumvent this problem[11,12]. In contrast to *in utero* electroporation not the whole embryo but only the head is used allowing therefore to place the electrodes in a more favorable way to direct the shRNA/DNA towards the hippocampus and dentate gyrus. Our group successfully employed *ex utero* electroporation to study the role of the transcription factor Bcl11b during dentate gyrus development[8]. Bcl11b has a dual role in dentate gyrus development by regulating progenitor cell proliferation as well as differentiation as was demonstrated by immunohistochemistry. To further define a mechanism for Bcl11b involvement in these processes, protocols of the Polleux group[11,12] were adjusted to study the dentate gyrus as described below in the protocol section. In a first approach the question was addressed whether Bcl11b is regulating neuronal cell differentiation cell autonomously. A second approach examined whether Desmoplakin, a direct target gene of Bcl11b, is sufficient to rescue the Bcl11b phenotype.

Protocol

NOTE: All animal experiments were carried out in accordance with the German law and were approved by the government offices in Tübingen.

1. Preparation of Micropipettes, Solutions and Membranes

1. Preparation of Micropipettes
    1. Pull glass micropipettes using a micropipette puller with the following program: Heat: 540, Pull: 125, Velocity: 20 and Delay: 140. The needle length amounts to 5.5 cm.
    2. Bevel needles using a microgrinder to obtain a suitable tip size of 4 mm. Store the needles in a box or 15 cm Petri dish to prevent damaging of the tips.

2. Preparation of Solutions
    1. Plasmid DNA Solution
        1. Prepare plasmid DNA containing the desired cDNA construct using an Endotoxin free Maxi-prep kit according to manufacturer's protocol.
        2. Adjust plasmid DNA solution to a final concentration of 3 µg/µl (without GFP spike vector) or 4 µg/µl (with 1 µg of GFP spike vector) in endotoxin free Tris-EDTA buffer containing Fast Green (final concentration 0.05%).

    2. Laminin Stock solution
        1. Dissolve 1 mg of laminin in sterile water to a final volume of 1 ml. Prepare 100 µl aliquots and store at -80 °C.

    3. Poly-L-Lysine Stock Solution
        1. Dissolve 50 mg of poly-L-lysine in 50 ml of sterile water to a final concentration of 1 mg/ml. Prepare 1 ml aliquots and store in -20 °C.

    4. Complete Hank's Balanced Salt Solution (Complete HBSS)
        1. Prepare Complete HBSS by combining 100 ml of 10x HBSS, 2.5 ml of 1 M HEPES buffer (pH 7.4), 30 ml of 1 M D-glucose, 10 ml of 100 mM $CaCl_2$, 10 ml of 100 mM $MgSO_4$, and 4 ml of 1 M $NaHCO_3$. Add sterile water up to 1 L and store at 4 °C.
        NOTE: Autoclave all solutions expect 1 M HEPES buffer and 1 M D-glucose, which are filter sterilized.

    5. Slice Culture Medium
        1. Prepare slice culture medium by adding 35 ml of Basal Medium Eagle, 12.9 ml of complete HBSS (1.2.4), 1.35 ml of 1 M D-glucose, 250 µl of 200 mM L-glutamine, and 500 µl of penicillin-streptomycin to obtain a final volume of 50 ml. Add horse serum to a final concentration of 5% and store at 4 °C.

    6. Low-Melting Point (LMP) Agarose
        1. Prepare a 4% LMP agarose solution by adding 2 g of LMP agarose to 50 ml of complete HBSS (1.2.4) followed by heating in a microwave for 1-2 min at high power. Keep this solution in a water bath at 37 – 39 °C. Store the solution at 4 °C and reuse.

    7. Paraformaldehyde Solution
        1. In a fume hood prepare a 4% paraformaldehyde (PFA) solution by adding 4 g of paraformaldehyde to 100 ml of 1x PBS. Heat the solution to 60 °C and add a few drops of 1 N NaOH until the solution becomes clear.

