Chapter 7

Discussion

The aim of this thesis was to identify the roles of miRNAs at the level of the whole transcriptome in neurons (Introduction, section 1.2). Through the profiling of mRNA and miRNA expression in primary neuronal cultures I established that the cultures were a suitable model system to study miRNAs in neurons (Chapter 3). Secondly, profiling miRNA abundance in the cultures allowed me to characterise the dynamic expression of miRNAs during neuronal growth and to contrast this with patterns of mRNA expression (Chapter 3, section 3.2). Additionally, it allowed me to select miRNAs for further study in primary neurons (Chapter 3, section 3.3).

Based on previously published works, it was known that transcripts destabilised upon miRNA overexpression and which contained seed matching sites in their 3′UTRs, were enriched in direct targets (Lim et al., 2005; Giraldez et al., 2006). I used this approach to identify putative direct targets of the selected miRNAs. Primary neuronal cultures were transfected with mimics and inhibitors of nine mouse miRNAs and with a mimic of one non-mouse miRNA (Chapter 5). Subsequent differential expression was detected using mRNA microarrays, and putative direct miRNA targets were derived using seed matching site enrichment as an indication of miRNA mediated regulation. With this strategy I identified hundreds of putative direct targets for six of the selected miRNAs (Chapter 5, section 5.2). These miRNAs were: neuronal miR-124 and miR-434-3p, non-neuronal miR-143, miR-145 and miR-25, and cel-miR-67 (a miRNA not present in the mouse). Functions associated with the lists of targets of these miRNAs were characterised through KEGG and GO enrichment analyses. This identified several biological processes associated with miRNA targets, including processes related to cell signalling, transport and cytoskeleton remodelling (Chapter 6, sections 6.1.3 and 6.1.5).

Further analysis of these targets lead to the conclusion that transfected miRNAs and most significantly the two neuronal miRNAs (miR-124 and miR-434-3p), act to inhibit genes induced during the transfection experiments (Chapter 6, Figure 6.2). Therefore, the role of exogenously added (transfected) miRNAs is context dependent, i.e. their targeting repertoires were to a significant extent defined by the context of genes that were induced in the cultures by the transfection procedure (Chapter 6, section 6.1.3).

The identified targets were enriched in multiple disease and stress related KEGG pathways (Chapter 6, section 6.1.3). Therefore, I tested whether transfected miRNAs inhibited genes that were induced by stresses other than transfection, but not necessarily associated with KEGG pathways. Indeed, genes that were induced in the brain by two types of stress (the kainate (Akahoshi et al., 2007) and ageing (Lu et al., 2004) stress), were inhibited by some miRNAs that were transfected in the primary cultures (Chapter 6, section 6.2.1). Importantly, this inhibition was most significant for the two neuronal miRNAs (Chapter 6, Figure 6.8). miRNA mediated regulation of stress inducible genes is likely to be of biological importance, because a significant fraction of these genes was previously genetically linked to neurological disease (Chapter 6, section 6.2.2).

Although the function of miRNAs as inhibitors of stress inducible genes was identified for the transfected miRNAs, several observations suggested that endogenous miRNAs can also inhibit (buffer) inducibly expressed genes. For example, if miR-124 were to act as a buffer, constraints to differential gene expression imposed by endogenous miR-124 can explain the observation that transfected non-neuronal miRNAs have a less significant impact on gene expression in relatively mature cultures (Chapter 5, section 5.1.3). In addition, upon transfection induced stress, the induced genes are specifically enriched in miR-124 seed matching sites; Furthermore, genes induced by kainate stress are specifically enriched in miR-124 and miR-434-3p seed matching sites (Chapter 6, section 6.2.1). The latter observation is consistent with inhibition of stress inducible genes by the endogenous miR-124 and miR-434-3p in normal conditions. This observation leads me to believe that endogenous miRNAs function to reduce variability in expression of stress inducible genes. Such a reduction was indeed detected upon transfection of the miR-124 mimic (Chapter 6, section 6.2.3). Analysis of variability in the expression of stress induced genes gives biological credence to miRNAs acting to buffer stress inducible genes (Chapter 6, section 6.2.2). Expression of these stress inducible genes was found to be more variable between biological replicates than that of other genes, and exogenous miR-124 reduced this variability. This observation suggests that stress inducible genes may require an additional level of post-transcriptional control, because their expression is inherently “noisy”.

