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**Publication date**

2015

**Document Version**

Final published version

[Link to publication](#)

**Citation for published version (APA):**

Schouten, M. (2015). *Epigenetic control of hippocampal stem cells: modulation by hyperactivation, glucocorticoids and aging*. [Thesis, fully internal, Universiteit van Amsterdam].

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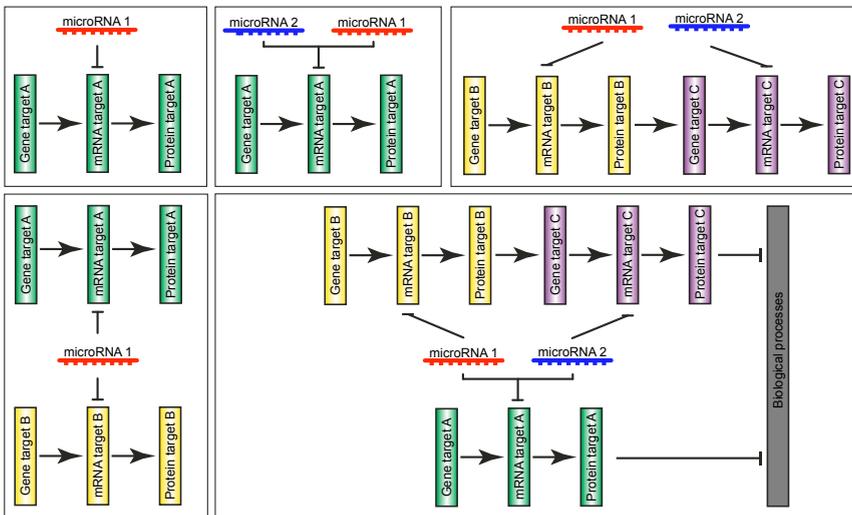
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## MicroRNAs and the regulation of neuronal plasticity under stress conditions

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*Neuroscience* (241) 188-205 (2013)

### Graphical abstract



Schematic illustration depicting different mechanisms of microRNA mediated molecular control, ranging from single microRNA-mRNA interactions (top left) to multiple microRNA and mRNA interactions regulating pathways and biological processes (bottom right).

**Abstract**

In the brain, the connection between sensory information triggered by the presence of a stressor and the organism's reaction involves limbic areas such as the hippocampus, amygdala and prefrontal cortex. Consequently, these brain regions are the most sensitive to stress-induced changes in neuronal plasticity. However, the specific effects of stress on neuronal plasticity in these regions largely differ. Despite these regional differences, in many cases the steps leading to brain adaptation to stress involve highly coordinated changes in gene expression affecting cell metabolism, neuronal plasticity and synaptic transmission.

In adult life the effects of stress on neuronal plasticity are largely reversible but stress in early life induces persistent changes in neuronal plasticity that increases vulnerability to develop psychopathologies and aging-related cognitive decline, suggesting the involvement of epigenetic mechanisms. A growing body of evidence demonstrates that microRNAs are key players in epigenetic regulation.

In this forefront review we present a critical look on the literature demonstrating the regulation of neuronal plasticity by microRNAs and the molecular mechanisms of target specificity in neurons. We propose that further progress in the identification of microRNA's function beyond single target identification would require a combination of developmental expression studies, bioinformatics and a deeper understanding of large networks of targets involved in epigenetic regulation. This will help to extend our understanding of the role microRNAs play in the regulation of stress-induced neuronal plasticity.

**Stress, adaptation and neuronal plasticity.**

Classically, stress is defined as the continuous struggle of living organisms to preserve an internal dynamic state of equilibrium defined as homeostasis. Therefore, physical and psycho-social factors that challenge homeostasis are defined as stressors. In the presence of a stressor, the organism's reaction is focused to counteract the potentially damaging effect of the stressor and restore homeostasis. This reaction is commonly known as the stress response. In the brain, the connection between the sensory information acquired in the presence of a stressor and the assessment/reaction mounted by the organism involves limbic brain structures, including the hypothalamus, hippocampus, amygdala and prefrontal cortex<sup>1,2</sup>.