    8. Permeabilization Solution
        1. Dissolve 9 g of BSA in 300 ml of 1x PBS containing 0.3% Trition X-100 and store at 4 °C. Add 10% sodium azide for long term storage.

3. Coating of Membrane Inserts
    1. Dilute one aliquot of laminin stock solution (1.2.2) and one aliquot of poly-L-lysine stock solution (1.2.3) in sterile water to a final volume of 12 ml.
    2. Place membrane inserts into 6 well plates with each well containing 2 ml of sterile water. Add 1 ml of coating solution on top of the membrane and incubate O/N at 37 °C in a 5% CO_2 incubator.
    3. After incubation wash the membrane inserts three times with 1 ml of sterile water and dry. Use coated membrane inserts on the same day or store at 4 °C for up to four weeks in a dry 6 well plate.

2. DNA Injection and Electroporation of E15.5 and E18.5 Embryos

1. Anesthetize a time mated female mouse by placing it into an anesthetizing chamber saturated with 5% isoflurane and connected to a vaporizer. Circulate isoflurane and oxygen at a rate of 1 L/min. Keep the animal in the box for 2-4 min or until unconscious which is tested by pinching between the paws of the mouse.
2. Euthanize the unconscious mouse by cervical dislocation on embryonic day (E) 15.5 or 18.5. Dissect the uterus containing the embryos[13] and place it into a Petri dish containing 15-20 ml of cold complete HBSS.
NOTE: From this point onwards, keep the embryos and tissues on ice.
3. Use a pair of scissors to separate each embryo from the uterine horn and place into a second Petri dish containing cold complete HBSS.

4. Under a dissecting microscope, sever the uterine muscle wall and the placenta using a pair of fine forceps (#55) and scissors. Carefully release the embryo from the yolk sac.
5. Use a pair of Bonn scissors to decapitate the embryos just above the forelimbs at a 60° angle. If the experiment requires genotyping of the embryos, collect a tissue sample for genomic DNA isolation (a small piece of tail).
6. Transfer the head to a clean and dry Petri dish. Because the head had been decapitated in a 60° angle, the head should tilt to one side when placed dorsal side up.
7. Place a needle carefully into the middle of the hemisphere close to the bregma (**Figure 1A**, **B**). Inject approximately 2-3 µl (at 3 or 4 µg/µl) of DNA solution by stepping on the pedal of the picospritzer III using 30 pounds of pressure for the duration of 10-15 msec per pulse, applying 5-8 pulses. The duration and number of the pulses depends on the diameter of the needle opening with smaller openings requiring more time. The interval between each pulse amounts to 1 sec.
8. Before placing the electrodes, apply a few drops of complete HBSS on the head of the embryo. Place the electrodes in such a way that the 'negative' terminal is on the same side as the injected ventricle and the 'positive' electrode on the opposite side of the injected ventricle below the ear of the embryo's head (**Figure 1C**, **D**). Apply 5 pulses of 50 V.
    1. Use 3 mm electrodes for E 15.5 and 5 mm electrodes for E 18.5.

3. Dissection of the Brain

1. After the electroporation, peel off the skin from the head with the help of a pair of fine forceps. Using a pair of spring scissors make a small incision in the middle of the cerebellum at the midline of the skull.
2. Insert the spring scissors into the incision and cut longitudinally along the sagittal suture. Peel off the skull and detach the brain from the skull by using fine forceps. Transfer the whole brain into 15-20 ml cold complete HBSS solution.
3. Meanwhile, pour 4% LMP agarose, kept at 37-39 °C in a water bath, into a peel-away mold.
4. Take the brain out of the complete HBSS by a small scoop spatula and drain excess of HBSS by using fine tissue paper or Kimwipes.
5. Place the whole brain gently into the agarose and adjust its position with a fine needle. Keep the mold on ice until the agarose is solidified and the block is sectioned (for coronal sections the olfactory bulbs point up).