The precise control of expression of these genes is likely to be of importance, because a significant proportion of the stress inducible genes were previously linked genetically to neurological diseases (Chapter 6, section 6.2.2).

Below I will discuss these results in more detail. Additionally I will outline further work to test hypotheses proposed in the course of this study.

7.1 Characterising the experimental system

Before proceeding to the identification of miRNA targets in primary neuronal cultures, I first established that E17.5 primary forebrain cultures were a suitable model to study functions of miRNAs in neurons[1] (Chapter 3).

First, I described trends in gene expression during development of the cultures at four timepoints (at 1DIV, 2DIV, 4DIV and 8DIV). I profiled abundance of mRNAs using microarrays and characterised the associated function of differentially expressed genes through GO term and KEGG pathway enrichment (Chapter 3, section 3.1.3) (Manakov et al., 2009). Among terms and pathways which were found to be significantly upregulated during the development of cultures were "synapse", "neurological system process" and "Long-term potentiation". The nature of these upregulated categories suggested that they were enriched in neuritic genes. At the same time, pathways "DNA replication" and "Cell cycle" were significantly downregulated (Chapter 3, section 3.1.3). These downregulated categories suggested that they were enriched in somatic[2] genes, as the associated biological process are taking place in the nucleus. Together these observations show that the development of cultures was dominated by neuritic expansion, and it was unlikely that proliferating secondary cell types (e.g. fibroblasts, endothelial cells and etc.) contributed significantly to the mRNA profiles of the cultures. When expression trends of downregulated genes were overlayed with upregulated genes over the course of development, the intersection was at the 4DIV timepoint (Chapter 3, Figure 3.7). In other words, after 4DIV, the overall abundance of neuritic transcripts was higher than the abundance of somatic transcripts. A ratio of neurtic to somatic transcript abundance above one, is likely to be similar to that of mature forebrain neurons, as they are characterised by extensive arborisation. Therefore, 4DIV can be viewed as a timepoint at which a developmental

[1] Before profiling gene expression there were good reasons to consider E17.5 as a predominantly neuronal culture, rather than glial. Performing brain dissections at 17.5 days of mouse prenatal development and using a specifically optimised cell culture protocol already favored survival of neurons over that of glial cells (Introduction, section 1.2.4).

[2] By somatic, I refer to the soma of a neuron.

switch occurs in the transition from immature to mature gene expression of primary cultures[3]. miRNAs were previously demonstrated to be of importance for developmental switch timepoints (Giraldez et al., 2005). I previously proposed that miRNAs play a similar role during neuronal development (Manakov et al., 2009).

I demonstrated that miRNA expression trends in the development of primary forebrain cultures were in agreement with previously published reports of miRNA activity in differentiated neurons (Chapter 3, section 3.2). Abundances of 362 mature miRNAs were profiled (at 1DIV, 2DIV, 4DIV and 8DIV) using a miRNA microarray platform, and three major expression trends were identified (Manakov et al., 2009): 1) The upregulated miRNAs (105 sequences); 2) The downregulated miRNAs (99 sequences); 3) The steady state highly expressed miRNAs (26 sequences) (Chapter 3, section 3.2.1). The steady state highly expressed category included several miRNAs that were previously shown to be induced upon neural differentiation (these included miRNAs of the let-7 family, miR-124 and miR-125) (Chapter 3, Table 3.3). The observation that these miRNAs are highly expressed as early as 1DIV was in agreement with the plating material being comprised, at least to a large extent, of committed neural cell types. Moreover, some of these miRNAs, for example miR-124, were previously shown to be neuron specific (Shkumatava et al., 2009; Hanina et al., 2010). Therefore their high steady state expression throughout the developmental timecourse was in agreement with these cultures consisting predominantly of differentiated neurons throughout the 1DIV to 8DIV developmental time-window. Analysis of differentially expressed miRNAs further supported the use of primary forebrain cultures as an accurate model system to study forebrain neurons (Chapter 3, section 3.2.2). For example, the most strongly downregulated miRNAs in culture (e.g. miR-143, miR-145, and etc.) were previously shown to be lowly expressed in forebrain synapses in comparison to the whole brain homogenate (Lugli et al., 2008; Siegel et al., 2009). On the other hand, some of the upregulated miRNAs were previously demonstrated to be significantly enriched in the adult brain (e.g. miRNAs of the mouse distal 12 cluster (Seitz et al., 2004)) or to be induced by neuronal activity (e.g. miR-132 (Klein et al., 2007)).

