Chapter 2

Methods

Equipment in continuous use

- Sparkfree laboratory refrigerator (+4 °C). Thermo Electron Corporation.
- Sparkfree laboratory freezer (-20 °C). Thermo Electron Corporation.
- Milli-Q® Gradient +A10 (Water purification system). EMD Millipore Corporation. Cat. no. QGARD00R1.
- Pipettes:
  - Gilson PIPETMAN® P2 (0.2 - 2 μl). Anachem Ltd. Cat. no. F144801
  - Gilson PIPETMAN® P10 (1 - 10 μl). Anachem Ltd. Cat. no. F144802
  - Gilson PIPETMAN® P20 (2 - 20 μl). Anachem Ltd. Cat. no. F123600
  - Gilson PIPETMAN® P100 (10 - 100 μl). Anachem Ltd. Cat. no. F123615
  - Gilson PIPETMAN® P200 (20 - 200 μl). Anachem Ltd. Cat. no. F123601
  - Gilson PIPETMAN® P1000 (200 - 1000 μl). Anachem Ltd. Cat. no. F123602
- Pippete tips (Ranin Aerosol Resistant Tips):
  - Capacity 10 μl. Anachem Ltd. Cat. no. RT-10F
  - Capacity 20 μl. Anachem Ltd. Cat. no. RT-20F
  - Capacity 100 μl. Anachem Ltd. Cat. no. RT-100F
  - Capacity 200 μl. Anachem Ltd. Cat. no. RT-200F
  - Capacity 1000 μl. Anachem Ltd. Cat. no. RT-1000F

2.1 Primary Neuronal Cultures

All mice were treated in accordance with the U.K. Animals Scientific Procedures Act of 1986, and all procedures were approved through the British Home Office Inspectorate.

Materials

Reagents

- C57BL/6 c/c mice at 18 or 19 days of gestation. Supplied on site.
- Fetal Calf Serum (FCS). Supplied on site.
- Dulbecco's Modified Eagle Medium (DMEM). Invitrogen[1]. Cat. no. 31330-038
- B-27 Supplement (optimised medium supplement for neurons (Brewer et al., 1993)). Invitrogen. Cat. no. 17504-044
- L-Glutamine 200 mM. Invitrogen. Cat. no. 31330-038
- Natural Mouse Laminin. Invitrogen. Cat. no. 23017-015
- Papain Vial. Worthington Biochemical Corprotation. Cat. no. PAP2
- Neurobasal Medium. Invitrogen. Cat. no. 21103-049.
- Penicillin Streptomycin (PenStrep). Invitrogen. Cat. no. 15140-122
- Dulbecco's Phosphate Buffered Saline 1× (1× DPBS). Invitrogen. Cat. no. 14190-094
- Poly-D-lysine hydrobromide (PDL). Sigma-Aldrich Corporation. Cat. no. 1000689047
- Ethanol 99.7 - 100% v/v. VWR International. Cat. no. 101707 2.5LT

Equipment

Dissection:

- 35 mm Petri dish. Corning Inc. Cat. no. 430588
- 55 mm Petri dish. Sterilin Ltd. Cat. no. PF55
- 100 mm Petri dish. Corning Inc. Cat. no. 430167
- 140 mm Petri dish. Sterilin Ltd. Cat. no. 501V
- 6-well cell culture plate. Corning Inc. Cat. no. 3156
- Pasteur pipette. Alpha Laboratories Ltd. Cat. no. LW4070
- Squirt bottle. Supplied on site.

[1]Invitrogen Corporation is a part of Life Technologies

- Leica MZ9.5 Stereomicroscope (binocular dissection microscope) with Leica CLS 150× light source. Meyer Instruments Inc.
- Thermo Scientific Holten HV Mini Laminar (sterile hood for dissections). Thermo Fisher Scientific. Cat. no. 54250130
- Scissors. Supplied on site.
- Dumont (curved) forceps. Fine Science Tools Inc. Cat. no. 11295-20
- Narrow Pattern Forceps, 2 pairs. Fine Science Tools Inc. Cat. no. 11002-12
- Dumont #5 Mirror Finish Forceps - Inox Biologie. Fine Science Tools Inc. Cat. no. 11252-23
- Iris Spatula - Slight Curve. Fine Science Tools Inc. Cat. no. 10093-13

Plating:

- 12 Well Cell Culture Cluster (12 well cell culture plate). Corning Inc. Cat. no. 3512
- 15 ml centrifuge tubes. Becton, Dickinson and Company. Cat. no. 4-2097-8
- AC1000 Improved Neubauer (cell counting chamber). Hawksley. Cat. no. AC1000
- Galaxy R CO_2 incubator. Wolf Laboratories Ltd
- Axiovert 200 (inverted microscope) with temperature control. Carl Zeiss AG
- 90 mm filter unit. Nalgene Nunc International. Cat. no. 450-0020
- Vacuum pump. Manufactured on site.
- Microbiological Safety Cabinet Class II. Holten.
- Sterile syringe filter, 0.2 μm. Nalgene Nunc International. Cat. no. 190-2520
- Swinging bucket centrifuge for 15-ml tubes. Eppendorf. Model: Centrifuge 5702
- Water bath. Grant Instruments (Cambridge) Ltd.
- Wide-bore glass tips. Manufactured on site.

Reagent and equipment setup

L-Glutamine solution L-Glutamine solution was aliquoted (1ml) and stored at –20 °C. *IMPORTANT:* Upon repeated freezing and thawing glutamine can spontaneously deaminate into excitotoxic glutamate, therefore re-freezing had to be avoided.

Laminin Laminin solution was thawed on ice, aliquoted (50 μl) and stored at -20 °C. For coating plates an aliquot of laminin was thawed on ice and mixed with 8 ml of ice-cold 1× DPBS. 600 μl of the mixture was used to coat one well of 12-well plate ($\sim$0.02 μg

$\cdot$ mm^{-2}). *IMPORTANT:* Upon rapid thawing or repeated freezing and thawing laminin may polymerize and form a gel, thus laminin solution was always handled on ice.

NeurobasalFull On the day of preparing the media, L-Glutamine solution (1ml aliquot) and B-27 (10ml) were added to 490 ml of Neurobasal. The mixture was filtered using the 90 mm filter unit and the vacuum pump. NeurobasalFull was stored at 4 °C and it was used until a change in its color was detected, which indicated a change in pH (approximately one month from the time of preparation).

DMEM + FCS An aliquot (50ml) of FCS was added to 450 ml of DMEM and filtered through 90 mm filter unit with vacuum pump. DMEM + FCS was used solely to terminate papain treatment and wash off the digested material. Similar to NeurobasalFull, DMEM + FCS was stored at 4 °C until change in pH was observed (approximately one month).

Papain Papain was used to partially digest dissected tissue, with the aim to dissolve extracellular matrix and preserve cell viability. A dry stock of papain was dissolved in 12 ml of 1× DPBS and stored at 4 °C. Maximal amount of the material that could be efficiently treated by a 1 ml papain aliquot was equal to that of two forebrains. An attempt to digest more material prevented an efficient digestion of extracellular matrix. Papain was filtered through a syringe 0.2 μm filter before use.

PDL Lyophylized PDL (5 mg) was dissolved in 50 ml of 1× DPBS to obtain 2× stock. Aliquots of 10 ml were stored at -20 °C. When defrosted, 10 ml of 1× DPBS were added to obtained 1× PDL. 1× PDL solution was stored at 4 °C for up to 2 weeks.

70% ethanol In order to decrease the risk of contamination of cells during dissection and plating, all work surfaces were treated with 70% ethanol (prepared in the squirt-bottle before the dissection procedure from 100% ethanol and Milli-Q water).

Coating cell culture plates 2 ml of 1× DPBS was added to the 10 outer wells of a 12 well cell culture plate in order to maintain humidity. 450 μl of 1× PDL solution was added into the two central wells and incubated for minimum 1 h (or overnight) at 37 °C in the incubator. During the incubation, a 50 μl aliquot of laminin was thawed on ice and diluted in 8 ml of ice cold 1× DPBS. After the incubation PDL was aspirated and wells were washed once with 1 ml of 1× DPBS. 600 μl of laminin solution was added to the two central wells and incubated for minimum 2 h (or overnight). After the incubation

laminin solution was aspirated and 950 μl of NeurobasalFull was added to the wells (this was always done within 2 h of plating cells). The cell culture plates were subsequently left in the 37 °C incubator to warm up the media.

Prewarming solutions Prior to dissections, papain (aliquots of 1 ml in 15 ml Falcon tube), DMEM+FCS and NeurobasalFull were prewarmed on the waterbath at 37 °C. This was necessary to eliminate an additional cold shock to the cells during plating.