4. Vibratome Sectioning and Slice Culture

1. Trim the LMP agarose blocks and glue them to the specimen stage using 'super glue'. After the glue is dry transfer the specimen stage to the buffer tray of the vibratome and fill with ice cold complete HBSS until the block is immersed in the solution.
   NOTE: Sterilize all instrument and equipment surfaces with 70% ethanol before sectioning.
2. Prepare 250 µm thick vibratome sections using a new blade as followed.
    1. Trim the block with the following settings; frequency – 60 Hz, amplitude – 0.7 µm, speed 16-18 mm/sec. Cut the sections containing the desired tissue with the above settings at slow speed (9 mm/sec). Starting the sectioning from the hindbrain, collect 5-7 sections from the cerebrum.
    2. Transfer the sections to a clean 6 well culture dish containing 5 ml of ice cold complete HBSS with the help of a bent spatula and keep on ice till all the sections are collected (**Figure 1E**).
3. Moisten the membrane in a cross fashion with 100 µl of complete HBSS before placing the sections on the membrane, to facilitate the orientation of the sections.
4. Transfer the sections using a bent spatula onto the membrane (pick up a corner of the section with forceps and pull onto the spatula and then use the forceps to push the section onto the membrane). Place up to five sections on one membrane and arrange by using forceps (**Figure 1F**). Do not overlap the sections with each other.
    1. Take the excess of HBSS off the membrane using a pipette. The specific membranes used here are attached to a frame and inserted into the tissue culture plate, which allows the tissue to be in contact but not covered by the medium.
5. Place the membrane inserts into a 6 well plate containing 1.8 ml of slice culture medium (1.2.5) (**Figure 1G**, **H**). Incubate the culture dish at 37 °C with 5% CO_2 for 11 DIV or 14 DIV. Change half the medium (0.9 ml) every second day.
   NOTE: At this stage, add reagents like Bromodeoxyuridine (BrdU; 10 µM final concentration) for labeling proliferating cells to the media for the first 20 hr of the culture time.

5. Fixation of the Sections Followed by Immunofluorescence Staining

1. Use a clean and sharp scalpel blade to cut and trim the membranes depending on the orientation of the sections.
2. Transfer the sections along with the membrane to a 24 well plate containing 1 ml of 4% PFA (1.2.7). Incubate the sections for 1 hr at RT followed by 3 washes with 1x PBS for 15 min each. Incubate the sections O/N with permeabilization solution at 4 °C with gentle agitation.
3. The following day, incubate the sections with appropriate primary antibodies, diluted in permeabilization solution, O/N or for 48 hr at 4 °C with gentle agitation.
4. Wash the sections 3 times for 15 min with 1x PBS and incubate O/N at 4 °C with the appropriate secondary antibodies diluted in permeabilization solution.
5. After the incubation with secondary antibodies, wash the sections once with 1x PBS for 15 min followed by DAPI staining for 10 min.
6. Wash the sections 3 times with 1x PBS for 15 min each and transfer to microscope slides. Add ImmunoMount and gently place a coverslip on top of the sections. Dry the slides O/N at 4 °C and seal with nail polish.
   NOTE: Keep the slides always at 4 °C.
    1. Analyze the slice cultures by confocal microscopy (**Figure 1I**).

Representative Results

Ablation of the transcription factor Bcl11b causes the impairment of progenitor cell proliferation and neuronal differentiation resulting in a reduced dentate gyrus size and cell number. Furthermore mutant neurons fail to integrate into the hippocampal circuitry causing learning and memory impairment[8]. To answer questions concerning the regulatory mechanism(s) of Bcl11b in these processes *ex utero* electroporation was employed.