Profiling miRNA and mRNA expression in the development of cultures suggested that miRNAs can directly shape gene expression in the cultures (Chapter 3, section 3.2.3). Significant depletion of seed matching sites for miRNAs highly expressed in the cultures (miR-124 and let-7 family miRNAs) was observed in 3′UTRs of highly abundant tran-

[3]Additionally, by characterising development of hippocampal and forebrain cultures in parallel, the development of forebrain cultures was shown to be highly similar to that of more commonly used hippocampal cultures (Chapter 3, sections 3.1.1 and 3.1.2). This observations supported the use of primary forebrain cultures as a model of growing neurons.

scripts (Chapter 3, Figure 3.9) (Manakov et al., 2009). This finding was in agreement with the reported role of miRNAs as major modulators of tissue and cell-type specific gene expression profiles (Farh et al., 2005; Sood et al., 2006). Significant biases in the distribution of the seed matching sites for miR-124 were also observed in differentially expressed genes. Transcripts that were upregulated early in development (between 1DIV and 2DIV) were depleted in miR-124 seed matching sites (Chapter 3, Figures 3.10c and 3.10d). Therefore, it was unlikely that endogenous miR-124 would inhibit genes upregulated in early stages of the development, at timepoints associated with the initial spurt of neurite growth and early synaptogenesis events (Valor et al., 2007). However, as cultures matured, opportunities appeared for endogenous miR-124 to constrain the expression of the upregulated genes: 3′UTRs of the transcripts that were upregulated in transition between 4DIV to 8DIV were either not depleted in miR-124 seed matching sites (forebrain cultures) or were enriched (hippocampal cultures) in these sites (Chapter 3, Figures 3.10a and 3.10b).

Lastly, profiling of trends in miRNA expression during the development of cultures enabled me to make a selection of miRNAs with distinct expression modes for the identification of miRNA targets. Two miRNAs from the steady state highly expressed category were selected (miR-124 and miR-103), three – from the downregulated category (miR-143, miR-145 and miR-25) and four – from the upregulated category (miR-434-3p, miR-370, miR-551b and miR-410). The selection procedure is described in Chapter 3, section 3.3. Additionally, one miRNA that was not related to any of the known mouse miRNAs (a *Caenorhabditis elegans* miRNA, cel-miR-67) was also selected. The non-mouse miRNA was selected in order to identify targets that were equivalent to a random sample of the transcripts that were susceptible to miRNA mediated regulation (without constraints imposed by the evolutionary selection). Profiling of mRNA expression in development of cultures identified timepoints at which to conduct experiments for the identification of miRNA targets: the 4DIV timepoint was selected because of its importance as a switch timepoint in the developmental gene expression program (see above), and two timepoints were picked around the 4DIV timepoint (3DIV and 6DIV) in order define a timepoint at which to derive optimal results (see below).