Preparing of chilled 1× DPBS (5% PenStrep) Before dissections 1× DPBS with 5% PenStrep was prepared and chilled on ice. Concentration of antibiotics in 1× DPBS (5% PenStrep) is approximately 500 units of penicillin and 500 μg of streptomycin per 1 ml. 1× DPBS (5% PenStrep) was used to store dissected foetuses and heads prior to dissection. 140 mm, 100 mm, 35 mm Petri dishes and a 6-well plate were placed on ice and filled with ice-cold 1× DPBS (5% PenStrep).

Procedure

Dissection:

- A pregnant mouse (17.5 days post coitus) was killed by cervical dislocation. The abdomen was disinfected by a squirt of 70% ethanol. The uterus with fetuses was dissected, using scissors, and narrow forceps. After a quick wash with 70% ethanol, the uterus with fetuses was placed into a 140 mm Petri dish with 1× DPBS (5% PenStrep), and the fetuses were killed by cooling.
- The fetuses were dissected from the uterus and decapitated. Holding one head at a time with curved forceps, the heads were quickly washed in a 100 mm Petri dish prefilled with ice-cold 1× DPBS (5% PenStrep). Afterwards the heads were distributed among the wells of a 6-well plate prefilled with DPBS (5% PenStrep).
- One head at a time was placed into the upper lid of a 55 mm Petri dish prefilled with ice-cold DPBS (5% PenStrep) and placed under a dissection binocular microscope. To maintain the sterile environment, the dissection was performed in a mini laminar.
- Using mirror finish forceps, the skin and the calvarium were removed from the head.
- The cranial nerves were severed with a spatula, and the brain was removed from the skull.
- The forebrains were dissected using mirror finished forceps and a spatula.

- The meningi were removed from each hemisphere, using mirror finished forceps. If necessary, the hippocampi were dissected at this stage using mirror finish forceps.
- After removing the meningi, either the whole forebrains or the hippocampi were transferred with a Pasteur pipette into a 35 mm Petri dish prefilled with ice-cooled 1× DPBS (5% PenStrep). If the material appeared intact after the transfer, then it was further shredded either with scissors or by trituration through a Pasteur pipette (shredding facilitated the papain digestion stage). The dissected material was kept on ice in 1× DPBS (5% PenStrep) until the end of the dissections.

Plating cells:

- The dissected tissue was taken out 1× DPBS (5% PenStrep) and placed into 1 ml of prewarmed papain solution (in 15 ml centrifuge tube), carrying over as little PBS as possible. The material was incubated in papain solution for 25 min in 37 °C waterbath. *IMPORTANT:* To achieve better disruption of extracellular matrix, the material equivalent to at most two embryonic forebrains was digested in one tube at a time.
- After the papain treatment, all further manipulations were performed in a Microbiological Safety Cabinet Class II. As much as possible papain solution was quickly removed using a wide-bore glass pipette and 1 ml of prewarmed DMEM+FCS was added to the tissue, which terminated the papain lysis.
- The tissue was macerated through a wide-bore glass pipette and P1000 tip (narrow-bore). Further DMEM+FCS was added to bring the total volume to 5 ml.
- After making sure that no clumps of the undisrupted tissue remained, the obtained cell suspension was centrifuged at 400 g (in a swinging bucket centrifuge) for 3 min 30 sec.
- As much as possible DMEM+FCS was removed. 1 ml of NeurobasalFull was added and the pellet was triturated through P1000 tip.
- The cell suspension was centrifuged at 400 g for 3 min 30 sec.
- The supernatant was removed and approximately 2 ml of NeurobasalFull was added to the pellet. *IMPORTANT:* If several papain digestions were carried out in parallel, all the pellets were mixed before counting at this stage.
- After trituration of the pellet (with P1000), the cells were counted using a cell counting chamber and an inverted microscope.

- After counting, NeurobasalFull was added to the cells so that the desired plating volume was approximately 50 μl if possible (as it made total $1,000\mu$l of the growth media per well).

Throughout this work, cells were plated at two densities: $1,850$ cells $\cdot$ mm^{-2} and 790 cells $\cdot$ mm^{-2} (referred to as high and low densities). Cells were cultured in a humidified incubator, with CO_2 concentration held at 5%, and temperature at 37 °C.

2.2 RNA extraction

Materials

Reagents

Extraction:

- Dulbecco's Phosphate Buffered Saline 1× (1× DPBS). Invitrogen. Cat. no. 14190-094
- miRNeasy®Mini Kit. Qiagen N.V. Cat. no. 217004
- QIAzol®. Qiagen N.V. Cat. no. 79306
- RNase-Free DNase Set. Qiagen N.V. Cat. no. 79254. Components:
  - DNase I (solid)
  - RDD buffer
  - Nuclease free water
- Liquid nitrogen. Supplied on site.
- Ethanol 99.7 - 100% v/v. VWR International, LLC. Cat. no. 101707 2.5LT
- Chlorophorm, $\geq$ 99%. Sigma-Aldrich Corporation. Cat. no. C2432-500ML
- Nuclease-Free Water. Ambion Inc.[1] Cat. no. AM9937
- RNase Zap® wipes. Ambion Inc. Cat. no. AM9786
- Azowipe®. Vernon-Carus Ltd. Cat. no. 81103

Quality Control:

- Agilent RNA 6000 Nano Kit. Agilent Technologies. Cat. no. 5067-1511

[1] Ambion Inc. is a part of Applied Biosystems Inc. (Life Technologies)

Equipment

Extraction:

- RNase-Free 1.5 ml Microfuge Tubes. Ambion Inc. Cat. no. 12400
- -90 °C freezer. SANYO Electric Co. Limited. Model: MDF-450V
- Table top centrifuge. Eppendorf AG. Model: Centrifuge 5415D
- Centrifuge with thermocontrol. Eppendorf AG. Model: 5417R
- Vortex. Fisons Scientific Equipment. Cat. no. SGP-202-0109

Quality Control:

- NanoDrop Spectrophotometer. Thermo Fisher Scientific. Model: ND-1000
- RNA Nano Chips for use with Agilent 2100 Bioanalyzer. Agilent Technologies. Cat. no. 5067-1511
- Agilent Technologies 2100 Bioanalyzer. Agilent Technologies
- Agilent 2100 Expert Software. Agilent Technologies

Reagent and equipment setup

Good laboratory practice of working with RNA

Performing biological experiments always requires following a set of rules that to prevent detrimental contamination of samples. This is especially important when working with samples of RNA, as any contamination can become detrimental to integrity of RNA samples due to the abundance of RNase in the environment. Thus, it was important to treat all work surfaces, gloves and pipettes with RNase inhibitor containing solution (such as RNase Zap) and to subsequently clean the surfaces with a fast evaporating alcohol liquid (in Azowipes). RNA was stored in water, thus it was important to use nuclease free water. Overtime, contamination with RNase is perhaps inevitable, thus RNA samples were stored in -90 °C freezer and always handled on ice when out of the freezer. There is a widely held view that RNA can lose its integrity due to mechanical shearing by ice crystals during freezing and thawing cycles. Therefore the number of these cycles was minimized when possible.

Preparation of DNase I

Before the RNA extraction procedure, DNase I was dissolved in 550 μl of water (can be stored at 4 °C for one month) and mixed with RDD buffer (in 1:7 ratio).

Preparation of the centrifuge

Prior to the beginning of extraction a centrifuge with a thermocontrol must be cooled down to 4 °C.

Procedure

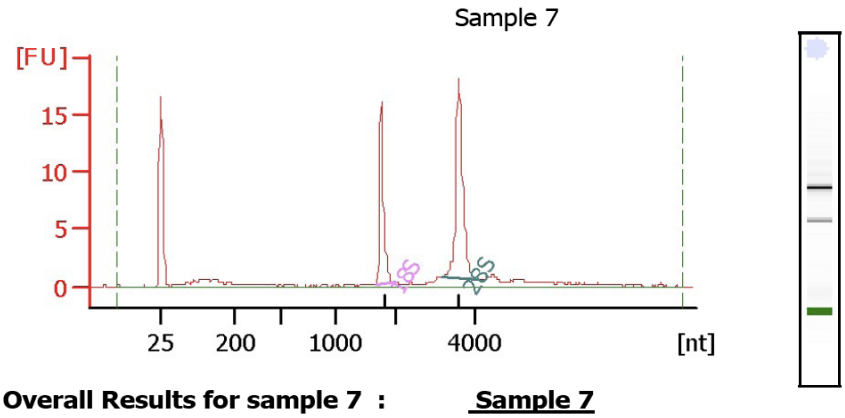
RNA extraction with RNeasy RNA extraction kit (Qiagen)

- Cultures from which RNA was to be extracted were transferred from the incubator onto the bench, the growth media was removed and the cultures were quickly washed once with 1× DPBS. After DPBS was removed, 700 μl of Qiazol was added per well and cells were scrapped off the bottom of the well using a P1000 tip. The suspensions were transferred into 1.5 μl microfuge tubes. *IMPORTANT:* It was possible that the stress stress associated with the removal from the controlled environment of the incubator could perturb gene expression in the cells. Therefore it was important to minimize the time between transportation and addition of Qiazol (usually, no more than 4 cultures were dealt with at a time).
- At this stage, it was possible to either snap-freeze the suspensions with liquid nitrogen and store at -90 °C or to immediately proceed with the rest of the extraction protocol. Freezing of the suspension was frequently more convenient when many cultures had to be dealt with at one time. For example, if 16 cultures were to be extracted, that would involve four rounds of Qiazol lysis (as only four cultures were taken out of the incubator at one time). Thus the lysates from the first batch of cultures would have remained significantly longer at the ambient temperature than the lysates from the last batch, which was not desirable.
- If the suspension was freshly obtained then it was incubated on a benchtop for 5 min. If the suspension was taken out of -90 °C freezer, it was incubated on benchtop until the lysate defrosted and appeared clear. Gentle shaking was found to increase the speed of defrosting.
- 140 μl of chloroform was added to the suspension and it was thoroughly vortexed for 15 s. Afterwards, the suspension was incubated on benchtop from 2 to 3 minutes.