Addressing the question whether Bcl11b cell-autonomously regulates neuronal cell differentiation, mosaic deletions of Bcl11b were generated by *ex utero* electroporation of a GFP-*Cre* recombinase construct or GFP alone[11] into $Bcl11b^{flox/flox}$ hippocampi at E15.5 followed by organotypic slice culture up to 18 days after electroporation (**Figure 2A**, **B**; this Figure has been modified from[8]). To determine whether Bcl11b regulates differentiation of granule cells cell-autonomously immunofluorescence staining was performed using specific antibodies recognizing NeuroD as well as GFP. NeuroD is expressed in mitotic stages 2b/3 and early postmitotic cells[14]. We have shown previously that the number of NeuroD positive cells is significantly increased in Bcl11b conditional mutants indicating an arrest of neuronal differentiation[8]. While the number of GFP positive cells alone and GFP/NeuroD positive cells did not differ in control and mutant cells a significant increase in NeuroD positive cells was observed in the dentate gyrus where cells had received Cre recombinase (**Figure 2C**; this figure has been modified from[8]). Finding NeuroD positive cells not only in cells which had received Cre recombinase but also in wild-type cells suggested that indirect mechanisms are involved in Bcl11b regulation of neuronal cell differentiation. From these data, however, additional cell-autonomous functions of Bcl11b cannot be excluded.

Previously, Desmoplakin was determined as a direct target gene of Bcl11b[8]. It was also shown that Desmoplakin is involved in the regulation of progenitor cell proliferation and differentiation of keratinocytes[15]. To demonstrate whether Desmoplakin is involved in the regulation of progenitor cell proliferation and/or neuronal differentiation of the dentate gyrus plasmid DNA expressing GFP alone or GFP and Desmoplakin under the control of the CMV promoter was electroporated into control and Bcl11b mutant brains (**Figure 3A**-**C**; this figure has been modified from[8]). The brain slices were cultured for the first 20 hr in the presence of BrdU (10 µM final concentration). At day 11 after electroporation the slice cultures were fixed followed by BrdU immunostaining and the number of BrdU positive cells was determined. Bcl11b mutant tissue electroporated with GFP only contained significantly fewer BrdU positive cells when compared to the control tissue. However, co-electroporation of GFP and Desmoplakin rescued the number of BrdU positive cells to control levels (**Figure 3D**; this figure has been modified from[8]). Taken together these data further confirm Desmoplakin as a direct target gene of Bcl11b and its role in the regulation of progenitor cell proliferation.

