Chapter 3

A model of neuronal development

Dissociated primary cultures are a popular model of neuronal development and function (Introduction, section 1.2.4) and it was used in this thesis to investigate the role of miRNAs in neurons. The cultures were studied in a time-window centered around 4 days of *in vitro* development (DIV): from **1DIV** to **8DIV**. The significance of the 4DIV timepoint as a switch point in development of primary neuronal cultures was previously reported (Valor et al., 2007). Valor *et al.* showed that before 4DIV the ratio of average abundances of neuritic to somatic transcripts was below one, while after 4DIV it was above one, which is similar to that of mature neurons. Appearance of early synapses at around 4DIV and detection of early events of electrical activity in the cultures at around 6DIV (Valor et al., 2007), also supported the proposition that neurons in primary neuronal cultures after 4DIV were similar to mature neurons. Studying miRNAs in the cultures between 1DIV and 8DIV, could, therefore, highlight functions of miRNAs in both immature and mature primary neurons.

In this chapter, I describe profiling of mRNA and miRNA abundances in 1DIV to 8DIV time-window in development of E17.5 primary cultures. This profiling demonstrated that primary forebrain cultures were a good model to study mRNA and miRNA expression during growth of committed (differentiated) neurons. The profiling of mRNA and miRNA expression in developing primary forebrain cultures was published (Manakov et al., 2009). Additionally, I found evidence that endogenous miRNAs shaped gene expression in primary forebrain cultures. Apart from being a novel observation on its own, this finding further supported the cultures to be a suitable model system to study roles of miRNAs.

Based on miRNA profiling results, nine mouse miRNAs and a control non-mouse miRNA were selected for functional perturbation experiments. The selection is described at the end of the current chapter.

3.1 A model of developmental gene expression

3.1.1 Gene expression changes in development of both hippocampal and forebrain primary cultures were highly correlated

To establish if gene expression in E17.5 primary forebrain cultures was sufficiently similar to that of better characterized hippocampal cultures (Introduction, section 1.2.4), gene expression was profiled in development of both forebrain and hippocampal cultures. Microarrays were used to profile gene expression changes throughout a timecourse of E17.5 primary cultures development. The cultures were grown as described in Methods (section 2.1). At four timepoints, 1DIV, 2DIV, 4DIV and 8DIV, total RNA was extracted and profiled on microarrays (Methods, sections 2.2 and 2.7).

To obtain comparable measurements across four developmental timepoints it was necessary to have all biological replicates derived from a single batch of cultures. For profiling of mRNA expression in hippocampal cultures, a batch of 12 cultures was plated (Methods, section 2.1), producing three biological replicates for each of the 4 timepoints. Forebrain cultures were plated in a separate experiment, i.e. dissociated forebrains were obtained from a set of embryos collected from a different group of pregnant mice. Because forebrains are much larger, it was possible to plate a batch of 23 cultures. This produced five biological replicates for 1DIV, and six replicates for each of the timepoints 2DIV, 4DIV and 8DIV. Total RNA was extracted (Methods, section 2.2) and profiled on mRNA microarrays (Methods, section 2.7).

Results of microarray profiling of development of cultures were highly consistent. Pearson correlation of within-timepoint biological replicates prior to normalisation was 0.99 or higher for both hippocampal and forebrain cultures at all timepoints (Supplementary Data Figures A.2 and A.1). Transformation and normalisation of raw data was performed as described in Methods (section 2.7). Hierarchical clustering of normalized expression values revealed that gene expression detected by microarrays was more similar between biological replicates within any of the timepoints than between different timepoints. At the same time, gene expression between consecutive timepoints was relatively closely related, with pairs of timepoints 1DIV, 2DIV and 4DIV, 8DIV, forming distinct outgroups. This consistent trend was true for profiles of both hippocampal and forebrain cultures (Supplementary Data Figure A.4).

Approximately 10,000 genes were detected with high confidence in primary cultures. Of 46,628 probes on the microarray platform, 16,408 probes in hippocampal culture ex-

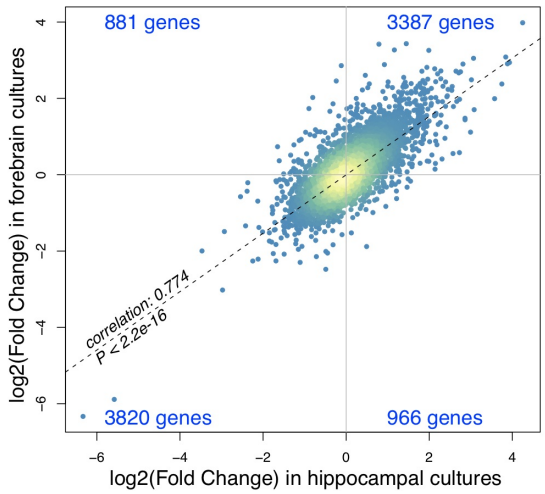


Figure 3.1: Correlation of forebrain and hippocampal cultures development. The points correspond to 9,054 genes detected in both the development of hippocampal and forebrain cultures (the x-axis corresponds to log_2 of the expression fold change between 1DIV and 8DIV in development of hippocampal cultures, the y-axis – to forebrain cultures). The colors of the points depend on the density of the points in a given region of the plot (yellow – highest, blue – lowest). The text in blue corresponds to gene counts in each quadrant of the plot, the italic in black gives Pearson correlation of the fold changes and P-value of the correlation. The dashed line is a linear model fitted through the points.

periment and 16,003 probes in forebrain culture experiment were reliably detected (using the standard Illumina detection call $P < 0.01$). The detected probes were mapped to 10,067 and 9,826 genes respectively. Detection, normalization and mapping are described in Methods (section 2.7).

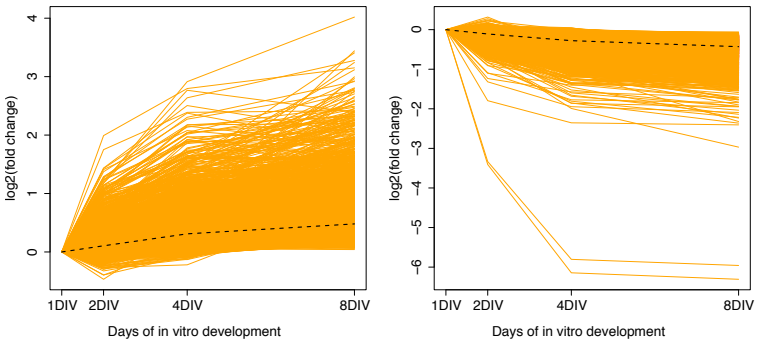
Comparison of differential expression between whole forebrain and hippocampal cultures, showed that gene expression trends in the two types of cultures were similar. Of 9,826 genes, whose expression was detected in forebrain cultures, 9,054 were also detected in hippocampal cultures development. When expression fold changes between 1DIV and 8DIV were compared between the two experiments, a Pearson correlation of 0.774 ($P < 2.2e-16$) was observed (Figure 3.1). This observation indicated that global trends in mRNA gene expression in development of forebrain cultures was similar to that of primary hippocampal cultures, an established model of neuronal development and function (Introduction, section 1.2.4).

3.1.2 The reciprocal trends of gene expression in development of hippocampal and forebrain primary cultures

Almost 90% of genes detected by microarrays in primary cultures were differentially expressed during development. Of all genes detected in hippocampal and forebrain culture development, 8,999 and 9,040 genes were differentially expressed between any of the two consecutive timepoints (adjusted $P < 0.1$, Methods, section 2.7). Between both experiments, the intersection of differentially expressed genes was 7,646 genes.