- The suspension was centrifuged at 12,000 g for 15 min at 4 °C.
- Aqueous upper phase was collected into a new tube and 1.5 volume 100% ethanol was added to it. Usually it was possible to collect approximately 300 μl of supernatant without risking contamination from the bottom phase.
- The solution was vortexed for 3 sec and immediately transferred onto a spin column (a part of RNeasy kit).
- The colum was centrifuged for 15 sec at 10,000 g and the flow through was discarded.
- 350 μl of RWT buffer (part of miRNeasy kit) was added to the spin column. The column was centrifuged for 15 sec at 10,000 g and the flow through discarded.
- 80 μl of prepared DNase I solution (see section 2.2) was added to the column. The column was incubated with DNase I for 15 min on a benchtop.
- 350 μl of RWT buffer was added to the column. The column was centrifuged for 15 sec at 10,000 g and the flow-through discarded.
- 500 μl of RPE buffer (part of miRNeasy it) was added to the column. After 15 sec centrifugation at 10,000 g the flow-through was discarded.
- 500 μl of RPE buffer (part of miRNeasy it) was added to the column. After 2 min centrifugation at 10,000 g the flow-through was discarded.
- The column was carefully placed into a new 1.5 ml tube and centrifuged at full speed for 2 min. This step was described as an "optional" in miRNeasy manual, but I found it to be important for the removal of the residual solvent.
- The column was transfered into a new 1.5 ml and RNA was eluted with 30 μl of nuclease free water (upon 1 min centrifugation at 10,000 g). This step was repeated with the flow-through solution to obtain higher final concentration of RNA.
- The solution of RNA in water was stored at -90 °C (either immediately following the extraction, or after measuring RNA concentration and quality control using Nanodrop).

Measuring concentration of RNA and quality control

Concentration of RNA was determined using Nandrop following the manufacturer's protocol. RNA integrity was assessed using the Bioanalyzer machine and Bioanalyzer 6000 Nano kit, following the manufacturer's protocol. The analysis of total RNA on bioanalyzer enables visual assessment of the integrity of peaks corresponding to 18S and 28S ribosomal RNA. No significant degradation was ever detected in any of the samples (a representative Figure 2.1).



Overall Results for sample 7 : Sample 7

RNA Area:	67.6
RNA Concentration:	40 ng/µl
rRNA Ratio [28s / 18s]:	1.8
RNA Integrity Number (RIN):	10.0 (B.02.05)
Result Flagging Color:	
Result Flagging Label:	RIN:10

Fragment table for sample 7 : Sample 7

Name	Start Size [nt]	End Size [nt]	Area	% of total Area
18S	1,694	1,983	14.6	21.5
28S	3,156	4,061	25.5	37.7

Figure 2.1: Example of an output of Bioanalyzer.

2.3 Quantitative RT-PCR. mRNA

Materials

Reagents

- Oligo(dT)$_{12-18}$ Primer. Invitrogen. Cat. no. 18418-012
- Random Primers (Nanomers). New England Biolabs Inc. Cat. no. S1254S
- Random Decamers RETROscript®. Ambion Inc. Cat. no. AM5722G
- 10 mM dNTP Mix (deoxyribonucleotides mix). Invitrogen. Cat. no. 18427-013
- SuperScript® II Reverse Transcriptase Kit. Invitrogen. Cat. no. 18064-014
- TaqMan® Universal PCR Master Mix, No AmpErase UNG (2×). Applied Biosystems Inc. Cat. no. 4324018
- Primers and probes mix for real-time PCR (TaqMan® Gene Expression Assays). Applied Biosystems Inc. Custom design
- Nuclease-Free Water. Ambion Inc. Cat. no. AM9937

Equipment

- 96-well plate (Thermowell 96 Well Plate Model (M)), Corning Inc. Cat. no. 6511
- 96-well plate cover (Microseal A film). MJ Research Inc. Cat. no. MSA-5001
- Optical 96-well plate (MicroAmp Optical 96-Well Reaction Plate). Applied Biosystems Inc. Cat. no. N8010560
- MicroAmp Optical Adhesive Film. Applied Biosystems Inc. Cat. no. 4311971
- Centrifuge with a 96-well plate rotor. DJB Labcare Ltd. Model: Heraeus Biofuge Stratos
- Peltier Thermal Cycler. MJ Research Incorporated
- NanoDrop Spectrophotometer. Thermo Fisher Scientific. Model: ND-1000
- 7500 Real Time PCR System. Applied Biosystems Inc.
- 7500 Real Time PCR System Sequence Detection Software v1.2.2. Applied Biosystems Inc.
- Primer Express® Software v3.0. Applied Biosystems Inc.

Reagent and equipment setup

Primers

Usage of internal controls is important for RT-PCR experiments, as the method is very sensitive to the amount of input cDNA. Thus in addition to profiling of the differentially expressed genes (Acta2 and Lass2), it was important to identify a stably expressed control gene. Microarray profiling of gene expression upon miR-124 over-expression and inhibition at 3DIV and 6DIV (Chapter 5, section 5.2.1) showed that some of the components of splicing machinery and translation factors were stably expressed. Two genes were picked from these categories as potential control genes: Sfrs7 (splicing factor, arginine/serine-rich 7) and Eif2b4 (eukaryotic translation initiation factor 2B, subunit 4 delta).

Primers and probes for the real-time PCR were designed to span two neighbouring constitutive exons (i.e. exons that were present in all annotated transcript of the gene, Ensembl version 56 (Flicek et al., 2008)). Primers were designed using Primer Express® Software v3.0 (Applied Biosystems Inc.) according to the manufacturer's protocol. Preference was given to neighbouring constitutively present exons at the 3′-end of the genes, as it maximized the chances of the regions of interest to be reverse transcribed with the oligo(dT) primer. The BLAST analysis (Altschul et al., 1990) confirmed that sequences of all primers were uniquely present in the mouse genome.

	Acta2	**Lass2**
Ensembl Gene ID	ENSMUSG00000035783	ENSMUSG00000015714
ExonA ID	4 ENSMUSE00000545192	3 ENSMUSE00000253057
ExonB ID	5 ENSMUSE00000545191	4 ENSMUSE00000253053
Forward Primer	CCCAGATTATGTTTGAGACCTTCAA	CAGACCAGCGGCAAGCA
Probe	TCCCCGCCATGTATGT	CCCAAGCAGGTGGAG
Reverse Primer	GGACAGCACAGCCTGAATAGC	CTCTGCCGTGACAAAAGGTCTA
	Eif2b4	**Sfrs7**
Ensembl Gene ID	ENSMUSG00000029145	ENSMUSG00000024097
ExonA ID	4 ENSMUSE00000186164	3 ENSMUSE00000138340
ExonB ID	5 ENSMUSE00000186166	4 ENSMUSE00000138342
Forward Primer	CAACAGGTTCCTACACGAAAGGA	CATCGCTATAGCCGACGAAGA
Probe	TACGGATCCAAAGTCA	AAGCAGGTCACGATCT
Reverse Primer	TGAGGCAGGTGGGAGAAGAG	CCCTGGATCGGGAATGG

Table 2.1: Real-time PCR primers and probes.

In a trial RT-PCR detection of Sfrs7 had lower standard deviation between and within treatments than Eif2b4. Therefore Sfrs7 was chosen as the control gene for the analysis of differential expression of Acta2 and Lass2.

Procedure

Reverse transcription: Generation of first strand cDNA

- Total RNA was mixed with dNTP (Table 2.2).

Component	per 12 μl mix
total RNA (40 ng $\cdot$ μl^{-1})	9.00
Random primers	1.00
Oligo dT	1.00
dNTP	1.00
TOTAL	12

Table 2.2: mRNA reverse transcription starting mix.