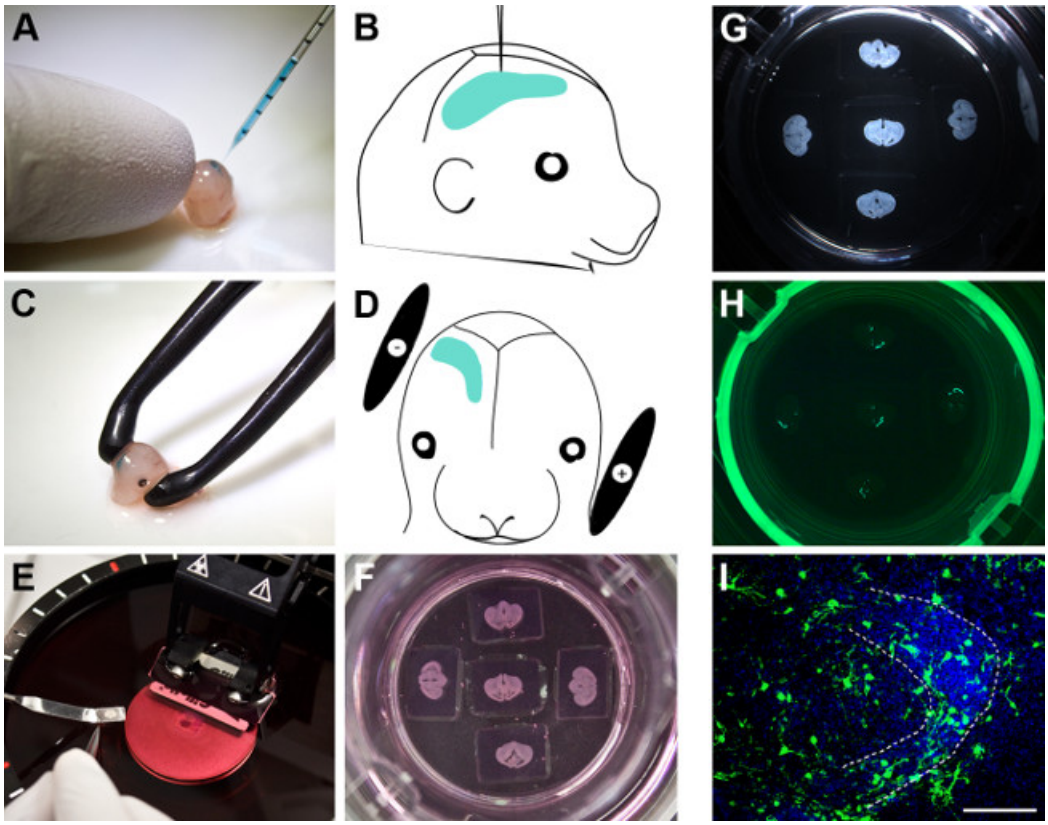

Figure 1. Set-up of *ex utero* electroporation and organotypic slice culture at E15.5. (A) Injection of DNA into one hemisphere. **(B)** Schematic drawing of DNA injection. **(C)** Positioning of the electrodes. **(D)** Schematic drawing of electrode placement. (E) Vibratome sectioning and handling of brain sections. **(F)** Placing brain sections on specific membranes. **(G-I)** Bright-field **(G)** and fluorescence (**H**, GFP) analysis of slice cultures on day 1 after electroporation. **(I)** Confocal image of the dentate gyrus at day 11 after electroporation using DAPI and GFP staining. Dashed line indicates the dentate gyrus. Scale bar = 100 µm. Please click here to view a larger version of this figure.

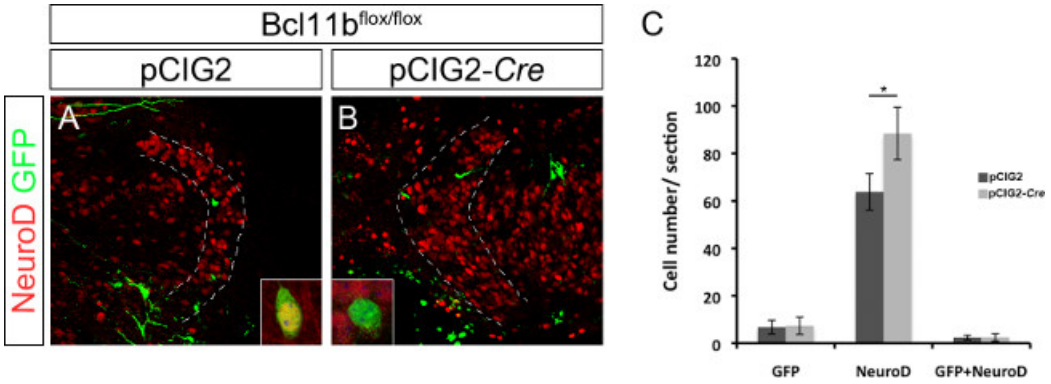

Figure 2. Mosaic deletion of Bcl11b by *ex utero* electroporation and organotypic slice culture (modified from [8]). Electroporation of control vector pCIG2 alone **(A)** and pCIG2-*Cre* construct **(B)** into the dentate gyrus at E15.5 followed by organotypic slice culture for 18 days. Sections were immunostained by using antibodies recognizing GFP (green) and NeuroD (red). Insets display GFP (green) and Bcl11b (red) staining at higher magnification to demonstrate loss of Bcl11b expression in cells expressing *Cre*-recombinase. **(C)** Statistical analysis of GFP, NeuroD and GFP/NeuroD positive cells. Dashed lines indicate the dentate gyrus. t-test, *p <0.005; error bars, s.d.; n = 5. Please click here to view a larger version of this figure.