To identify trends of differential expression, differentially expressed genes (adjusted $P < 0.1$) were clustered using MCL (van Dongen, 2000) (see Methods, section 2.7). Clustering of gene expression trends identified 32 distinct clusters in hippocampal and 28 in forebrain culture experiments. The two largest clusters of genes encompassed a majority of all differentially expressed genes in both experiments (66.67% in hippocampal and 72.62% in forebrain cultures). The third largest cluster in both cases included less than 5% of genes. Median expression trends of the two largest clusters were approximately inverse, with median trends of expression being gradual upregulation and downregulation (Figure 3.2). A significant overlap was observed between genes in the two major clusters of upregulated and downregulated genes between hippocampal and forebrain cultures. Of genes that were expressed both in hippocampal and forebrain cultures, approximately 67.40% and 71.68% of hippocampal genes in upregulated and downregulated clusters were in the respective forebrain clusters (Figure 3.3). The P-value for these intersections exceeded the precision limit for the hypergeometric test as implemented in the R *stats* package (equivalent to $P < 1e - 45$) (RTeam, 2008).

(a) Upregulated cluster (HP) **(b)** Downregulated cluster (HP)



(c) Upregulated cluster (FB) **(d)** Downregulated cluster (FB)

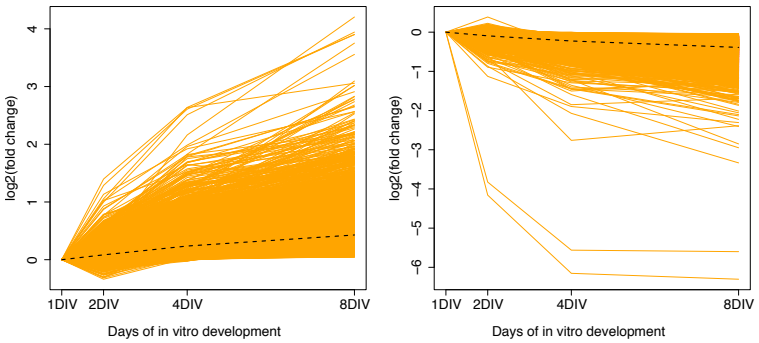


Figure 3.2: Gene expression clusters of down- and upregulated genes.
The orange lines correspond to the fold change of gene expression (log_2) starting from 1DIV and across the other three developmental timepoints (2DIV, 4DIV and 8DIV). The x-axis shows time (DIV, days of *in vitro* development), the y-axis shows fold change in gene expression (log_2)). The subfigures show: 3.2a – trends in the biggest gene expression cluster in developing hippocampal cultures (dashed line – median trend); 3.2b – trends in the second biggest expression cluster; 3.2d – trends in the biggest gene expression cluster in developing forebrain cultures; 3.2c – in the second biggest expression cluster. Abbreviations: *HP* – hippocampal cultures; *FB* – forebrain cultures. Clustering is described in Methods (section 2.7).

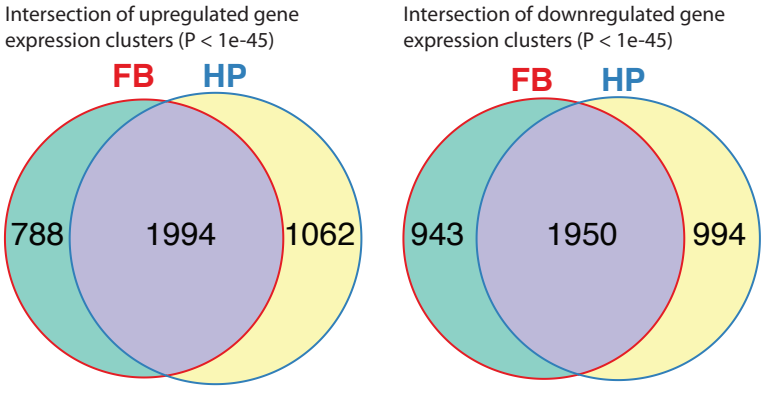


Figure 3.3: Intersection of gene expression clusters in hippocampal and forebrain cultures development.
FB – forebrain cultures; *HP* – hippocampal cultures. The genes detected as differentially expressed in hippocampal cultures (8,999 genes) were used as a gene universe for calculation of the hypergeometric P-values.

3.1.3 Cell growth, not proliferation, was a predominant ongoing process in development of primary cultures

Enrichment analysis of Gene Ontology (GO) (Ashburner et al., 2000) and KEGG terms (Kanehisa et al., 2008, 2000) was used to determine whether development of primary forebrain cultures was predominantly characterized by cell growth and neuronal activity, or by cell proliferation. If gene expression trends in development of cultures were consistent with increasing cell growth, then such observations would have been consistent with the growth of neurites being a predominant process in development of cultures. On the other hand, if gene expression trends were consistent with ongoing cell proliferation, then such a result would be suggestive of proliferating non-neuronal cell types being a dominant component of cultures. To test this, significantly differentially expressed genes (adjusted $P < 0.1$, Methods, section 2.7) between 1DIV and 8DIV were separated into two groups: down and upregulated in development. The separation of differentially expressed genes in two groups was done solely based on the direction of change during development without considering magnitude of change. Based on this criterion, 4,426 genes were defined as downregulated and 4,098 as upregulated in forebrain cultures development. Enrichment of GO terms of "Biological Process" and "Cellular compartment" types and of KEGG terms (also known as KEGG pathways) was then evaluated to describe function and localization of proteins encoded by down- and upregulated genes.

Analysis of GO term enrichment implied that in development of primary cultures there was an increase in cell growth, and not in proliferation. GO terms describing nuclear localization and biological processes taking place in the nucleus were enriched in downregulated genes (Figure 3.4). At the same time, terms describing extracellular, plasma membrane and other non-nuclear localizations were enriched in upregulated genes. Terms relating to biological processes not taking place in the nucleus were also overrepresented in upregulated genes. Importantly, some of the terms specifically related to neuronal biology and activity (e.g. "synapse" and "neurological system process") were among enriched terms in upregulated genes. Gene counts of down and upregulated genes in a representative selection of 40 most enriched GO terms is shown in Figure 3.4 (the full lists of the top 40 most enriched GO terms is in Supplementary Data Tables A.2 to A.5).

In agreement with the GO enrichment results, KEGG pathway analysis showed that biological processes taking place in the nucleus and cell cycle related pathways were downregulated while processes taking place not in the nucleus, as well as specifically neuronal pathways, were upregulated. For example, among the top 10 most enriched pathways

in downregulated genes were "spliceosome" ($P < 3.04e-20$), "DNA replication" ($P < 8.61e-10$) and "Cell cycle" ($P < 1.14e-09$). At the same time pathways "Neuroactive ligand-receptor interaction" ($P < 9.14e-05$), "Long-term potentiation" ($P < 0.00134$), "Calcium signalling function" ($P < 8.5e-05$) and "Cell adhesion molecules (CAMs)" ($P < 0.00134$) were in top 10 pathways enriched in upregulated genes. A complete list of the top 25 most enriched KEGG pathways is in Supplementary Data, Tables A.6 and A.7.