- The starting mix was transferred into 96-well PCR plate and incubated at 65 °C for 5 min in Peltier Thermal Cycler.
- The plate was transferred on ice. 4 μl of 5× 1 st strand buffer and 2 μl of DTT (0.1 M dithiothreitol from SuperScript® II Reverse Transcriptase Kit) were added to the samples.
- The plate was incubated at 42 °C for 2 min in Peltier Thermal Cycler.
- 1 μl of SuperScript II reverse transcriptase was added to the samples.
- The sample was incubated for 50 min at 42 °C, followed by 15 min incubation at 70 °C for 15 min.
- The product of the reaction (cDNA) could be stored at 4 °C overnight.

Real time PCR

Concentration of cDNA in all samples was normalized to 67.5 ng·ml^{-1}. Initially, several dilutions were tested and 67.5 ng· ml^{-1} was found to consistently produce the Ct values (see below for the definition of Ct) between 23 and 30 for amplification of Acta2, Lass2 and Sfrs7 transcripts, which is within the optimal range of 7500 Real Time PCR Sequence Detection System.

- 15 μl of 2× TaqMan Universal RT-PCR Mix was pipetted into the wells of optical 96-well plates. *IMPORTANT:* Pipetting errors are a very common source of variability in RT-PCR results. In order to minimize pipetting errors, a new pipette tip for each

well of the 96-well reaction plate was used for addition of PCR components, as I found that variable amounts of liquid may adhere to the walls of the tip and lead to inconsistencies.

- 10 μl of cDNA (at 67.5 ng $\cdot$ μl^{-1}), 5 μl of appropriately diluted Assay Mix (see above) was added to the wells to make 30 μl of total reaction volume. *IMPORTANT:* Pipetting of small volumes was found to be relatively imprecise and lead to additional variability in RT-PCR results. This was especially important for addition of primers-probe mix as the amount of the probe determined the detection of real time PCR progression. Thus the dilutions were scaled to obtain the volumes that could be pipetted relatively accurately.
- The plate was sealed with optical plate cover and centrifuged briefly in a centrifuge with a rotor for 96-well plates in order to bring all liquid to the bottom of the wells and remove large air bubbles.
- The real time PCR was conducted in the thermal cycler using the default 40 cycle program (Table 2.3).

HOLD	**HOLD**	**CYCLE (40 cycles)**	
2 min	10 min	15 sec	60 sec
50 °C	95 °C	95 °C	60 °C

Table 2.3: mRNA real time PCR program.

Analysis of expression by RT-PCR

TaqMan probes were used to evaluate the rate of PCR in real time. The probes were designed to anneal to the internal part of the amplification product. The probes had a fluorescent reporter dye at the 5′ and a quencher at the 3′-end. During the amplification, DNA polymerase hydrolysed the probe with its 5′ nuclease activity. This decoupled the reporter from the quencher and lead to the increase in the fluorescence with each cycle. During the initial stages of a typical correctly set-up PCR, the fluorescence stays at a baseline. As PCR progresses and the amount of product approximately doubles every cycle, the increase in the fluorescences eventually becomes detectable and to raise exponentially. Eventually, the pool of the available probe depletes and the fluorescence levels at a plateau.

If primers and a probe are designed correctly, the stage of PCR at which the fluorescence starts to grow exponentially correlates with the amount of the starting material,

i.e. it correlates with the abundance of transcripts of the gene in question. Even though this estimate is not immediately indicative of the absolute level of the transcript, it is straightforward to make a comparison across several samples and draw conclusions on their relative abundance.

It is difficult to identify the exact cycle at which the fluorescence starts to grow exponentially. Instead, it is more reliable to estimate the fractional cycle number at which the fluorescence reaches a threshold value above the baseline. The fractional cycle number at which the fluorescence reaches a threshold value is called the Ct value. The rate of amplification of the same transcript is approximately identical between different samples, even if the starting amounts of the transcript are different. Thus the slopes of the curves describing the exponential growth in the fluorescence are approximately parallel for the same transcripts. Therefore, the threshold can be chosen at any level of the fluorescence for relative comparison, as long as it is above the baseline and below the plateau.

The fluorescence threshold is chosen arbitrarily and it cannot be immediately used to describe absolute levels of the transcript in question. However, the difference between the Ct values of different samples is indicative of the relative difference in abundance of the transcript. To make a comparison between different samples, the within sample differences in Ct values (ΔCt) between a control transcript and a transcript of interest are usually compared (instead of comparing actual Ct values between different samples). This is done because PCR progression is dependent on the amount of the starting material, and having an internal control is a way of normalizing differences of input RNA across the different samples. Because the difference of the differences is compared across the samples, the estimate is called $\Delta\Delta$Ct.

The Ct values for each of the primers-probe mixes was obtained separately with the threshold automatically defined by the software. Therefore, one threshold was applied to all samples probed with one probe, but the threshold was different for samples probed with a different set of primers and probes. For example, at 36 h of incubation, the threshold of 0.24 was used to define the Ct values analysis of Lass2 (including samples that were transected with mimics and inhibitors of miR-124), but a different threshold of 0.42 was used to define the Ct value of for Sfrs7 in the same samples. Such an approach optimised the detection of relative differences in gene expression between samples treated with mimics and inhibitors. The disadvantage of this method was that it did not allow for estimation of absolute expression levels.

2.4 Quantitative RT-PCR. miRNA

Materials

Reagents

- Reverse transcription primers and real time PCR primers and probes:
  - snoRNA202 (mouse). Applied Biosystems Inc. Cat. no. 4380914
  - hsa-miR-143[2]. Applied Biosystems Inc. Cat. no. 4373134
  - hsa-miR-let7c. Applied Biosystems Inc. Cat. no. 4373167
  - hsa-miR-370. Applied Biosystems Inc. Cat. no. 4373031
- TaqMan® MicroRNA Reverse Transcription Kit. Applied Biosystems Inc. Cat. no. 4366596. Components of the kit:
  - 100 mM dNTPs (deoxyribonucleotides)
  - MultiScribe Reverse Transcriptase, 50U/μl
  - 10 x Reverse Transcriptase Buffer
  - RNase Inhibitor, 20U/μl
- Nuclease-Free Water. Ambion Inc. Cat. no. AM9937
- TaqMan® Universal PCR Master Mix, No AmpErase UNG (2×). Applied Biosystems Inc. Cat. no. 4324018

Equipment

The same equipment as used for mRNA RT-PCR (see section 2.3).

Reagent and equipment setup

Primers

Primers for synthesis of the first strand cDNA and primers and probes for real time PCR were purchased from Applied Biosystems Inc. (the sequences were proprietary). For the reasons discussed in section 2.3 it was important to use an internal control. Measurement of expression of snoRNA202 was chosen as the internal control, as its use was previously reported in miRNA quantification experiments (Bak et al., 2008; Elia et al., 2009; Judson et al., 2009; Quintavalle et al., 2010).

[2]The same primers were sold for profiling of mouse and human miR-143, miR-let7c and miR-370

Procedure

Reverse transcription: Generation of cDNA

- The components of the kit were thawed on ice
- The master mix for reverse transcription was produced according to Table 2.4.

Component	per 15 μl reaction
100mM dNTPs	0.15
MultiScribe Reverse Transcriptase, 50U/μl	1.00
10x Reverse Transcriptase Buffer	1.50
RNase Inhibitor, 20U/μl	0.19
Nuclease-free water	4.16
TOTAL	7.00

Table 2.4: miRNA reverse transcription master mix.

- The reverse transcription primers were thawed on ice and mixed with the reverse transcription master mix and total RNA (20 ng/μl) in 3:7:5 ratio. The mixture was gently mixed and centrifuged to bring down the droplets. The total volume for reverse transcription was 20 μl and it was performed in a 96-well plate. *IMPORTANT:* Centrifugation speed at this stage must not exceed 400 g.
- The 96-well plate with reaction mixes was incubated on ice for at least 5 min before the start of the reverse transcription. The reverse transcription was performed in Peltier Thermal Cycler according to the program in Table 2.5.

Step Type	Time(min)	Temperature(°C)
HOLD	30	16
HOLD	30	42
HOLD	5	85
HOLD	∞	4

Table 2.5: miRNA reverse transcription program.

Real time PCR

- The product of the reverse transcription reaction (cDNA) was diluted with nuclease free water in 1:15 ratio.
- Real time PCR reactions were set according to Table 2.6.

Component	per 20 μl reaction
primers-probe mix for real time PCR	1.00
appropriately diluted cDNA	1.33
TaqMan 2X Universal PCR MasterMix	10.00
Nuclease Free Water	7.67
TOTAL	20

Table 2.6: miRNA real time PCR master mix.

- The real time PCR (20 μl reaction volume) was conducted in optical 96-well plates using 7500 Real Time PCR System (see section 2.3) with the program described in Table 2.7.

HOLD	CYCLE (40 cycles)	
10 min	15 sec	60 sec
95°C	95°C	60°C

Table 2.7: miRNA real time PCR program.

Analysis of expression by RT-PCR

As in section 2.3.

2.5 Transfection protocol

Primary forebrain cultures for transfection experiments were obtained and cultured as described in section 2.1.