7.2 Identification of miRNA targets

It has previously been demonstrated that the introduction of exogenous miRNAs directly target transcripts whose 3′UTRs contain seed matching sites for that miRNA (Lim et al.,

2005; Giraldez et al., 2006). Conversely, inhibition of a miRNA through transfection of an inhibitor, causes upregulation of its targets (Conaco et al., 2006). Hence, I decided to transfect primary neuronal cultures with miRNA mimics and inhibitors and attempt to derive lists of putative direct targets from these experiments. miRNA targets are identified by selecting transcripts that are differentially expressed upon miRNA perturbation and also contain seed matching sites for the perturbed miRNAs.

Before conducting experiments to identify miRNA targets, it was necessary to identify the developmental timepoints which would enable the most efficient identification of the targets. To do this, I conducted a series of transfection experiments[1] with the mimics of miR-124, miR-143, miR-145, cel-miR-67 and also with the inhibitor of miR-124 (Chapter 5, sections 5.1.1 and 5.1.2). These transfection experiments were performed at either 3DIV, 4DIV or 6DIV (see above). The transfection of miR-124 elicited differential gene expression, characterised by significant enrichment of miR-124 seed matching sites in 3′UTRs of downregulated transcripts (Chapter 5, sections 5.1.2 and 5.2.1). This was an indication that the exogenously added miR-124 directly caused a significant proportion of the observed changes in gene expression.

In the case of miR-124 transfection experiments, the enrichment of miR-124 seed matching sites was most significant at 6DIV (Chapter 3, section 5.1.2). This observation suggested that the 6DIV timepoint was the stage at which the direct contribution of miR-124 to differential gene expression was most significant. Therefore 6DIV was selected to be the optimal timepoint for the identification of putative direct targets of miR-124 and of other neuronal miRNAs (see below). Transcripts, which were downregulated by the transfection of the mimics of non-neuronal miRNAs (miR-143, miR-145 and cel-miR-67), were significantly enriched in seed matching sites for the transfected miRNA in a majority of the experiments (Chapter 5, section 5.1.1). However, these enrichments were more significant at 3DIV and 4DIV, rather than at 6DIV (Chapter 5, Figure 5.2). The maximal enrichment of seed matching sites was detected at 4DIV, therefore this was selected as the best timepoint at which to identify targets of non-neuronal miRNAs.

The enrichment of the seed matching sites for miR-124 was more significant in bidirectional perturbation experiments (Chapter 4, section 4.2.1). These experiments directly contrast overexpression and inhibition of the same miRNA. This method was favoured over the alternative approach involving contrasting mimic with mock transfected cultures

[1]These transfections were performed with an siRNA transfection protocol (Maclaren et al., 2011). I confirmed that this protocol was efficient for transfection of neurons in primary forebrain cultures by imaging cultures transfected with eGFP expressing plasmid and a fluorophore labelled oligonucleotide (Chapter 4, section 4.1.1).

(the unidirectional contrast). From the bidirectional experiment at 6DIV, I compiled a list of 399 putative direct miR-124 targets in primary neurons (Supplementary Data, Table A.9). The targets were defined as significantly downregulated genes (differential expression $P < 0.01$) that encode transcripts harbouring miR-124 seed matching sites in 3′UTRs. This approach is further validated by significant intersections of identified miR-124 targets and targets from previously published works (Chapter 5, section 5.3). Therefore, I believe that both the bidirectional transfection strategy and the identified list of 399 miR-124 targets are useful beyond the scope of this work, and publication of these data will be of use to scientific community.

Using data from the miR-124 bidirectional transfection experiments, I optimised the original siRNA transfection protocol (Maclaren et al., 2011) by adjusting the posttransfection incubation time and the cell plating density. These adjustments improved detection of differential expression of seed matching site containing transcripts in the bidirectional contrast (Chapter 4, section 4.2.2). With the adjusted protocol I performed the bidirectional transfection experiments on the remaining selected mouse miRNAs[4]. As a result of these bidirectional experiments, lists of putative targets were compiled for four miRNAs: 251 targets of miR-143 (Supplementary Data, Table A.10), 301 targets of miR-145 (Supplementary Data, Table A.11), 169 targets of miR-25 (Supplementary Data, Table A.12) and 101 targets of miR-434-3p (Supplementary Data, Table A.14). To my knowledge, this is the first report of direct targets of miR-434-3p, despite the fact that this miRNA is transcribed from the mouse chromosome 12 distal region (Davis et al., 2005), a region highly expressed in the adult brain relative to other organs (Seitz et al., 2004), and which has previously been implicated in cognitive disfunction (Lewis and Redrup, 2005).