Abundant neurite outgrowth during the developmental timecourse was also evident upon visual inspection of primary cultures. On the day of plating (0DIV) cells almost completely lacked any appendages (Figure 3.5a) while by 3DIV the outgrowth was already evident (Figure 3.5b). To confirm that cultured cells were indeed neurons, I immunostained a neuronal marker, β3-tubulin (Lee et al., 1990), at 3DIV and 8DIV (Methods, section 2.6). By combining this immunostaining with visualisation of all nuclei (DAPI staining, see Methods, section 2.6) it was established that throughout the developmental timecourse the population of cells was comprised almost entirely of neurons (Figure 3.5 and Table 3.1). Additionally, I confirmed viability of cells in primary neuronal cultures with Trypan assay (Altman et al., 1993) at 3DIV (Figures 3.6a and 3.6b, Table 3.2) and at 8DIV (Figures 4.6c and 4.6d, Table 3.2), see for a comparison cultures treated with sodium azide (Figures 4.6e and 4.6f). Trypan assay is described in Methods (section 2.6).

As described in the Introduction (section 1.2.4) in the work by Valor *et al.*, which investigated the gene expression program of developing primary E17.5 hippocampal cultures, the 4DIV timepoint was identified as a switch point in maturation of the cultures (Valor et al., 2007). A similar effect was observed in developing primary E17.5 forebrain cultures: if expression trends of the upregulated genes were overlaid on the same plot with the downregulated genes, their median trends intersected at almost exactly the 4DIV timepoint (Figure 3.7). Therefore, in this thesis 4DIV was treated as a developmental switch point, when gene expression of neuritic genes became, on average, higher than that of nuclear genes (i.e. many of the somatic genes). In other words, after 4DIV the ratio of neuritic and somatic genes in developing cultures was similar to that of mature neurons. This central position of the 4DIV timepoint in gene expression program of cultures was important for selection of experimental timepoints for transfection experiments that are described in Chapters 4 and 5.

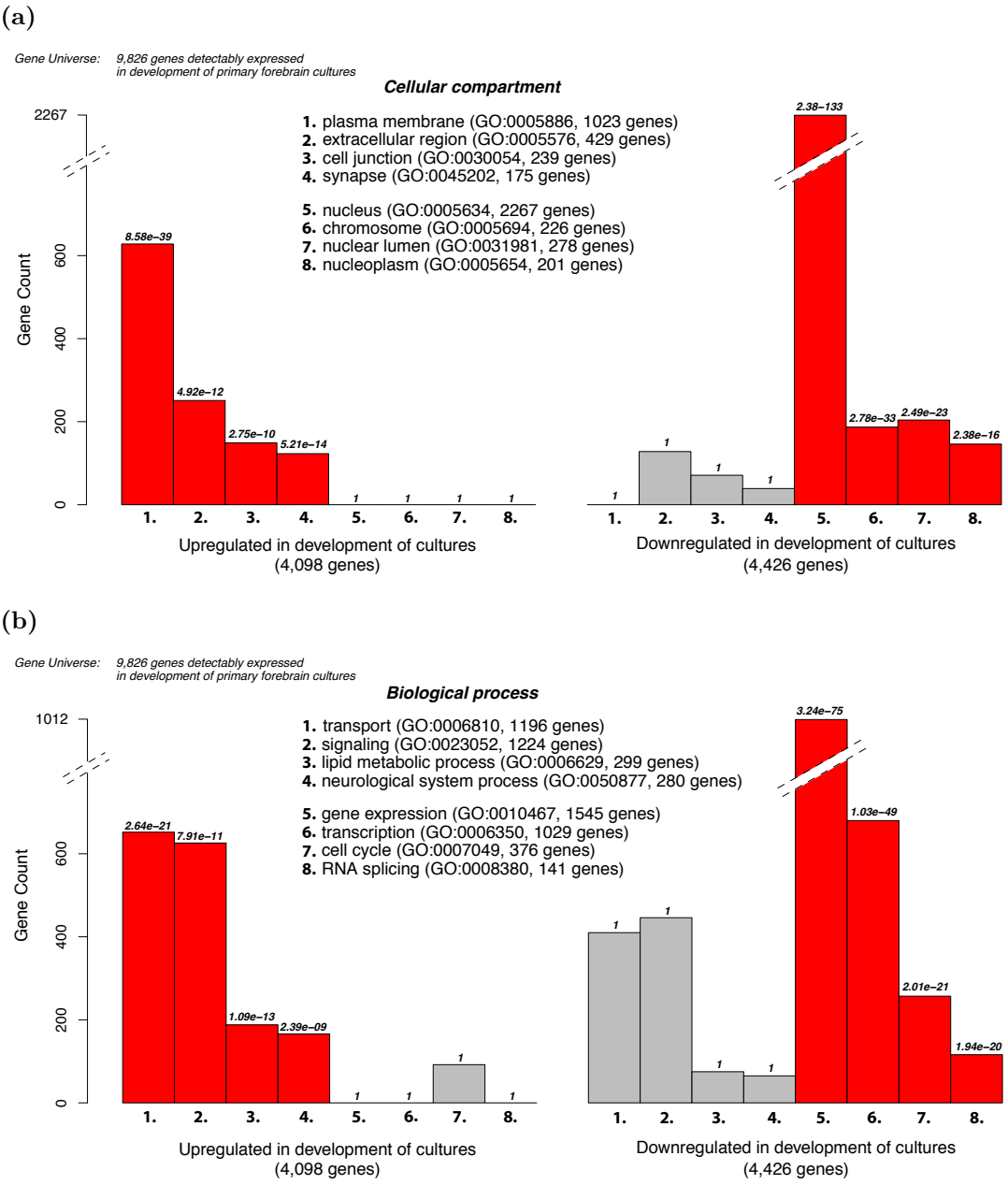
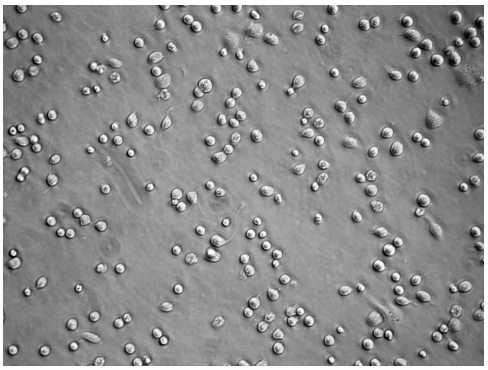


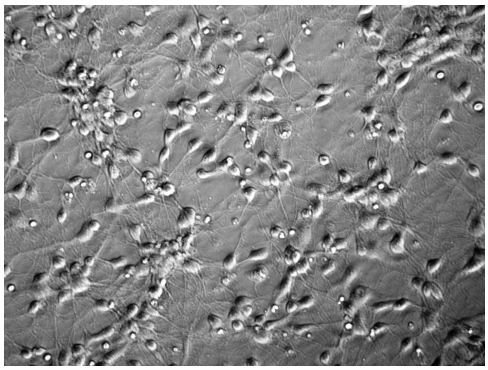
Figure 3.4: Enrichment of Gene Ontology (GO) terms in differentially expressed genes during primary cultures development.

The y-axes show the number of genes from a GO category that were identified among the up- or down-regulated genes. The enrichment P-values for each of the terms is given at the top of each bar (Methods, section 2.10). The numbers on the x-axes correspond to the GO terms listed in the plot areas. The bars for the significantly enriched GO terms is shown in red and not significantly enriched – in grey.