Materials

Reagents

- DharmaFECT 3 siRNA Transfection Reagent. Dharmacon[1]. Cat. no. T-2003-01
- miRNA mimics:
  - mmu-miR-143. Dharmacon, cat. no. MI0000257 / MIMAT0000247 or Qiagen N.V. Cat. no. MSY0000247
  - mmu-miR-145. Dharmacon, cat. no. MI0000169 / MIMAT0000157 or Qiagen N.V. Cat. no. MSY0000157

[1]Dharmacon is a part of Thermo Fisher Scientific

  - mmu-miR-451. Dharmacon Cat. no. MI0001730 / MIMAT0001632
  - cel-miR-67. miRIDIAN microRNA Mimic Negative Control #1). Dharmacon. Cat. no. CN-001000-01-05
  - mmu-miR-25. Qiagen N.V. Cat. no. MSY0000652
  - mmu-miR-410. Qiagen N.V. Cat. no. MSY0001091
  - mmu-miR-551b. Qiagen N.V. Cat. no. MSY0003890
  - mmu-miR-370. Qiagen N.V. Cat. no. MSY0001095
  - mmu-miR-434-3p. Qiagen N.V. Cat. no. MSY0001422
  - mmu-miR-124. Qiagen N.V. Cat. no. MSY0000134
  - mmu-miR-103. Qiagen N.V. Cat. no. MSY0000546

- miRNA inhibitors:

  - Anti-mmu-miR-124. Qiagen N.V. Cat. no. MIN0000134
  - Anti-mmu-miR-434-3p. Qiagen N.V. Cat. no. MIN0001422
  - Anti-mmu-miR-145. Qiagen N.V. Cat. no. MIN000157
  - Anti-mmu-miR-103. Qiagen N.V. Cat. no. MIN0000546
  - Anti-mmu-miR-551b. Qiagen N.V. Cat. no. MIN0003890
  - Anti-mmu-miR-370. Qiagen N.V. Cat. no. MIN0001095
  - Anti-mmu-miR-410. Qiagen N.V. Cat. no. MIN0001091
  - Anti-mmu-miR-25. Qiagen N.V. Cat. no. MIN0000652
  - Anti-mmu-miR-143. Qiagen N.V. Cat. no. MIN0000247

Procedure

The transfection protocol described here was developed by Eric MacLaren (Maclaren et al., 2011) for transfection of siRNA into primary neuronal cultures. This protocol was developed in order to reduce levels of several genes encoding components of the post-synaptic density and to study if such pertrubations affected electrical activity of neurons.

For the first round of miRNA transfections, MacLaren's protocol was followed precisely. The cultures were plated at a high density ($\approx$ 1,850 cells $\cdot$ mm^{-2}) and the incubation time after transfection was 48 h. After a round of test transfections (Chapter 4, section 4.2.2) lower plating density ($\approx$ 790 cells$\cdot$mm^{-2}) and shorter incubation time (36h) were used in some of the experiments (the exact settings for each of the experiments conducted in this thesis project are listed in Table 2.8). The steps of the transfection procedure are listed below:

- Before starting the transfection procedure, the growth media was removed from a cell culture well to leave only 400 μl.
- 3.5 μl of 20 μM stock RNA was diluted in 98 μl of Neurobasal in Tube 1. In parallel, 2.4 μl of DharmaFECT 3 were added to 12 μl of Neurobasal in Tube 2. The tubes were incubated on a benchtop for 5 minutes. For 20 mM stock of RNA this produced approximately 115 nM concentration of the mimic in the final volume of approximately 600 μl. In order to achieve a different concentration in the final volume, a different amount of RNA could be added (adjusting the amount of Neurobasal appropriately). For mock transfection no RNA was added.
- Contents of Tube 1 and Tube 2 were combined in Tube 3 and gently mixed by pipetting up and down. Tube 3 was incubated on benchtop for 20 minutes.
- 80 μl of Neurobasal was added to Tube 3, the content was gently mixed and transferred drop by drop to the cell culture well (evenly distributing the content across the culture well).
- After the addition of the reaction mixture, the culture was transferred back to the incubator (set to 37 °C).
- After 36 h or 48 h incubation time (see Table 2.8) total RNA was extracted from cultures as described in Methods, section 2.2. On all occasions transfection experiments were carried out in four biological replicates.

miRNA ID	**DIV**	**Mimic**	**Inhib**	**Mock**	**Dens**	**Inc**	**M**	**Batch**
cel-miR-67	3DIV	115nM		available	1,850	48h	D	i
cel-miR-67	4DIV	115nM		available	1,850	48h	D	ii
cel-miR-67	4DIV	115nM		available	1,850	48h	D	iii
cel-miR-67	6DIV	115nM		available	1,850	48h	D	iv
cel-miR-67	6DIV	115nM		available	1,850	48h	D	v
miR-143	2DIV	250nM	250nM	available	790	36h	Q	vi
miR-143	3DIV	115nM		available	1,850	48h	D	i
miR-143	4DIV	250nM	250nM	available	790	36h	Q	vii
miR-143	6DIV	115nM		available	1,850	48h	D	iv
miR-145	3DIV	115nM		available	1,850	48h	D	i
miR-145	4DIV	115nM		available	1,850	48h	D	ii
miR-145	4DIV	250nM	250nM	available	790	36h	Q	vii
miR-145	6DIV	115nM		available	1,850	48h	D	iv

Continued on the next page

miRNA ID	DIV	Mimic	Inhib	Mock	Dens	Inc	M	Batch
miR-25	4DIV	250nM	250nM	available	790	36h	Q	vii
miR-103	4DIV	250nM	250nM	available	790	36h	Q	viii
miR-124	3DIV	115nM	230nM	available	1,850	48h	Q	ix
miR-124	4DIV	115nM	230nM		1,850	48h	Q	x
miR-124	6DIV	115nM	230nM	available	1,850	48h	Q	xi
miR-370	6DIV	250nM	250nM		790	36h	Q	xii
miR-410	6DIV	250nM	250nM		790	36h	Q	xii
miR-551b	6DIV	250nM	250nM		790	36h	Q	xii
miR-434-3p	6DIV	250nM	250nM	available	790	36h	Q	xiii
Mock only	4DIV			available	790	36h	na	vii

Table 2.8: Parameters of transfection experiments.
miRNA ID - miRBase Release 14 mature miRNA identifiers (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006, 2008); *DIV* - the timepoint at which the experiment was conducted; *Mimic* - the concentration of a miRNA mimic in the transfection mixture; *Inhib* - the concentration of a miRNA inhibitor in the transfection mixture; *Mock* - was the mock transfection *available* or not for the unidirectional contrasts (in case of the *Mock only* experiment, mock transfected cultures were contrasted with untransfected; *Dens* - the cell density that was used for plating of cultures for the experiments; *Inc* - the post-transfection incubation time (i.e. time from the transfection until cells were killed during miRNA extraction); *M* - the manufacturer of miRNA mimics and inhibitors (*D* - Dharmacon, *Q* - Qiagen N.V.); *Batch* - the batch identifier (cultures for experiments that have the same batch numbers were extracted from the same set of mice on the same day).

2.6 Microscopy

Materials

Reagents

- Dulbecco's Phosphate Buffered Saline 1× (1× DPBS). Invitrogen. Cat. no. 14190-094
- 4% Paraformaldehyde (PFA). Supplied on site.
- Methanol. VWR. Cat. no. 20847.320
- Albumin, from bovine serum (BSA). Sigma-Aldrich Corporation. Cat. no. A2153-100G
- Triton® X-100. Sigma-Aldrich Corporation. Cat. no. 93443-100ML
- Dulbecco's Phosphate Buffered Saline 1× (1× DPBS). Invitrogen. Cat. no. 14190-094

- Nuclease-Free Water. Ambion Inc. Cat. no. AM9937
- Prolong® Gold antifade reagent. Invitrogen. Cat. no. P36934
- Prolong® Gold antifade reagent with DAPI (4′,6-diamidino-2-phenylindole). Invitrogen. Cat. no. P36935
- Enhanced GFP (eGFP) expressing plasmid (pML40-CAG), 410 ng·ml^{-1} (obtained from Meng Li, personal communication)
- Fluorescently labelled oligonucleotide (AllStars Neg. siRNA AF 488). Qiagen N.V. Cat. no. 1027284
- Trypan blue stain 0.4%. Invitrogen. Cat. no. 15250-061
- β3-tubulin rabbit polyclonal antibody (primary antibody). Synaptic Systems. Cat. no. 302 302g
- Goat anti-rabbit IgG (H+L) AlexaFlour 633 (secondary antibody). Molecular Probes. Cat. no. A21070

Equipment

- Super Frost® Plus Slides. VWR International. Cat. no. 631-0108
- Coverslips. Supplied on site.
- Digital camera (AxioCam MRm). Carl Zeiss Ltd. Cat. no. 000445-554
- Light microscope (Axioplan 2 Imaging). Carl Zeiss Ltd.
- Imaging software (AxioVision Release 4.6). Carl Zeiss Ltd.
- Adobe Photoshop, CS4 Extended V11.0. Adobe Systems Inc.