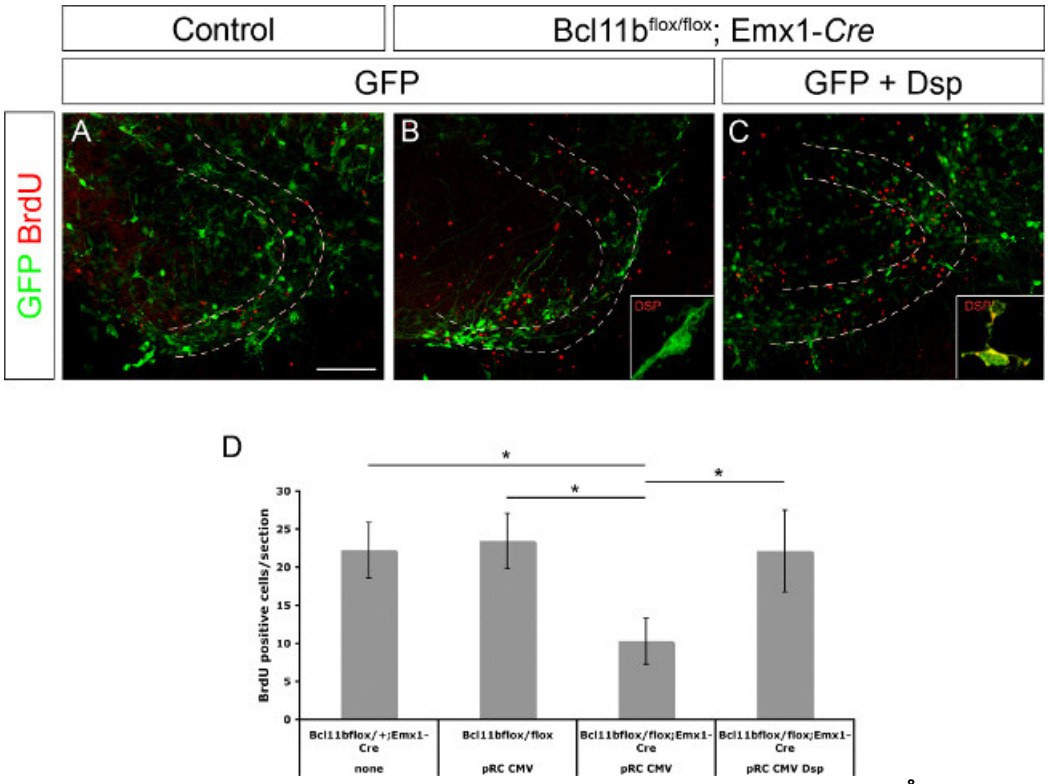

Figure 3. Bcl11b phenotype is rescued by re-introduction of Desmoplakin (modified from [8]). GFP only **(A, B)** as well as GFP and Desmoplakin **(C)** were electroporated into the dentate gyrus of control **(A)** and mutant **(B, C)** brains at E15.5 followed by organotypic slice culture for 11 days. Sections were immunostained by using antibodies recognizing GFP (green) and BrdU (red). **(D)** Statistical analysis of BrdU positive cells. Dashed lines indicate the dentate gyrus. Insets display Desmoplakin staining (green) at higher magnification. t-test, *p <0.01; error bar, s.e.m; n = 4. Please click here to view a larger version of this figure.