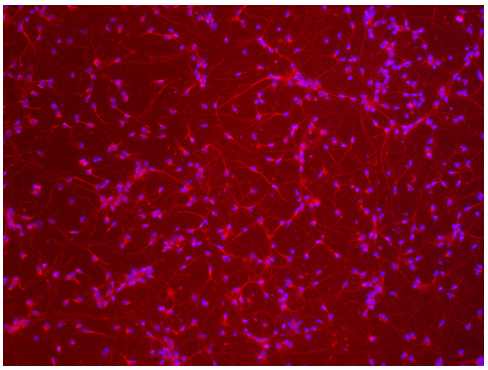
(a) 0DIV (DIC)



(b) 3DIV (DIC)



(c) 3DIV (β3-tubulin and DAPI)



(d) 8DIV (β3-tubulin and DAPI)

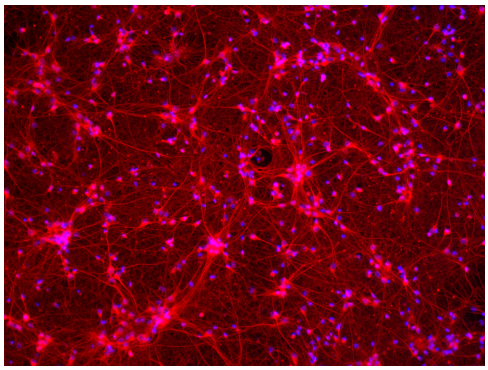
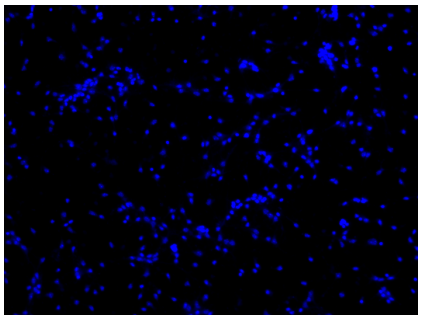


Figure 3.5: Neurons in primary cultures.
Cells in primary cultures were visualised using differential interference contrast (DIC) settings at 0DIV (Figure 3.5a) and 3DIV (Figure 3.5b). Immunostaining of a neuronal marker, β3-tubulin, is shown in red and DAPI staining is shown in blue (at 3DIV in Figure 3.5c and at 8DIV in Figure 3.5d). See Methods (section 2.6) for details.

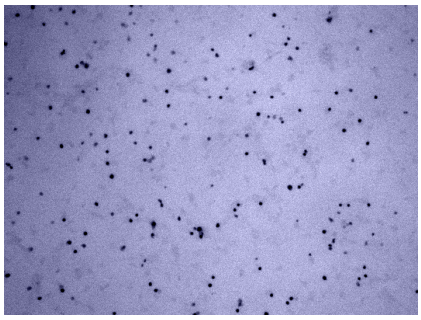
Days *in vitro*	**Percent of neurons**	**Standard deviation**
3DIV	99.7%	± 0.24%
8DIV	99.5%	± 0.34%

Table 3.1: Neurons in primary neuronal cultures.
Percent of neurons was estimated based on three non-overlapping 10× objective images (∼ 500 cells per image, e.g. Figures 3.5c and 3.5d). Total numbers of cells were estimated by counting DAPI stained nuclei, neurons – by counting β3-tubulin positive cells (Methods, section 2.6).

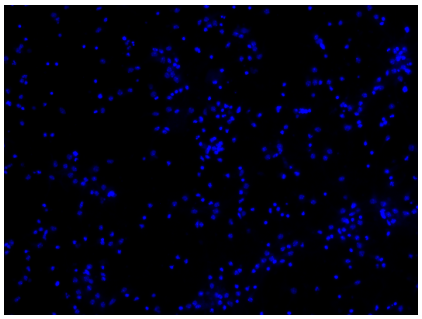
(a) 3DIV (DAPI)



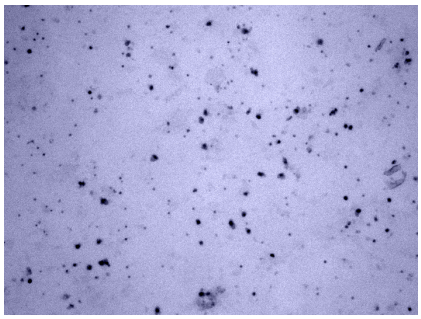
(b) 3DIV (Trypan)



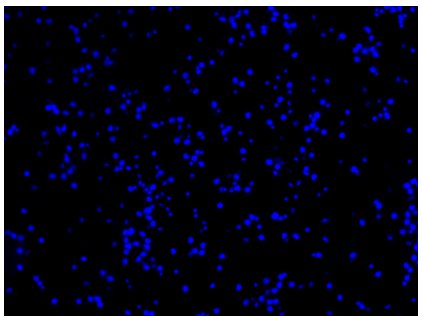
(c) 8DIV (DAPI)



(d) 8DIV (Trypan)



(e) killed by sodium azide (DAPI)



(f) killed by sodium azide (Trypan)

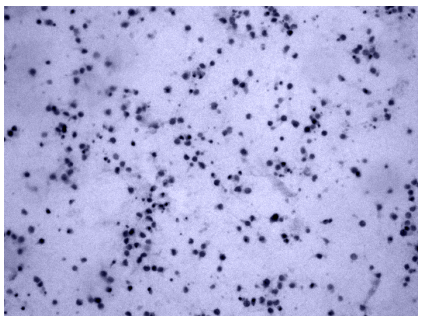


Figure 3.6: Viability of cells in primary neuronal cultures.
Pairs of figures show the same areas of cultures stained with DAPI or Trypan blue (see titles of the subfigures). Figures 3.6a and 3.6b show a culture at 3DIV; Figures 3.6c and 3.6d show a culture at 8DIV; Figures 3.6e and 3.6f show a culture treated at 8DIV with sodium azide (0.03%, 24 h incubation). See Methods (section 2.6) for details.

Days *in vitro*	**Viability**	**Standard deviation**
3DIV	68.8%	± 5.58%
8DIV	66.4%	± 8.05%

Table 3.2: Viability of cells in primary neuronal cultures.
Viability was estimated based on three non-overlapping 10× objective images (∼ 500 cells per image, see for example Figure 3.6). Total numbers of cells were estimated by counting DAPI stained nuclei, numbers of dead cells – by counting Trypan stained cells (Methods, section 2.6).

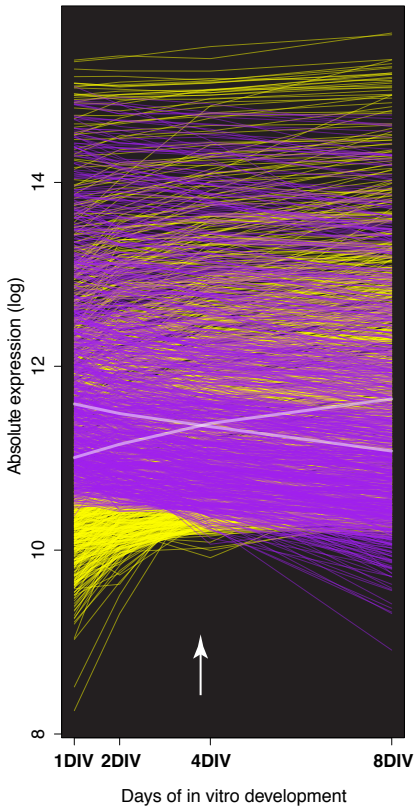


Figure 3.7: A gene expression switch point in development near 4DIV.
The thin lines represent trends of expression (median between replicates) of 2,000 most highly expressed genes from the downregulated (purple lines) and upregulated (yellow lines) categories. The y-axis shows *log* transformed and normalized absolute expression values (Methods, section 2.7), the x-axis shows time (DIV, days of *in vitro* development). The thick white lines are equivalent to the median trends of the 1,000 genes in each of the two categories. The arrow points to the crossing of the median trends (near 4DIV).