Additionally, identification of targets of a non-mouse, non-neuronal miRNA have not previously been reported in mouse primary neuronal cultures. Therefore, targets of cel-miR-67 will be useful for researchers of neuronal miRNAs as a control of specificity for neuronal miRNA targets identified in the future. Indeed, I have been approached by many researchers already interested in these datasets. Although these targets are described within this thesis, I also intend to publish these data in the near future to be made available as a resource to the community.

[4]Targets of cel-miR-67 were derived from a unidirectional overexpression experiment (i.e. a contrast of cultures transfected with the mimic of cel-miR-67 with mock transfected cultures), because it was not represented in the mouse genome and so its inhibition was not possible. The experiment at 4DIV (marked with the index “A”, Figure 5.2d), which resulted in the highest enrichment of the seed matching sites for cel-miR-67, was used to derive the list of 394 putatively direct targets of cel-miR-67 in mouse primary neurons (Chapter 5, section 5.2.2 and Supplementary Data, Table A.13).

7.3 Context dependent function of miRNAs

Analysis of miRNA targets identified in this thesis showed that transfected miRNAs inhibited transfection induced genes, and that the identification of targets was dependent on the context of genes induced during the transfection experiments. The targeting repertoires of neuronal miRNAs were found to be better adapted for the inhibition of genes that were induced by stress in primary cultures and the brain, than targeting repertoires of random miRNAs. In the first part of this section I will discuss this context dependent inhibition of inducible genes by transfected miRNAs. In the second part, I will describe a line of evidence that supports a similar role for endogenous neuronal miR-124 and miR-434-3p in neurons and the brain.

Exogenously added (transfected) miRNAs are context dependent inhibitors of inducibly expressed genes

The hypothesis of context dependent miRNA-mediated regulation was prompted by significant intersections between targets of six unrelated miRNAs and genes induced by the transfection reagent (Chapter 6, sections 6.1.1, 6.1.2 and 6.1.3). Additionally, various GO terms and KEGG pathways were identified to be frequently enriched in both transfection induced genes and in targets of multiple miRNAs. Terms related to cell signaling, molecular transport and cytoskeleton remodelling were frequently enriched in targets and in the transfection induced genes (Chapter 6, sections 6.1.3 and 6.1.5).

The genes induced by the transfection reagent were also enriched in published miR-124 targets derived similarly (Chapter 3, section 6.1.4). At the same time, these genes were not enriched in a large independent set of miRNA targets (the Ago HITS-CLIP set) identified in P13 neocortex with a transfection-free method (Chapter 6, Figure 6.4). This observation shows that the repertoire of miRNA targets identified in transfection experiments was dependent on the context of genes induced by the experimental procedure.

The targets for miR-124 and miR-434-3p were the most significantly enriched in the genes induced by the transfection reagent. In total, 34.3% (3.3 times more than expected by chance alone, if the intersections were determined within the experimental test universe) and 37.5% (3.5 times more than expected) of miR-124 and miR-434-3p targets were induced by the transfections (enrichment P-values were $2.34e-41$ and $3.56e-14$, respectively). At the same time, enrichment was weakest in targets of a non-mouse miRNA, cel-miR-67, and an oncogenic miRNA, miR-25 (Poliseno et al., 2010) (Chapter 6, Figure 6.2).