During the acute stress response, initially a rapid activation of the sympathetic nervous system takes place, resulting in the release of noradrenaline (NA) in synapses and adrenaline (ADR) from the medulla of the adrenal glands. Subsequently, neuropeptides such as corticotropin-releasing hormone (CRH) and vasopressin (AVP) are released in the hypothalamus, resulting in the activation of the hypothalamic-pituitary-adrenocortical (HPA) neuroendocrine axis and in a rise in blood concentrations of adrenal glucocorticoids (GCs). All these neurochemical mediators play a key role in allostasis, the process of maintaining homeostasis through change, and promote beneficial adaptation to the environment. However, to allow effective coping with stressors, the stress reaction has to be adequately terminated. When the stress response becomes abnormal and deviates from its normal temporal course or is excessive, changes accumulate resulting in an allostatic load, understood as the cost the body and brain must pay for adaptation to adverse conditions<sup>3,4</sup>. Four types of allostatic load have been proposed: (1) repeated challenges represented by chronic stress, (2) failure to habituate with repeated challenges, (3) failure to shut off the response after the challenge is past, and (4) failure to mount an adequate response<sup>1</sup>.

Not surprisingly, the limbic brain structures that participate in mounting and terminating the stress response are the most affected by stress. Indeed, careful examination on the microdistribution of corticosterone receptors

in rats showed the exclusive expression of the mineralocorticoid receptors (MR) in the hippocampus. Although broadly expressed in the brain, the highest density of glucocorticoid receptors (GR) was observed, amongst others, in the hippocampus and amygdala<sup>5</sup>. These data suggest those regions are primary targets of GCs in the brain.

Neuronal plasticity has been defined as an intrinsic property that enables the brain to escape the restrictions imposed by the genome and thus adapt to environmental pressures, physiologic changes, and experiences<sup>6</sup>. Under this definition, we will consider here short-term 'dynamic' structural adaptations as well as long-term ones associated with early-life experience, aging and even transgenerational genome-independent changes. Indeed, the response of brain areas such as the PFC, the amygdala and the hippocampus to GCs in terms of plasticity have been studied extensively and includes from structural, synaptic and molecular (epigenetic) plasticity to even trans-generational changes in brain plasticity, which we discuss in following sections. Both acute and chronic stress induce strong changes in neuronal plasticity in the hippocampus, prefrontal cortex and amygdala, changing crucial neuronal structural parameters including dendritic spine density and dendritic length and branching<sup>7</sup>. However, chronic stress induces contrasting patterns of neuronal plasticity in different brain areas. While in the hippocampus a marked atrophy of dendritic trees is observed, particularly in the cornu ammonis 3 (CA3) area, in the basolateral amygdala chronic stress induces dendritic growth<sup>8,9</sup>. Similarly, chronic stress induces a selective retraction of apical dendritic arbors in the medial prefrontal cortex (mPFC), while apical dendritic arborization in the orbital frontal cortex is increased<sup>10</sup>. Mechanistically, the effects of stress on structural plasticity involve GCs, excitatory amino acids and other neurochemical mediators released during the stress response<sup>2,7</sup>. In the dentate gyrus (DG), the absence of GCs induced by complete adrenalectomy results in dendritic atrophy of granule cells<sup>11</sup>, and knockdown of the glucocorticoid receptor (GR) in individual granule cells leads to an increase in dendritic complexity and abnormal distribution of dendritic spines<sup>12</sup>. Importantly, the effects of chronic stress on structural plasticity are largely reversible in young adult animals<sup>13</sup>.

More recent observations have demonstrated that stress and its neurochemical mediators regulate synaptic structure and morphology also at a much more subtle level. In the mPFC, chronic stress alters dendritic spine morphology, resulting in a reduction in large spines and an increase in smaller spines suggesting a failure in spine maturation and stabilization following chronic stress<sup>14</sup>. In particular, GCs are critical regulators of dendritic spine development and plasticity *in vivo*. In the barrel cortex, GCs increase spine turnover and inhibition of GCs action results in a substantial reduction in spine turnover rates. This reduction in spine turnover could then be reversed by corticosterone replacement<sup>15</sup>. Consistently, in the hippocampus, knockdown of the GR in newborn granule cells of the DG leads to an increase in the number of mushroom-shaped mature dendritic spines<sup>12</sup>, overall indicating that stress and GCs are broad regulators of dendritic spine maturation and stabilization.