Summary of section 3.1

Analysis of GO and KEGG enrichment characterised the development of E17.5 primary forebrain cultures (1DIV to 8DIV time-window) in terms related to cell growth and neuronal activity, and not related to cell division (section 3.1.3). Genes associated with nuclear localisation and function (i.e. many of the somatic genes) appeared to be downregulated in the development of cultures, while genes associated with presumably neuritic localisation (e.g. plasma membrane and synaptic GO terms) were upregulated. After 4DIV the average abundance of the somatic genes remained lower than that of the neuritic genes, which indicated the importance of the 4DIV timepoint a switch timepoint in maturation of primary neurons. Also, these observations meant that a contribution of mRNA from proliferating secondary cell types (e.g. fibroblasts, endothelial cells and etc.) to the overall gene expression profile of the cultures was relatively small. These findings validated E17.5 primary neuronal cultures to be a suitable model to study gene expression in growing neurons. Additionally, I found that profiles of gene expression programs of E17.5 primary hippocampal and forebrain cultures were very similar (section

3.1.1 and 3.1.2). Therefore, primary forebrain cultures could be used to study neuronal gene expression in a way similar to primary hippocampal cultures.

3.2 A model of miRNA activity in neurons

3.2.1 Identification of three categories of differentially expressed miRNAs in forebrain cultures development

In addition to mRNA profiling (section 3.1), the samples of total RNA extracted from developing forebrain primary cultures were used to profile miRNA abundance using the *Illumina Universal Sentrix Array Matrix*. Micorarray analysis and mapping of array probes to official miRBase Release 13 miRNA symbols (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006, 2008) was performed as described in Methods (section 2.7). Overall, expression of 362 miRNAs was assessed in primary cultures, and 204 miRNAs were found to be differentially expressed between the first and last developmental timepoints (adjusted $P < 0.1$). As in the case of mRNA coding genes analysis (see section 3.1.3), all differentially expressed miRNAs were separated into two categories: Downregulated (99 miRNAs) and upregulated (105 miRNAs). Interestingly, of the 30 most highly expressed miRNAs (based on average expression between 1DIV and 8DIV), only 4 were differentially expressed, which was approximately 4.2 times less than expected by chance alone (hypergeometric $P < 5.04e-07$). Therefore, in addition to down- and upregulated categories, a third category was singled out, which was named as the steady state highly expressed. In summary, differential expression analysis identified three non-overlapping groups or categories of miRNAs with distinct patterns of expression in development of primary cultures:

- Steady state highly expressed (26 miRNAs)
- Downregulated (99 miRNAs)
- Upregulated (105 miRNAs)

A full listing of miRNAs per expression category, together with the ranks of their expression at the final developmental timepoint (8DIV), is in Supplementary Data (Table A.8).

To validate profiling of miRNA expression by microarrays, one miRNA was selected from each category (let-7c from the steady state, miR-143 from the downregulated and miR-370 from the upregulated categories) and their expression at 1DIV and 8DIV was

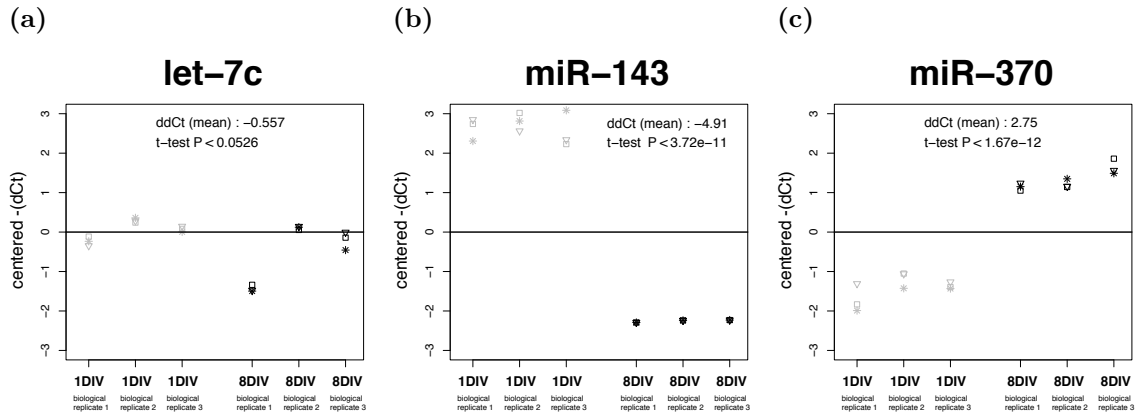


Figure 3.8: Validation of miRNA expression profiles with qRT-PCR.
The x-axes correspond to biological replicates (three per each of the two timepoints, 1DIV and 8DIV), the y-axes correspond to inverse ΔCt values centered around experimental medians (*"centered -(dCt)"*). The styled points correspond to ΔCt values for technical replicates per one biological replicate. The text gives provides the following information: 1) The mean of ΔΔCt values; 2) The t-test P-value for the differential expression. ΔΔCt method is described in Methods (section 2.3). The styled points correspond to ΔCt values of technical replicates for each biological replicate.

assessed with qRT-PCR (Methods, section 2.4). Results of qRT-PCR analysis were consistent with microarray results for all the three miRNAs (Figure 3.8).

3.2.2 miRNA expression in cultures development was similar to that in the brain and neurons

The dynamics of mRNA abundance, as described by gene expression analysis, were consistent with the development of primary E17.5 embryonic forebrain cultures being a model of neuronal development (section 3.1.3). It remained unknown, however, if miRNA expression and activity in primary cultures was similar to that in neuronal development. This section describes the analysis of miRNAs observed in primary cultures in the light of previously published research of neuronal miRNAs, which showed that expression of miRNAs in cultures was similar to that in the brain and neurons.

Several miRNAs that are known to be involved in inhibition of neural progenitor proliferation and promotion of neuronal differentiation were found to be among the top 20 most highly expressed miRNAs in primary cultures. These were six let-7 miRNAs, miR-124, miR-125b-5p, miR-9 and miR-137. Expression of these miRNAs along with references to relevant literature on their function is summarised in Table 3.3. Interestingly, all of these miRNAs belonged to the steady state highly expressed category (see section 3.2.1) and were highly expressed starting from 1DIV. Their high expression both at 1DIV and 8DIV

was consistent with E17.5 cultures to be comprised predominantly of developing differentiated neurons, as in published literature these miRNAs were shown to be implicated in neuronal differentiation.

miRNA	**#1DIV**	**#8DIV**	**Function**	**Reference**
miR-9	1st	1st	Promotes neurogenesis in the MH	(Leucht et al., 2008)
			Promotes differentiation of NSCs	(Zhao et al., 2009)
			Promotes differentiation of NPGs	(Shibata et al., 2008)
let-7 family	2nd	3rd	Inhibits Lin28, a plurepotency factor	(Rybak et al., 2008)
miR-125b-5p	11th	4th	Promotes neuronal differentiation of neuroblastoma	(Le et al., 2009)
miR-137	12th	9th	Promotes neuronal differentiation of glioblastoma	(Silber et al., 2008)
miR-9*	9th	14th	Inhibits BAF53a, a chromatin remodelling factor of NPGs	(Yoo et al., 2009)
miR-124	17th	10th	Inhibits SCP1, a partner of REST	(Visvanathan et al., 2007)
			Inhibits PTBP1, a repressor of neuronal splicing	(Makeyev et al., 2007)
			Promotes neuronal differentiation of glioblastoma	(Silber et al., 2008)
			Promotes neuronal differentiation of adult NPGs	(Cheng et al., 2009)
			Inhibits BAF53a, a chromatin remodelling factor of NPGs	(Yoo et al., 2009)

Table 3.3: Ranking of expression of pro-neuronal miRNAs.
miRNA - miRNA identifier as of miRBase Release 13; *#1DIV* - rank of expression at 1DIV; *#8DIV* - rank of expression at 8DIV; *Function* - published function in neurogenesis and/or establishing of neuronal identity; *Ref* - references to corresponding literature. Abbreviations: "NPGs" - neural progenitors (*in vivo*); "NSCs" - neural stem cells (*in vitro*); "MH" - midbrain-hindbrain domain. For let-7 family expression rank of let-7a is provided. Additional five let-7 miRNAs (let-7b, let-7d, let-7g, let-7c and let-7f) were among top 20 most highly expressed miRNAs at both of the timepoints (for 8DIV expression see Supplementary Data, Table A.8).