To test if miRNAs inhibited genes that were induced by stresses other than transfection, I compiled lists of genes that were induced by two additional types of brain stress: the injection of a kainate into the mouse hippocampus (kainate stress) (Akahoshi et al., 2007), and ageing of the human brain (ageing stress) (Lu et al., 2004). Transfection of primary cultures with neuronal miRNAs, miR-124 and miR-434-3p, was found to significantly downregulate genes that were induced in kainate and ageing stresses (as well as the genes induced by the transfection reagent). At the same time, transfection of the non-mouse miRNA, cel-miR-67, did not significantly downregulate any of the sets. This observation suggested that the targeting repertoire of neuronal miRNAs was better adapted to inhibit genes inducible by stress in the brain than targets of a random miRNA.

In summary, the observations discussed above showed that a significant proportion of the targets of different transfected miRNAs were shared, and that almost all transfected miRNAs converged on the inhibition of genes that were induced by the transfection reagent. Therefore, inhibition of inducible genes may be a common feature of miRNA mediated regulation as a whole. At the same time, the neuronal miRNAs, miR-124 and miR-434-3p, were most efficient in causing the widespread inhibition of genes induced by the transfection reagent in primary cultures, as well as inhibition of the genes induced by the other two types of stress. Therefore, targeting repertoires of neuronal miRNAs may be specifically adapted to inhibit genes that can be induced in neurons and the brain.

Endogenous miR-124 and miR-434-3p as buffers of inducible genes in neurons and the brain.

Although the experiments in this thesis directly studied the activity of only transfected miRNAs, several observations indirectly provide insights into the function of endogenous miRNAs in neurons and the brain (see above). Endogenous neuronal miRNAs appear to be buffers of perturbations to the equilibrium in the neuronal transcriptome. I discuss these propositions below.

1. Endogenous miR-124 buffers differential gene expression in mature neurons. In the development of untransfected cultures, transcripts with 3′UTRs not depleted in miR-124 seed matching sites are upregulated relatively late in development (in transition from 4DIV to 8DIV), while transcripts upregulated early (1DIV to 2DIV) are depleted in miR-124 sites (Chapter 3, section 3.2.3). This means that the scope for endogenous miR-124 mediated inhibition of developmentally upregulated transcripts normally appears only in more mature neurons.

Transfections of non-neuronal miRNAs had a pronounced effect on gene expression in primary cultures at 3DIV and 4DIV, but the effect diminished at 6DIV (Chapter 5, sections 5.1.1). At the same time, a significant enrichment of seed matching sites for miR-124 was observed in transcripts upregulated upon transfection of non-neuronal miRNAs at 6DIV, while this was not the case in experiments at 3DIV or 4DIV (Chapter 5, Figure 5.2). The increasing scope for miR-124 mediated inhibition can account for differences between transfection experiments at later developmental timepoints. It is possible that in more mature cultures (6DIV) endogenous miR-124 buffers the induced transcripts and reduces the extent of their upregulation. This potentially leads to an overall decrease in differential expression (Chapter 5, section 5.1.3). In agreement with the bigger scope for miR-124 action at 6DIV, transfections of the mimic of miR-124 had the greatest effect on the transcriptome at the 6DIV timepoint (Chapter 5, section 5.2.1).

The proposition of endogenous miR-124 imposing constraints on differential gene expression in mature neurons is speculative, however it can be experimentally tested. These experiments will be suggested later.

2. Endogenous miR-124 and miR-434-3p buffer genes inducible by stresses

Buffering of changes to the transcriptome by miR-124 may be a general phenomenon. In order to study this, I assessed the distribution of seed matching sites for miRNAs in the 3′UTRs of transcripts after the injection of a kainate into the mouse hippocampus (Akahoshi et al., 2007). Kainate stress was found to upregulate transcripts with 3′UTRs enriched in seed matching sites for miR-124 and miR-434-3p, but not for other miRNAs (Chapter 6, section 6.7b). This enrichment, together with the observation that exogenous miR-124 and miR-434-3p can significantly inhibit genes induced by the kainate stress (Chapter 6, Figure 6.8), suggests that genes induced by kainate stress are enriched in targets of miR-124 and miR-434-3p. This observation, in conjunction with recent reports that targets of miR-124 are normally co-expressed with miR-124 in the same cells (Shkumatava et al., 2009; Clark et al., 2010), suggests that normally (before stress) miR-124 and miR-434-3p buffer expression of genes that can be induced by the stressful condition.