Reagent and equipment setup

eGFP expressing plasmid The plasmid pML40-CAG expressed enhanced GFP (eGFP) (Zhang et al., 1996) under CAGGs promoter (cytomegalovirus immediate-early enhancer sequence connected to a modified (AG) chicken β-actin promoter) (Alexopoulou et al., 2008).

Fluorescently labelled oligonucleotide Fluorescently labelled oligonucleotide (AllStars Negative control) was recommended by Qiagen N.V. as a way of controlling the transfection efficiency. This oligonucleotide had a proprietary sequence and it was labelled by AlexaFlour 488 fluorophore at the 3′-end. AllStars Neg. control was diluted in nuclease-free water to obtain 20 μM stock, and stored at -20 °C.

Growing cultures Cultures for microscopy were grown as described in section 2.1, with the only difference that prior to addition of PDL (see section 2.1) one glass coverslip was placed into culture wells. For transfections (see below) cultures were grown at relatively low density ($\sim$ 790 cells $\cdot$ mm^{-2}), and for the immunostaining and the trypan blue assay cultures were grown at intermediate density ($\sim$ 1,250 cells $\cdot$ mm^{-2}).

Transfection of cultures Cultures were transfected as described in section 2.5. The concentration of AllStar Neg. control in the final transfection volume was 115 nM. Approximately 1.5 ng of the plasmid was used for the transfection.

The blocking solution To reduce non-specific binding of antibodies, and to dilute antibodies to appropriate concentrations, I used the following blocking solution: 0.2% triton X-100 and 3% BSA (in 1$\times$ DPBS).

Procedure

Imaging cultures transfected with the eGFP expressing plasmid or the AlexaFlour 488 labelled oligonucleotide

- The growth media was removed from culture wells and replaced with 1$\times$ DPBS.
- 1$\times$ DPBS was aspirated and replaced with 0.5 ml of 4% PFA solution for 20 min.
- 4% PFA solution was aspirated, coverslips removed from the culture wells. Remainder of 4% PFA was removed from the coverslips by daubing off.
- The coverslips were rinsed by 5 dips into Milli-Q water.
- Small drops of antifade mounting reagent were placed on a microscope slide. The coverslips were carefully placed on top of the drops. The slides were stored either at 4 °C or -20 °C.

Slides were visualised using Axioplan 2 Imaging microscope 495 nm light emission or differential interference contrast (DIC) settings. For DIC images, all parameters were set automatically with the microscope software. The fluorescence of both eGFP and AlexaFlour 488 transfected cultures was detected with 495 nm light for excitation of the fluorophores. The exposure time was controlled either manually or automatically (see figure legends). For imaging of cultures transfected with the plasmid, the exposure was set to the level that produced a maximum contrast between fluorescence of the cells and the background. For imaging of cultures transfected with the AlexaFlour 488 labelled oligonucleotide, the exposure was manually set in all cases to 220 ms to make images

comparable. For better visual contrast, the spectrum was inverted in all photographs of eGFP and the labelled oligonucleotide transfections using Photoshop. (No other parameters of the original images were changed).

Immunostaining β3-tubulin

Staining of a neuronal marker, β3-tubulin (Lee et al., 1990), was performed to evaluate abundance of neurons in primary cultures. Below are the steps of the procedure that was taken for the immunostaining:

- Coverslips were removed into 1× DPBS at room temperature.
- Coverslips were placed in a dish (pre-cooled on ice) with methanol (at -20 °C) for 7 min incubation.
- Coverslips were rehydrated in 1× DPBS briefly (for less than 1 min).
- Coverslips were drained and 100 μl of the blocking solution was added for 1 h.
- Coverslips were washed briefly in 1× DPBS and the primary antibody (100 μl of the supplier stock diluted at 1:1000 in the blocking solution) was added for 1 h.
- Coverslips were washed in 2 ml 1× DPBS for 5 minutes (this was repeated three times).
- Coverslips were drained and the secondary antibody (100 μl of of the supplier stock diluted at 1:1000 in the blocking solution) was added for 20 min (incubation in the dark).
- Coverslips were washed in 2 ml 1× DPBS for 5 minutes (this was repeated three times).
- Coverslips were washed briefly in distilled water.
- A drop of the antifade reagent (with DAPI) was placed onto slides and coverslips were placed on top (cells down). The slides were left overnight at 4 °C before imaging.
- Slides were visualised using Axioplan 2 Imaging microscope 358 nm (for DAPI) and 632 nm (for β3-tubulin staining) light emission with default settings.
- False colors were added to photographs using Photoshop.

Counts of DAPI stained nuclei were assumed to correspond to the number of all cells in a culture, counts of cells stained for β3-tubulin were assumed to correspond to the number of neurons. The percentage of neurons was estimated in the same fashion as viability in Trypan assay (see below).

Trypan assay

Dead cells were visualised in cultures with Trypan blue stain (Altman et al., 1993). Below are the steps of the procedure that was taken for Trypan blue staining:

- 100 μl of Trypan blue stain (0.4% solution, as supplied) was added per cell culture (1000 μl of a growth media, see section 2.1) for 6min.
- All liquid was aspirated and replaced with 0.5 ml of 4% PFA solution for 20 min.
- Coverslips were washed briefly in distilled water.
- A drop of the antifade reagent (with DAPI) was placed onto slides and coverslips were placed on top (cells down). The slides were left overnight at 4 °C before imaging.
- Slides were visualised using Axioplan 2 Imaging microscope 358 nm light (for DAPI) and white light for Trypan with default settings.
- False colors were added to photographs using Photoshop.

Counts of DAPI stained nuclei were assumed to correspond to the number N of all cells in a culture, counts of Trypan blue stained cells were assumed to correspond to the number D of dead cells. Photographs obtained at a low magnification (10× objective) were used for counting ($\sim$ 500 cells per photograph), counts from three non-overlapping images were averaged per each treatment (see text). Viability was defined by the following formula:

$$\frac{N - D}{N} \cdot 100\%$$

2.7 Microarray profiling of mRNA and miRNA expression

Acquisition and analysis of two types of microarray data are described in this section: the in-house data and the external data. The in-house data was generated by microarray facilities at the Wellcome Trust Sanger Institute to profile mRNA or miRNA abundances in total RNA samples that I extracted as a part of experiments described in Chapters 3, 4, 5 and 6. The external data was generated elsewhere in independent experiments.

In-house microarray data: Experimental procedures

Microarray profiling of mRNA and miRNA abundances in total RNA (extracted as described in Methods, section 2.2) was performed by the staff of the microarray facility at the Wellcome Trust Sanger Institute (Naomi Hammond [nh4@sanger.ac.uk], Peter Ellis [pde@sanger.ac.uk] and Cordelia Langford [cfl@sanger.ac.uk]). All procedures were carried out according to the standard Illumina protocols (http://www.illumina.com/support/literature.ilmn). Below is a summary of the procedures.

Reagents

- Illumina® TotalPrep RNA Amplification Kits. Illumina Inc. AMIL1791

Equipment

- Nanodrop
- BeadArray reader
- BeadStudio software
- For mRNA expression profiling: Illumina Sentrix BeadChip Array Mouse-WG6_v1.1 (used in the hippocampal and forebrain developmental timecourse experiments, Chapters 3) or Illumina Sentrix BeadChip Array Mouse-WG6_v2 (used in all miRNA perturbation experiments, Chapters 4, 5 and 6).
- For miRNA expression profiling: Illumina Universal Sentrix Array Matrix (used in the forebrain developmental timecourse experiments, Chapter 3)
- Thermal cycler
- Hybridisation oven

Illumina mRNA microarray profiling assay

- The total RNA was reverse-transcribed with oligo(dT) primers. The oligo(dT) primers had a T7 RNA polymerase binding site (promoter) at the 5′-ends, which was necessary for the *in vitro* transcription step (see below).
- The RNA was digested with RNaseH.
- The cDNA was converted to double-stranded cDNA with a DNA polymerase.
- The purified double-stranded cDNA was incubated with the T7 RNA polymerase and rNTPs (including biotin-tagged rUTP) to produce biotinylated single-stranded anti-sense RNA (called aRNA or cRNA). This step was equivalent to *in vitro* transcription, and amplification was achieved at this step (Gelder et al., 1990).

- The cRNA was purified, quantitated (using Nanodrop), mixed with the hybridisation buffer and applied to the array slides.
- The slides were washed and labelled with streptavidin-Cy3.
- The arrays were scanned using BeadArray reader and the image data was processed by BeadStudio.
- Quality control of loading and hybridisation efficiencies was performed using sample dependent and sample independent control measurements.
- The raw data output (not normalized) of BeadStudio was used for the next stage (**Data processing**).