Additionally, the miRNAs in the downregulated category were in agreement with their reported depletion from the synaptic fraction in the adult mouse forebrain (Table 3.4) (Lugli et al., 2008; Siegel et al., 2009). Strikingly, the four miRNAs identified by Lugli *et al.* as the most depleted from the synaptic fraction of the mouse forebrain (in comparison to the whole forebrain homogenate) were exactly the same four miRNAs that were the most strongly downregulated in cultures development (Lugli et al., 2008). Additionally, Siegel and colleagues also identified four miRNAs that were significantly depleted from forebrain synaptic fraction, all of which were among 12 most downregulated miRNAs in cultures (Table 3.4).

miRNA	**# depletion** (Lugli et al., 2008)	**# depletion** (Siegel et al., 2009)	**# DR**	**× FC**	**adj. *P* downreg in cultures**
miR-143	1st	1st	1st	× 9.65	$P < 8.49e-14$
miR-451	2nd	*na*	4th	× 4.03	$P < 3.36e-09$
miR-150	3rd	2nd	3rd	× 7.36	$P < 5.11e-10$
miR-145	4th	3rd	2nd	× 7.27	$P < 2.35e-13$
miR-301	5th	*na*	38th	× 1.51	$P < 1.29e-04$
miR-153	6th	*na*	*na*	*na*	*not sign.*
miR-126-5p	7th	4th	21st	× 1.89	$P < 6.67e-07$
miR-126-3p	8th	*na*	6th	× 2.79	$P < 2.77e-10$

Table 3.4: Deption of downregulated miRNAs from synaptosomes.
miRNA - miRNA identifier as of miRBase Release 13; *# depletion* - rank of synaptic depletion (Lugli et al., 2008; Siegel et al., 2009); *# DR* - rank of downregulation during development of forebrain cultures (of significantly downregulated in the development miRNAs, see text); × *FC* - fold downregulation during development of forebrain cultures; *adj. P downreg in cultures* - adjusted P-value of downregulation during development of forebrain cultures (Methods, section 2.7). Abbreviations: "*na*" - not applicable; "*not sign.*" - not significant.

Lastly, miRNAs with a known function and/or expression in the adult brain were found to be upregulated in development of primary cultures. For example, miR-132, a miRNA induced by neuronal activity and implicated in homeostatic regulation of neuronal function (Klein et al., 2007), was upregulated in development of cultures (differential expression adjusted $P < 0.01$). In addition, upregulation was detected for miRNAs transcribed from the distal end of mouse chromosome 12. Expression of these miRNAs was shown to be restricted to the brain in the adult mice (Seitz et al., 2004). The distal 12 region encodes 54 miRNA hairpins (i.e. pre-miRNAs) from which 80 distinct mature miRNAs are transcribed and processed (according to miRBase Release 13 (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006, 2008)). Of these mature miRNAs, 53 were profiled by microarrays in development of cultures (Methods, section 2.7) and 41 were found in the upregulated category (approximately 2.7 times more than expected by chance alone, $P < 2.12e-15$). At the same time, only three miRNAs of the distal 12 region were attributed to the downregulated category of miRNAs (significant depletion, $P < 2.4e-05$).

3.2.3 miRNAs were active in primary cultures: miR-124 and let-7 miRNAs shaped gene expression

The previous section demonstrated that expression of miRNAs in the development of cultures was similar to that previously reported in the brain and neurons. In addition, it was possible to obtain evidence of direct miRNA effects on gene expression in primary

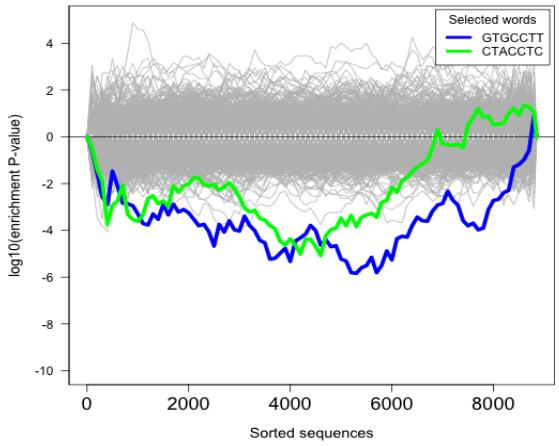
cultures. Sylamer, a method of word distribution analysis across sorted sequences[1] (van Dongen et al., 2008), was applied to estimate occurrence biases of miRNA seed matching sites in 3′UTRs of genes expressed in cultures (Methods, section 2.8). Results of the analysis were consistent with miR-124 and let-7 miRNAs (which were highly expressed in cultures, see Supplementary Data Table A.8) playing a direct role in shaping gene expression in the primary cultures.

Seed matching sites for miR-124 and let-7 miRNAs were significantly depleted from 3′UTRs of highly expressed genes in both forebrain and hippocampal cultures (Sylamer $P < 1e{-}04$ in all cases), which was consistent with the direct role of these miRNAs in regulation of mRNA levels in the primary cultures (Figure 3.9). Significant depletion of the seed matching sites (of the 7(2)-type, see Methods, section 1.2.1) was observed throughout the developmental timecourse: at 1DIV (Figures 3.9a and 3.9b), as well as at 8DIV (Figures 3.9c and 3.9d). The depletion in 3′UTRs of highly expressed genes suggested that miR-124 and let-7 miRNAs from the beginning of the developmental timecourse participated in shaping gene expression in primary cultures. Since miR-124 expression was shown to be specific to neurons (Christodoulou et al., 2010; Clark et al., 2010; Shkumatava et al., 2009), this result also meant that neuronal gene expression had a major contribution to the gene expression profile of the cultures.

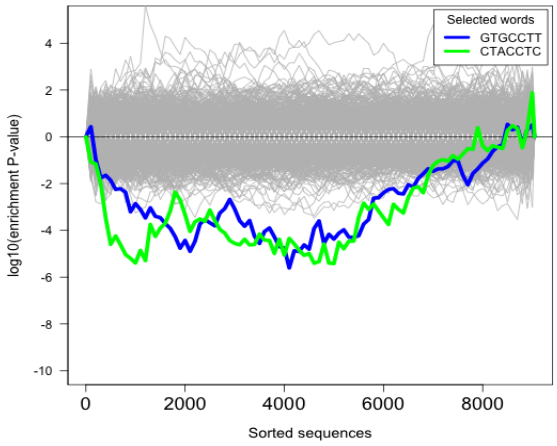
Apart from modulation of the level of gene expression, activity of miR-124 would likely to have a direct impact on dynamics of differential gene expression in cultures at the later stages in development of cultures. Sylamer analysis of 3′UTRs of genes expressed in the early stages of development, in transition from 1DIV to 2DIV, showed that the upregulated genes were significantly depleted from miR-124 seed matching sites (Figures 3.10a and 3.10b). This can be interpreted as miR-124 being permissive to upregulation in gene expression at this early stage, when the upregulated early genes faced little inhibition from miR-124. However, at the later stage, in transition from 4DIV to 8DIV, this depletion disappeared (forebrain primary cultures, Figure 3.10c) or even changed to enrichment (hippocampal primary cultures, Figure 3.10d). Therefore, it is conceivable that genes, which were upregulated later in the development, faced moderation by miR-124.