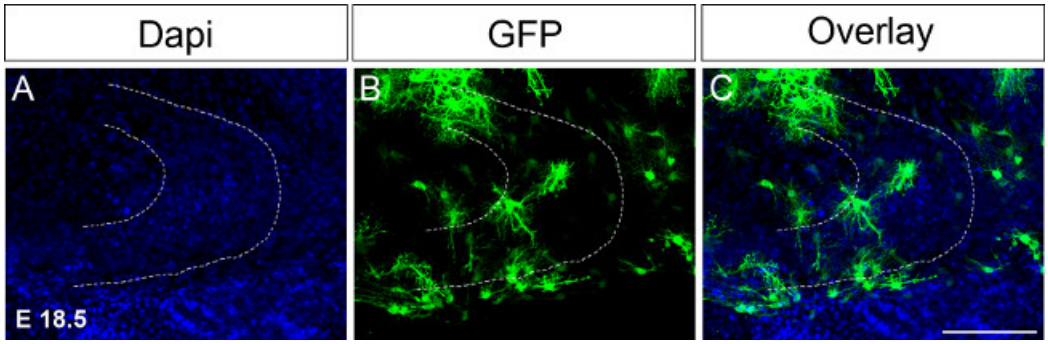

Figure 4. *Ex utero* electroporation at E 18.5 followed by organotypic slice culture. Injection of DNA expressing GFP into one hemisphere followed by immunostaining at day 16 after electroporation. **(A)** DAPI staining; **(B)** GFP staining; **(C)** merged image. Dashed line indicates dentate gyrus. Scale bar = 100 µm. Please click here to view a larger version of this figure.

Discussion

The hippocampus has an important function in learning and memory. The dentate gyrus is also one of two brain regions where neurogenesis occurs not only during development but also throughout adulthood. Postnatal and adult hippocampal neurogenesis proceeds in a similar way involving many common factors. Defining the regulatory mechanisms of these factors will be very helpful in understanding neurodegenerative diseases which in turn will lead to new therapies and preventive measures. To obtain this information one requires a system to manipulate single cells and observe them in their native environment as demonstrated by *ex utero* electroporation followed by organotypic slice culture.

Ex utero electroporation was first successfully applied in studying cortex development[11,16]. To our knowledge examining the role of Bcl11b in hippocampal development is the first describing *ex utero* electroporation of DNA into dentate gyrus tissue[8]. We based our protocol on the methods published by the Polleux group studying cortex development[11,12]. To successfully apply this method to study the dentate gyrus the size and position of the electrodes had to be adjusted. Placing the negative electrode at the cortex near the injection site and the positive electrode at the opposite site below the ear changed the polarity of the electroporation and succeeded in introducing DNA into cells of the dentate gyrus. Placing the electrodes correctly and applying a current of 0.06 to 0.08 mA turned out to be very crucial steps to achieve satisfactory electroporation results. To obtain the most adequate current depends mainly on the correct placing of the electrodes as described above. Additional critical steps of the protocol are injecting the material into the ventricle as well as handling the slices after vibratome sectioning. The brain tissue is not fixed throughout the whole procedure and therefore the tissue is very soft and easily destroyable when transferring from the vibratome onto the membrane for cultivation. Sections also have to be handled with great care when starting the immunostaining. With these modifications and considerations manipulations of single cells of the dentate gyrus were successfully performed answering questions concerning Bcl11b regulation of early hippocampus development (**Figure 2** and **3**; this figures have been modified from[8]).

As mentioned above manipulating single cells and observe their fate is the major advantage of *ex utero* electroporation. A major drawback of the *ex utero* approach in comparison with *in utero* electroporation is the limited time to keep slices in culture. It is also possible that *ex utero* cultivation conditions might cause a delay in development. In our experiments so far we were able to keep the slices in culture up to 18 days which corresponds to the age of P14. Cultivation of organotypic slices beyond this time lead to disintegration of the tissue. An additional disadvantage of the *ex utero* approach is the limited number of embryos that can be electroporated per mother. In contrast to *in utero* electroporation where up to 8 embryos can be treated this number is limited to 4 embryos in the *ex utero* approach. Dissection of the embryos, electroporation, vibratome sectioning and transfer of the slices into culture conditions have to be performed in a short time to ensure successful slice cultures.

Because major events of dentate gyrus development occur between P14 and P30 it would be necessary to keep organotypic slices in culture for prolonged time periods. In a first attempt to extend the culture time electroporations were performed at E 18.5 adjusting the conditions accordingly (**Figure 4**; see protocol). Electroporating at this time point allows keeping the brain sections in culture up to P17 or 18. In addition the hippocampal formation is further developed at E 18.5 when compared to E 15.5, *e.g.*, hippocampal structures are already formed and more easily recognizable. In the future we would like to perform *ex utero* electroporation at even later time points, *e.g.*, P0 to P4 to examine changes during dentate gyrus development up to P30.

Disclosures

The authors declare that they have no competing financial interests.

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