In addition to genes induced by the kainate stress, I obtained a list of the mouse homologs of genes induced by ageing of the human brain (Lu et al., 2004). These genes were inhibited significantly in primary forebrain cultures by both transfected miR-124 and miR-434-3p (Chapter 6, Figure 6.8). This suggests that endogenous miR-124 and miR-434-3p can buffer expression of genes induced by ageing.

Results of the transfection experiments in this thesis show that miRNAs are targeting genes induced by stress. This raises the question: what is the biological purpose for buffering stress inducible genes under normal conditions? One possible explanation comes from assessing variability in the expression of genes inducible by stresses in normal (untransfected) primary neuronal cultures. Genes inducible by transfection, kainate and ageing stresses were found to have greater variability in their expression between replicates of cultures at 8DIV than other genes (Chapter 6, section 6.10). Interestingly, transfection of cultures with miR-124 reduced this variability, while transfection with a non-mouse miRNA, cel-miR-67, did not (Chapter 6, Figure 6.11). Therefore, endogenous miR-124 may also normally act to reduce variability in expression of genes inducible by stress. One would expect precise control of gene expression to be important for neurons. My data indicates that miRNA mediated regulation of stress inducible genes is necessary, because precise control of expression of these genes is of particularly importance for neurons: the mutations in stress inducible genes are significantly more frequently linked to neurological disorders, than in genes on average ($P < 3.67e-05$, see Chapter 6, section 6.2.2). I believe the results presented here are a starting point for establishing a more general model of miRNA buffering of gene expression in neurons. The next section will detail some proposed experiments that can further test this hypothesis.

7.4 Directions for future work

In this thesis I directly demonstrated that transfected miRNAs inhibited genes that were induced by the transfection reagent (i.e. the transfection stress). The proposition of that endogenous miR-124 and miR-434-3p may also inhibit genes inducible by stress merits further investigation. This could be tested by studying mutant neuronal cultures that do not express miR-124 and/or miR-434-3p. The hypothesis that these miRNAs act as buffers of genes inducible by stress leads to the following predictions that could be tested in these cultures:

- The inter-replicate variability in the expression of genes inducible by stress is expected to be higher between cultures of mutant neurons. This prediction is based on the observation that exogenous (transfected) miR-124 reduces variability in expression of these genes in wild type cultures (Chapter 6, section 6.2.3).
- Transfection of mutant cultures at 6DIV with non-neuronal miRNAs is predicted to lead to similarly significant miRNA mediated changes in differential gene expression, as in 3DIV and 4DIV transfections. This prediction is based on the observation that

the decrease in the effect of non-neuronal miRNAs at 6DIV was associated with simultaneous enrichment of miR-124 seed matching sites in upregulated transcripts (Chapter 5, section 5.1.2).

Once mutant mouse lines that lack miR-124 and miR-434-3p become available, it will allow to test other predictions of the hypothesis of endogenous miRNAs as buffers of perturbations in the transcriptome. For example, in Chapter 6 I described the innate bias in the distribution of miR-124 and miR-434-3p seed matching sites upon kainate injection into mouse hippocampus. Therefore, it seems likely that these two miRNAs are particularly important in mouse neurons for buffering changes in the transcriptome that are associated with neuronal activity. Based on the results of a recent study by Konopka and colleagues (Konopka et al., 2010), in which mice with reduced abundance of nearly all miRNAs displayed enhanced learning, it is possible that mice lacking just miR-124 and/or miR-434-3p will display a similar enhancement. On the other hand, genes induced by ageing of the brain were efficiently inhibited by transfections of both miR-124 and miR-434-3p (Chapter 6, Figure 6.8). Therefore, it is possible that earlier than normal induction of genes associated with ageing will take place in the mutant mice.