[1] A full description of Sylamer is in the Introduction (section 2.8).

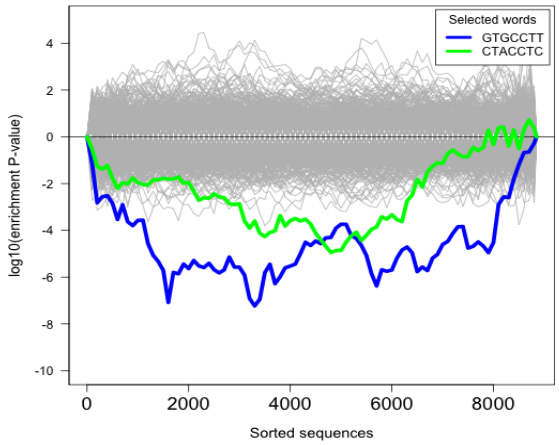
(a) miR-124 and let-7 at **1DIV**, FB culutres



(b) miR-124 and let-7 at **1DIV**, HP cultures



(c) miR-124 and let-7 **8DIV**, FB cultures



(d) miR-124 and let-7 **8DIV**, HP cultures

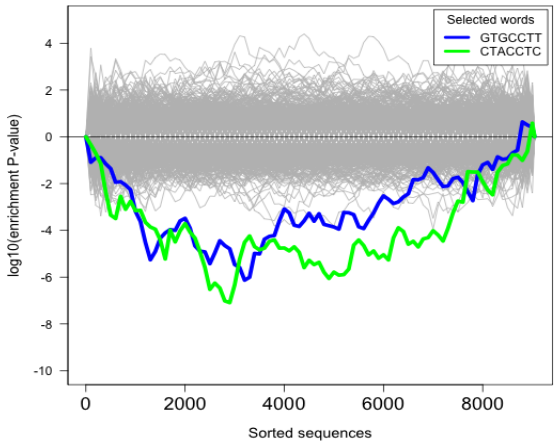
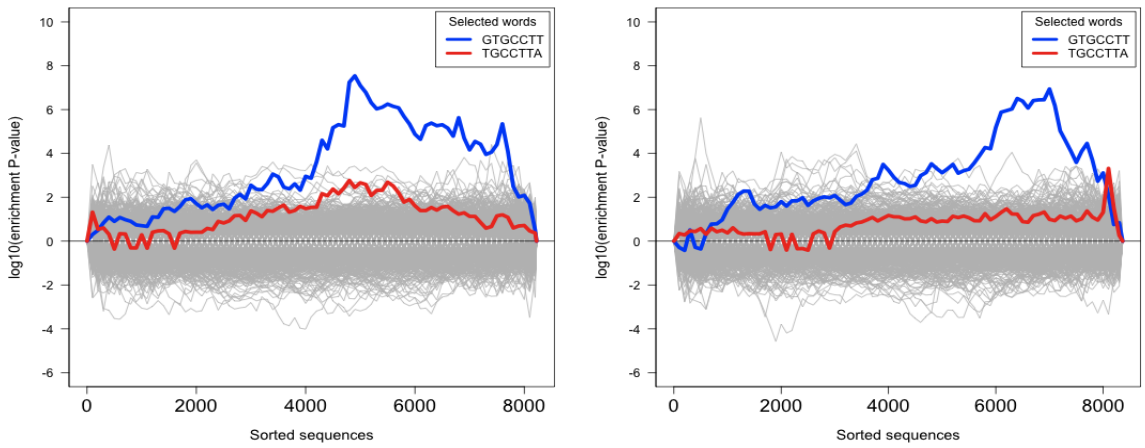


Figure 3.9: Signature of miR-124 and let-7 regulation of the level of gene expression in primary cultures.
The x-axes represent sorted 3′UTRs corresponding to detectably expressed genes (detected with the standard Illumina detection call $P < 0.01$, see Methods, section 2.7). These genes are ordered **from the most abundant to the least abundant** in replicates: 3.9a – of forebrain cultures at 1DIV; 3.9b – of hippocampal cultures at 1DIV; 3.9c – of forebrain cultures at 8DIV; 3.9d – of hippocampal cultures at 8DIV. The y-axes represent the hypergeometric P-values for occurrence biases of 876 nucleotide words complementary to the seed regions (7(2) and 7(1A)-types) of the complete set of 581 distinct mouse miRNAs, according to miRBase Release 14 (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006, 2008). Positive values on the y-axes correspond to an enrichment ($+|log_{10}$(P-value)|) and negative values to a depletion ($-|log_{10}$(P-value)|). The blue and the green lines show enrichment profiles of 7(2)-type seed matching sites complementary, respectively, to miR-124 and miRNAs of let-7 family. The grey lines show profiles of the rest of the distinct seed matching sites. The mapping of microarray probes to mRNA transcripts, and transcripts to genes, is described in Methods (section 2.7). The identification of the seed regions and parameters of Sylamer (van Dongen et al., 2008) is in Methods (section 2.8). The full description of the Sylamer method is in the Introduction (section 2.8). Abbreviations: *FB cultures* – primary forebrain cultures; *HP cultures* – primary hippocampal cultures.

(a) miR-124 in **1DIV** → **2DIV**, FB culutres **(b)** miR-124 in **1DIV** → **2DIV**, HP cultures



(c) miR-124 in **4DIV** → **8DIV**, FB cultures **(d)** miR-124 in **4DIV** → **8DIV**, HP cultures