Illumina miRNA microarray profiling assay

- The 3′-ends of total RNA were polyadenylated and the total RNA was reverse-transcribed using biotin-tagged oligo(dT) primers. The oligo(dT) primer had a universal sequence at the 5′-end which was necessary for PCR step (see below).
- The cDNA was attached to streptavidin beads and hybridised to miRNA-specific oligos.
- The miRNA-specific oligos were extended using a DNA polymerase.
- The extended products were eluted and PCR was performed using fluorescently labelled primers.
- Single-stranded PCR products (ssDNA) were prepared, quantitated (using Nanodrop) and hybridised to the arrays.
- The arrays were scanned using BeadArray reader and the image data was processed by BeadStudio.
- Quality control of loading and hybridisation efficiencies was performed using sample dependent and sample independent control measurements.
- The raw data output (not normalized) of BeadStudio was used for the next stage (**Data processing**).

In-house microarray data: Data processing

Data processing and normalization. mRNA arrays

Analysis of the array data was performed in R environment (RTeam, 2008) with Bioconductor packages (Gentleman et al., 2004). The output of BeadStudio was imported into R using *lumi* package functions (Du et al., 2008). In addition to the essential quality

control steps performed by the microarray facility (see above), additional quality control steps were performed using *lumi* package functions. First, correlation of probe intensities levels was examined between pairs of biological replicates. Second, all samples were clustered hierarchically to examine their relation. In case of mRNA profiling, pairwise correlations of all replicate samples was above 0.99, and clustering of samples corresponded perfectly to the design of the experiments (Supplementary Data Figure A.2, A.1 and A.4). Therefore all replicate samples were used for further steps of the analysis. Detection call P-values were obtained with the *detectionCall* method, which is available via the *lumi* package. If the P-value was < 0.01 (the default threshold), the probe was considered "Present", otherwise it was considered "Absent". The "Absent" probes were removed, and expression values of the "Present" probes were transformed with the Variance Stabilizing Transformation (VST) method and normalized with the robust spline normalization (RSN) method (Lin et al., 2007), both available in the *lumi* package (Du et al., 2008).

Data processing and normalization. miRNA arrays

The output of BeadStudio was imported into R using *lumi* package functions (Du et al., 2008). Furthermore, using methods in the *lumi* package, the correlation of probe intensity values was assessed between the samples as a way of QC (to complement the QC steps performed by the microarray facility, see above). Some miRNA array samples stood out as poorly correlated with the rest of the samples (Supplementary Data Figure A.5 and A.6a). When raw probe intensities were visualised, it became apparent that probe intensities in the poorly correlated samples displayed a global downward shift (Supplementary Data Figure A.6b). In order to minimise the biases to the subsequent steps of analysis, seven poorly correlated samples were removed from further analysis (specified in the legend to Supplementary Data Figure A.6b). After the removal of these samples, there remained 4 samples for 1DIV, 2 samples for 2DIV, 4 samples for 4DIV and 5 samples for 8DIV timepoints (see Chapter 3, section 3.2). Raw values of the remaining samples were transformed using log_2 transformation and normalized using quantile normalization, as these methods of transformation and normalisation were shown to be optimal for Illumina miRNA microarray data (Rao et al., 2008).

Mapping the Illumina probes. mRNA arrays

Mapping of Illumina mRNA probes to gene and transcript identifiers was performed by my colleague, Cei Goodger-Abreu [cei@langebio.cinvestav.mx]. A summary of the steps involved is given below.

Illumina microarray probe sequences were taken from the Illumina mRNA array annotation files (BGX files) available from the Illumina website[1] http://www.illumina.com/support/annotation_files.ilmn. Illumina mRNA array probes were aligned to the complete set of the full-length Ensembl v56 mouse transcripts (Hubbard et al., 2009) with SSAHA2 (Ning et al., 2001). All categories of Ensembl transcripts, were retrieved using the Ensembl Perl API, which enabled access to Core, Vega and OtherFeatures mouse transcripts (Hubbard et al., 2009). A transcript from the highest scoring SSAHA2 alignment was chosen for each probe (at least 30 perfect consecutive matches were required). If more than one alignment had an equally high score, manually curated Vega transcripts were preferred. In order to resolve ambiguity of multiple transcripts from the same source aligning equally well, the "biotype" annotation was considered (protein coding transcripts were preferred to pseudogenes, and nonsense-mediated decay had the lowest preference). If the ambiguity was still not resolved, transcripts with the longest 3′UTR or cDNA were selected. When probes did not align to any of the Ensembl transcripts, mapping of the probes to RefSeq 38 (Pruitt et al., 2009) transcript identifiers was taken from the Illumina BGX files. After probes were uniquely mapped to the best transcript identifiers (either from Ensembl or from the Illumina BGX files), the corresponding GeneBank gene identifiers (in this thesis they are referred to as Entrez gene IDs) were matched to the probes. For probes mapped to Ensembl or Vega transcripts, the Entrez gene IDs were retrieved using Ensembl API, and for probes mapped to RefSeq IDs via the BGX annotation files, the Entrez gene IDs were taken from the BGX file.

Mapping the Illumina probes. miRNA arrays

Sequences of miRNA microarray probes were taken from the Illumina annotation file (the BGX file) available from the Illumina website[2] http://www.illumina.com/support/annotation_files.ilmn. Illumina miRNA array probes were aligned to the full set of mature miRBase Release 13 miRNA sequences (Griffiths-Jones et al., 2008, 2006; Griffiths-Jones, 2004) using SSAHA2 (Ning et al., 2001). Full length perfect matching to mature sequences

[1]The name of the annotation file for v1.1 beadarrays: MouseWG-6_V1_1_R4_11234304_A, for v2 beadarrays: MouseWG-6_V2_0_R2_11278593_A

[2]The name of the annotation file for the miRNA array matrix: mouseMI_V1_R0_XS0000127-MAP

was required. This requirement alone resolved all ambiguity and resulted in mapping of 362 Illumina miRNA probes to unique mature mouse miRNAs in the miRBase.

Analysis of differential expression

The differential expression was estimated for all probes (VST transformed and VSN normalised probe intensity values, see above) using the R package *limma* (Smyth, 2004). A linear model was fitted and coefficients were estimated for every probe according to the design of the experiments. Moderated t-statistics for each probe were evaluated with the empirical Bayes method. The P-values associated with the t-statistics were multiple-test corrected with Benjamini and Hochberg method (Benjamini and Hochberg, 1995). Each probe was paired up with a transcript and Entrez gene ID using probe mapping procedure that was described above. Probes that could not be mapped were excluded at this stage. Ambiguity in the mapping was resolved by keeping one probe (with the best adjusted P-value of differential expression) per gene. The remaining probe set usually contained from 9,000 to 11,000 probes, each uniquely corresponding to one Entrez gene ID. Fold-changes (in log_2 scale), t-statistics, P-value and adjusted P-value for the probes were used to describe differential expression of the corresponding genes.

For the miRNA arrays, the analysis of differential expression for all probes (log_2 transformed and quantile normalised values) that mapped to miRBase Release 13 mature miRNA identifiers (see above) was performed with *limma* (Smyth, 2004). Fold-changes (in log_2 scale), t-statistics, P-value and adjusted P-value for the probes were used to describe differential expression of the corresponding miRNAs.

Clustering of genes according to gene expression trends

The probes that were uniquely mapped to genes and which were differentially expressed in the timecourse experiments between any pair of consecutive timepoints or between the first and the last timepoint[3] (adjusted $P < 0.1$) were clustered according to their expression. The clustering was done using the Markov Cluster Algorithm (MCL) software package[4] (van Dongen, 2000; Freeman et al., 2007). Probes were described by measurements on the four timepoints, with each measurement defined as the median of the intensity values taken over the replicates. For each pair of the probes, their similarity was computed as the Pearson correlation coefficient over their intensity values. A graph was defined where

[3] four developmental timepoints, see Chapter 3, section 3.1
[4] freely available for download at http://www.micans.org/

the nodes are probes, and two nodes (probes) are connected if the correlation between them was at least 0.9, with the weight of the edge set to that correlation value. This graph was processed with the MCL algorithm, which naturally partitions the graph into separate clusters. The inflation parameter was set to 3.

External microarray data

Data retrieval

External microarray data (not normalised) was obtained directly from Gene Expression Omnibus (GEO) website http://www.ncbi.nlm.nih.gov/geo/. Accession identifiers for three analysed external data sets were GSM210760 (Makeyev et al., 2007), GSE6388 (Akahoshi et al., 2007) and GSE10246 (Lattin et al., 2008).

Normalisation, differential expression analysis and mapping

The external data sets were generated using Affymetrix microarray platforms. A method that was specifically designed for transformation and normalisation of Affimetrix data, called RMA (Irizarry et al., 2003a,b), was used in analysis of the external data. RMA was implemented via *affy* Bioconductor package (Gautier et al., 2004),

For the external datasets, differential expression was estimated for all probes using *limma* (Smyth, 2004) in the same way as was described for the in-house data (see above). As a result, each probe was assigned fold changes (on the RMA scale, wich is approximately equal to log_2) moderated t-statistics and corresponding P-values (both unadjusted and adjusted with Benjamini and Hochberg method).