The observation that miRNA targets are upregulated in stressful conditions (Chapter 6, section 2.7) raises a question concerning the mechanism behind this upregulation. One possibility is that upregulation was caused by miRNA independent mechanisms (for example, activation of transcription). However, an alternative explanation is a relief of miRNA mediated regulation in stress, or even a switch of miRNA mediated regulation to an activatory mode. Intriguingly, both relief of miRNA mediated regulation (Bhattacharyya et al., 2006) and a switch to activation (Vasudevan et al., 2007) were previously reported in stress, however this subject has not been studied extensively. My observations suggest a similar effect to possibly take place in neuronal cultures which makes them a suitable model system to study this enigmatic phenomenon. According to reports from the laboratory of Philip Sharp, localisation of components of RNA silencing machinery may be important for miRNA function (Leung and Sharp, 2006; Leung et al., 2006). Neuronal cultures, like other cell culture systems, allow direct access to cells which makes the study of subcellular localisation of miRNAs and RNA silencing machinery possible. Additionally, it was shown that the activity of several miRNAs, most notably of miR-124, is likely to play a major role in shaping gene expression in the brain (Farh et al., 2005; Sood et al., 2006) and in primary neuronal cultures (Manakov et al., 2009). Therefore, primary neuronal cultures allow us to make use of global changes in the transcriptome as a robust marker of changes in neuronal miRNA mediated activity.

Inhibition of targets by miR-25 in the transfection experiment described in Chapter 5 (section 5.2.2) was extremely efficient. In fact, enrichment of miR-25 seed matching sites in 3′UTRs of downregulated transcripts was more significant than in miR-124 experiments (Chapter 5, section 5.2.1). Interestingly, expression of miR-25 was found to be induced in tumours (Poliseno et al., 2010), while expression of a majority of miRNAs is downregulated in tumours (Thomson et al., 2006; Lotterman et al., 2008). Therefore, the high impact of miR-25 on transcriptome of neurons may be related to its potential role in reprogramming cells during carcinogenesis. I propose that transfection of mimics of other miRNAs that are co-expressed with miR-25 in tumours will have a similarly strong effect on the transcriptome of differentiated cell types, such as the cells of primary neuronal cultures. Further research of miR-25 and other oncogenic miRNAs in neuronal cultures may help to understand mechanisms of carcinogenic reprogramming of differentiated cell types.

7.5 Conclusion

In this thesis I identified hundreds of putative direct miRNA targets for six different miRNAs (both neuronal and non-neuronal) in primary neuronal cultures. This large resource of novel miRNA targets allows an in-depth analysis of the roles of miRNAs in neurons. Analysis of these target lists indicates that the major function of miRNA mediated regulation is buffering of gene expression. This effect is context dependent. The targets in this thesis were identified using chemical transfections of miRNA mimics and inhibitors, therefore the functions of miRNAs were elucidated in the context of stress associated with transfection. In this context, I identified that the targeting repertoire of neuronal miRNAs is adapted for the inhibition of genes induced by different stresses in neurons and the brain. I have identified lists of putative direct miRNA targets (see Supplementary Data), which I hope will be a useful resource for future research into both the function of miRNAs and their role during stress.

Context dependent inhibition of inducibly expressed genes at the level of the whole genome is a novel concept. However, taking the experimental context into account is necessary for understating the results of previously published miRNA transfection experiments and for the design of the future experiments into miRNA function. If context dependent inhibition of inducibly expressed genes is confirmed for endogenous miRNAs, it will make miRNAs guardians of transcriptional equilibrium. This would contribute to our understanding of the role of miRNAs in general and of neuronal miRNAs in particular. It would also provide an explanation for the mechanism of inhibition of neuronal plasticity and learning by neuronal miRNAs (Rajasethupathy et al., 2009; Gao et al., 2010; Konopka et al., 2010).