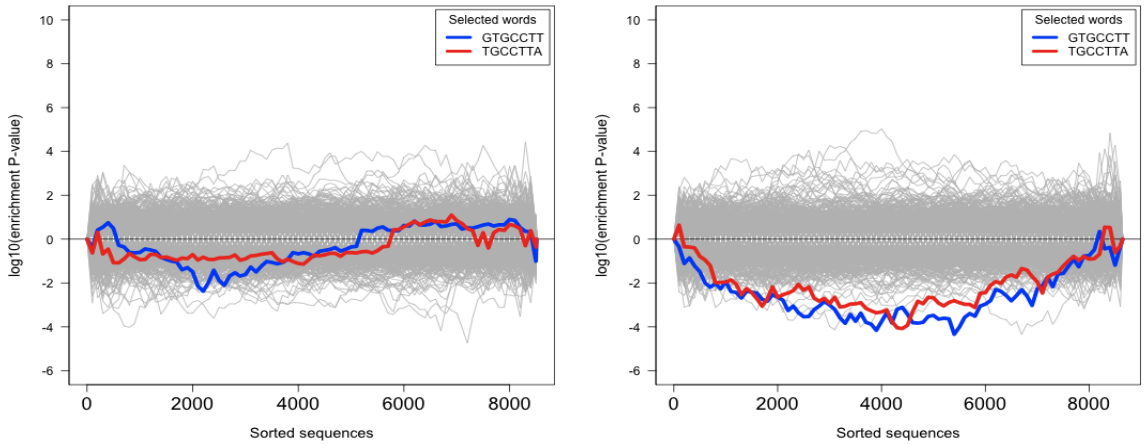


Figure 3.10: Signature of miR-124 regulation of differential gene expression in primary cultures.
The x-axes represent sorted 3′UTRs corresponding to detectably expressed genes (detected with the standard Illumina detection call $P < 0.01$, see Methods, section 2.7). These genes are ordered **from the most downregulated to the most upregulated** by fold change t-statistic for differential expression between replicates: 3.10a – of forebrain cultures at 1DIV compared to 2DIV; 3.10b – of hippocampal cultures at 1DIV compared to 2DIV; 3.10c – of forebrain cultures at 4DIV compared to 8DIV; 3.10d – of hippocampal cultures at 4DIV compared to 8DIV. The y-axes represent the hypergeometric P-values for occurrence biases of 876 nucleotide words complementary to the seed regions (7(2) and 7(1A)-types) of the complete set of 581 distinct mouse miRNAs, according to miRBase Release 14 (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006, 2008). Positive values on the y-axes correspond to an enrichment ($+|log_{10}$(P-value)|) and negative values to a depletion ($-|log_{10}$(P-value)|). The blue and the red lines show the enrichment profiles of 7(2) and 7(1A)-type seed matching sites for miR-124. The grey lines show the enrichment for the rest of the distinct seed matching sites. The mapping of microarray probes to mRNA transcripts, and transcripts to genes, is described in Methods (section 2.7). The identification of the seed regions and parameters of Sylamer (van Dongen et al., 2008) is in Methods (section 2.8). The full description of the Sylamer method is in the Introduction (section 2.8). Abbreviations: *FB cultures* – primary forebrain cultures; *HP cultures* – primary hippocampal cultures.

Summary of section 3.2

Profiling of miRNAs in developing E17.5 primary forebrain cultures (1DIV to 8DIV time-window) showed that expression of several miRNAs with a known role in differentiation of neuronal progenitors was high from the early stages in development of the cultures and remained so until the end of the time-window in question. This suggested that from 1DIV to 8DIV the cultures consisted predominantly of committed neurons. Composition of down- and upregulated categories of miRNAs was consistent with developing cultures being a model of neuronal growth: miRNAs previously reported as depleted from synapses were downregulated in the cultures, while miRNAs that were reported as enriched in the adult brain and neurons were upregulated. Additionally, it was also possible to establish, using the example of miR-124 and let-7 miRNAs, that miRNAs were likely to have been biologically active in cultures. Therefore I concluded that the forebrain cultures were a good model to study miRNA regulation of gene expression in neurons.

3.3 Selection of miRNAs for functional experiments

The goal of this work was to describe the role of miRNAs in the development and function of neurons (Introduction, section 1.2.2). Identification of three different modes of miRNA expression during development of primary forebrain cultures posed a question if miRNAs from different classes had similarly important roles in neurons.

Based on results of published works, miRNAs of the downregulated category were least likely to be functionally important for neurons. For example, miRNAs that were most downregulated in the cultures development were found to be most strongly depleted miRNAs from synapses in the adult mouse forebrain (Lugli et al., 2008) (Table 3.4). Additionally, a phenotype of a stable knock out mouse line lacking two of the four most downregulated miRNAs, miR-143 and miR-145 (Table 3.4), was published (Elia et al., 2009), and no significant abnormalities in brain development and function were reported. Therefore miRNAs of the downregulated category were assumed to be non-neuronal and non-functional in neurons under normal circumstances.

On the other hand, multiple miRNAs from the steady state highly expressed category were described as functionally important for neuronal development (Table 3.3). The upregulated in development miRNAs could also *a priori* be important for neuronal biology, as at least one of these miRNAs, miR-132, was shown to be induced by neuronal activity (Klein et al., 2007). Additionally, 41 miRNAs transcribed from the region in the distal end of chromosome 12 were among upregulated in cultures miRNAs (section 3.2.2). Previously, miRNAs from that region were shown to be highly expressed in the brain relative to other organs (Seitz et al., 2004), and misregulation of expression of that region was implicated in a mental disorder (Lewis and Redrup, 2005). Therefore miRNAs from the upregulated category were assumed to be functional and neuronal.

In total ten miRNAs were selected for functional experiments. Selection of miRNAs from down- and upregulated categories was based on two criteria: the level of expression during the developmental timecourse of primary cultures and the magnitude of change in expression between the first and the last developmental timepoints (i.e. between 1DIV and 8DIV). Table 3.5 summarises this information about the selected miRNAs from down- and upregulated categories.

Two miRNAs were selected from the steady state highly expressed category: **miR-124** and **miR-103**. Selection of miR-124 was due to its reported role in neuronal differentiation (summarised in Table 3.3) and also because of the indication of its direct role in development of primary cultures (Figures 3.9 and 3.10). Additionally, experiments

miRNA	# expression	# fold change
miR-143	*at 1DIV:* 16th	*Downregulated:* 1st
miR-145	*at 1DIV:* 12th	*Downregulated:* 2nd
miR-25	*at 1DIV:* 3rd	*Downregulated:* 10th
miR-551b	*at 8DIV:* 4th	*Upregulated:* 1st
miR-370	*at 8DIV:* 33rd	*Upregulated:* 2nd
miR-410	*at 8DIV:* 7th	*Upregulated:* 10th
miR-434-3p	*at 8DIV:* 2nd	*Upregulated:* 36th

Table 3.5: Selection of down- and upregulated miRNAs.
 # expression - rank of a miRNA by the level of expression at 1DIV or 8DIV among all miRNAs comprising a relevant category (either down- or upregualted); *# fold change* - rank of a miRNA by the level of fold change between 1DIV or 8DIV among all miRNAs comprising a relevant category (either down- or upregualted).

with this miRNA could serve as a positive control of methodology, because of the strong prior information arguing for the importance of miR-124: if the methods were suitable for studying neuronal function of miRNAs, such a function should be observable for miR-124. On the other hand, miR-103, although it was the second most highly expressed miRNAs at 8DIV (Supplementary Data, Table A.8), was not previously reported as having neuronal function. This miRNA was selected in order to establish if a high miRNA abundance was a good indication of importance and function of a miRNA.

To estimate the effects to be expected from an undoubtedly non-neuronal miRNA in neurons, a non-mouse miRNA, **cel-miR-67**, was selected for functional experiments. This miRNA was identified in *Caenorhabditis elegans*, and its seed region was different from any known mature mouse miRNA, as of miRBase Release 14 (Griffiths-Jones, 2004; Griffiths-Jones et al., 2006, 2008). Despite cel-miR-67 not being naturally expressed in mouse primary cultures, the same methods that were used for overexpression of mouse miRNAs could be used for cel-miR-67 (Methods, section 2.5). Delivery of cel-miR-67 into mouse cells was expected to result in its loading and guiding mouse RISC to targets of cel-miR-67 in the same way as the endogenous miRNAs, because cases of functional activity of ectopically expressed miRNAs in a mammalian cell culture system were previously reported (Lim et al., 2005). It was assumed that cel-miR-67 could act similarly to mouse miRNAs and use its seed region for RISC guidance. Mouse neuronal mRNA transcripts had not evolved to avoid targeting by cel-miR-67. Therefore the effect of this miRNAs could serve as an estimate of effects expected from a "generic" miRNA, which targets a random sample of mouse transcripts susceptible to miRNA mediated destabilisation.