Bioconductor annotation libraries (Gentleman et al., 2004) of microarray platforms were used in analysis of the external data ("mouse4302.bd" (Makeyev et al., 2007; Lattin et al., 2008) and "mgu74bv2.db" (Akahoshi et al., 2007)). These libraries provided mapping of microarray probes to RefSeq transcript and Entrez gene identifiers. For RefSeq transcript IDs, 3′UTR sequences were obtained from Ensembl, using Ensembl API (Hubbard et al., 2009). The length of 3′UTRs was used to resolve the ambiguity in mapping of probes to RefSeq transcript IDs: when the probes were mapped to more than one RefSeq transcript ID, the ID corresponding to the transcript with the longest 3′UTR was selected. In the Bioconductor annotation files, each RefSeq transcript ID corresponded to one Entrez gene ID. Therefore, by uniquely matching the probes to RefSeq transcript IDs, each probe was also uniquely matched to an Entrez gene ID. The adjusted P-values of differential expression were used for selection of the best probe per Entrez gene ID (i.e.

a probe with the most significant adjusted P-value). By selecting one probe per Entrez gene ID, the total number of probes was reduced to the total number of genes represented on the platform.

2.8 Seed enrichment analysis

Obtaining sequences

3′UTRs

The 3′UTR sequence for the transcripts mapped to each of the microarray platforms (see section 2.7) were obtained from Ensembl v56 with the Ensembl API (Hubbard et al., 2009). Three FASTA files were created using the retrieved sequences (referred here to as raw sequences): one for the two versions of the in-house Illumina mRNA arrays[1], and one file for the external data (see section 2.7). For Sylamer analysis (see below) it was recommended to remove regions of low complexity and repetitive (redundant) sequences, therefore the FASTA files with raw sequences were processed as previously described (van Dongen et al., 2008). The low complexity regions were masked out using DUST (Tatusov R.L. and Lipman D.J., personal communication) and redundant sequences were masked out using purge-sequence from the RSA-tools (Thomas-Chollier et al., 2008). The processed sequences are referred in this thesis to as dusted/purged sequences.

Seed matching sites for miRNAs

The sequences of all mature mouse miRNAs were downloaded directly from miRBase Release 14 (Griffiths-Jones et al., 2008, 2006; Griffiths-Jones, 2004). For each miRNA, two sequences complementary to the seed region (i.e. the seed matching sites) were produced: the sequence complementary to bases 2-8 from the 5′-end of the miRNA (7(2)-type seed matching site), and to bases 1-7 with an A opposite to position 1 (7(1A)-type). This resulted in 876 distinct seed matching oligonucleotide words. The seed matching sites were stored as a flat file and used for the enrichment analyses (see below).

[1] Retrieval of sequences for the in house arrays was done by Cei Goodger-Abreu [cei@langebio.cinvestav.mx]

Seed matching site enrichment

Simple hypergeometric test of enrichment

The hypergeometric test was used to evaluate the enrichment of transcripts with seed matching sites for a particular miRNA among the 3′UTRs of transcripts[2] differentially expressed beyond a certain threshold. The urn model is a popular way to describe the hypergeometric test. In this model an urn contains N balls of which K are black and $N-K$ are white. We draw a sample of n balls from the urn without replacement and observe k black balls. The probability of such an event follows the hypergeometric distribution, and is given by

$$\frac{\binom{K}{k}\binom{N-K}{n-k}}{\binom{N}{n}}$$

The one-sided test of enrichment asks for the cumulative probability of finding at least k black balls in a sample of size n randomly drawn from the urn according to the formula given above, by summing all probabilities over the range $k \ldots K$. This test, computed using standard R functions (RTeam, 2008), was used to test enrichment of seed matching site containing transcripts by equating the set of all transcripts with the set of all balls, by equating transcripts that contain at least one seed-matching site with black balls (and all other transcripts with white balls), and by considering a sample to be a set of transcripts differentially expressed beyond a certain threshold T. Denoting the sample size by n, when observing k transcripts containing at least one seed-matching site in the sample, the one-sided test thus gives the probability of observing as least as many as k transcripts containing seed-matching sites.

Sylamer

Sylamer tests for nucleotide word occurrence biases in a sorted list of sequences using hypergeometric test (van Dongen et al., 2008). In this thesis, Sylamer was applied to test for miRNA effects by searching sorted lists of 3′UTRs[3] for enrichment or depletion of miRNA seed matching sites. The mechanism of Sylamer is described in the Introduction (section 1.2.3). Below is the description of the internal parameters that were used for Sylamer analyses in this thesis:

[2]The matching of 7(2) and 7(1A)-type seed sites to the raw 3′UTR sequences was done using Perl.
[3]Transcripts without 3′UTR sequence were excluded from the test.

- Sequences were sorted either by t-statistic (see section 2.7) or by the intensity values (i.e. the level of expression) of corresponding microarray probes (see the figure legends).
- In each test distribution of 876 seed matching sites of length 7 bases corresponding to mouse mature miRNAs[4] (see above) was assessed.
- The sample size (bin size) of selected sequences was incremented by 100 at each step.
- The level of Markov-correction was set to 4.
- Sequences of 3′UTRs were dusted/purged (see above).

2.9 Neuron specific genes

Neuron specific genes were defined as genes with expression significantly higher in the mouse brain than in other organs, and which at the same time encoded proteins of post-synaptic density (PSD). These two characteristics were combined, because on its own they did not guarantee specificity of expression in neurons, while selecting the intersection between the two types of genes increased the likelihood of the specificity. The list of 1,634 mouse PSD genes was obtained experimentally by my colleague Alex Bayes (personal communication). The list of genes with expression significantly higher in the mouse cortex relative to other tissues and organs was obtained through analysis of "mouse gene expression tissue atlas" from "BioGPS" gene annotation portal (http://biogps.gnf.org/) (Lattin et al., 2008; Wu et al., 2009a) of the Genomic Institute of the Novartis Research Foundation.

The raw microarray data that comprised the "mouse gene expression tissue atlas" was downloaded from GEO (Barrett et al., 2007), accession number GSE10246 (Lattin et al., 2008)). This dataset consisted of a microarray gene expression profile of 91 mouse tissues and cell cultures. The raw microarray data was normalized as described in section 2.7. Of the 91 tissues in the data set, there were three that represented the cortex ("cerebral cortex", "cerebral cortex prefrontal", "hippocampus"). These tissues were labelled as "cortex". The expression in these cortical structures was contrasted to the expression in a selection of unrelated to the cortex tissues ("bone", "bone-marrow", "epidermis", "heart", "intestine large", "intestine small", "kidney", "lens", "liver", "lung", "lymph

[4]Additional two seed matching sites were included in analysis of experiments where the mimic of a *Caenorhabditis elegans* miRNA, cel-miR-67, was transfected. These words corresponded to 7(2) and 7(1A)-type seed matching sites for cel-miR-67 (*GGTTGTG* and *GTTGTGA*)

nodes", "mammary gland (lact)", "mast cells", "NK cells", "ovary", "pancreas", "placenta", "prostate", "salivary gland", "spleen", "stomach", "testis", "umbelical cord", "uterus"). These tissues were labelled as "non-cortex".

The tissues with the label "cortex" were contrasted against samples with the label "non-cortex" and differentially expressed probes were identified using the *limma* R package (Smyth, 2004), as described in section 2.7. Subsequently, the probes were uniquely mapped to Entrez gene and RefSeq transcript identifiers as described in section 2.7.

Genes that were upregulated in the cortex by more than two fold (with adjusted P-value of differential expression below 0.05) comprised the list of 2,732 genes with expression enriched in the cortex. The intersection of these genes with the PSD genes (Alex Bayes, personal communication) produced the list of 732 genes, which were putatively neuron-specific (referred in this thesis to as neuron-specific). Of these genes, 725 were represented on Illumina Mouse-WG6 v2 microarray platform that was utilised in functional miRNA experiments in this thesis (Methods, section 2.7). These putatively neuron-specific genes are listed in Supplementary Data (Table A.1).

2.10 Enrichment of GO and KEGG terms

Analysis of the enrichment of GO (Ashburner et al., 2000) and KEGG (Kanehisa et al., 2008, 2000) terms was performed in R environment (RTeam, 2008) with Bioconductor packages (Gentleman et al., 2004). Annotation of the Entrez gene IDs to GO and KEGG terms was obtained from "illuminaMousev2.db" library, available via the Bioconductor website http://www.bioconductor.org/packages/2.6/data/annotation/. The test of enrichment of GO and KEGG terms in the selected categories of genes, in comparison to the gene universes, was performed using the *GOstats* package (Falcon and Gentleman, 2007).