The observations described above indicate that in the hippocampus, stress is a key modulator of structural plasticity particularly in the DG-CA3 system. Besides the atrophy of dendritic trees observed in CA3 pyramidal cells<sup>8</sup>, chronic stress affects the mossy fiber terminals from dentate granule neurons, providing a major excitatory input to the CA3 proximal apical dendrites. Mossy fiber terminals in chronically stressed rats show marked rearrangement of synaptic vesicles and an increased area of the synaptic terminal occupied by mitochondria, however, these changes were not observed after acute stress<sup>16</sup>. Additionally, chronic stress and administration of high GCs concentrations result in profound changes in the morphology of the mossy fiber terminals and significant loss of synapses on CA3 pyramidal neurons. Again, these effects were largely reversible and the accompanying impairments in spatial learning and memory were undetectable following rehabilitation<sup>17</sup>. Interestingly, knockdown of the GR in newborn granule cells of the DG leads to a substantial increase in the size of mossy fiber terminals in the CA3 area<sup>12</sup>. Together, these observations suggest that stress and GCs acting through the GR regulate structural plasticity in the DG-CA3 system to control the excitatory input of DG granule cells onto CA3 pyramidal neurons.

The presence of ongoing neurogenesis is another interesting and rather exceptional form of structural plasticity, present in among others the DG-CA3 system. In distinct areas of the adult brain new neurons continue to be generated for the whole life of the individual. The first description of postnatal neurogenesis in the adult rat brain was made by Altman and Das<sup>18</sup>. In the early 1990s, multiple groups rediscovered this phenomenon taking place in both the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus in rodents<sup>19-21</sup>, and in humans<sup>22</sup>. In the SGZ, astrocyte-like cells divide and generate new granule neurons and astrocytes<sup>23-25</sup>. Recent observations indicate that newborn neurons participate in behavioral tasks that specifically involve the DG<sup>26,27</sup>. Regarding stress and its mediators, early studies showed that acute stress in the form of a single episode of psychosocial stress and GCs inhibit proliferation in the SGZ<sup>28,29</sup>. Although some contradictory observations have been reported, the overall result appears to be that stress inhibits adult neurogenesis by lowering the cell proliferation rate<sup>30,31</sup>. Some studies have shown that neural precursor cells (NPC) and some of their progeny in the DG express intracellular receptors for GCs<sup>32-35</sup>. In particular, blockade of the GR with the antagonist mifepristone *in vivo* rapidly recovers GC-induced inhibition of proliferation and induction of apoptosis observed in the DG after chronic and acute stress, respectively<sup>36-38</sup>. Additionally, knockdown of the GR in newborn granule cells of the DG accelerates their neuronal differentiation and migration<sup>12</sup>. Therefore, the elevation in GCs associated with activation of the HPA axis seems to be a central mechanism for regulation of all aspects of neurogenesis by stress in the SGZ. However, GC levels are not always inversely correlated with levels of SGZ proliferation, suggesting the existence of additional mechanisms, including excitatory neurotransmitters and other cellular mediators<sup>39</sup>. In terms of reversibility, some studies have shown that the reduction in proliferation and apoptotic changes induced by acute and chronic stress in the DG are largely reversible<sup>40,41</sup>, while others have demonstrated that they are long-lasting<sup>42,43</sup>. Possibly, this discrepancy could be explained by the use of different stressor and stress modes in these studies, or in the cell type examined as indicator of hippocampal neurogenesis.

### Mechanisms involved in the regulation of neuronal plasticity by stress.

Stress induces significant changes in neuronal plasticity in specific brain regions, thereby locally affecting neuronal circuits in these areas. How are these effects brought about? In many cases, the initial steps leading to the ultimate effects of stress on brain adaptation involve highly coordinated and region specific changes in gene expression<sup>44</sup>. One likely explanation for these region specific effects of steroids on the brain could be the local expression of factors such as steroid co-activators<sup>44,45</sup>. Gene-expression profiling studies have shown that in the hippocampus, chronic stress and GCs coordinate the expression of subsets of genes involved in energy metabolism, signal transduction, neuronal structure, vesicle dynamics, neurotransmitter catabolism, cell adhesion, genes encoding neurotrophic factors, and their receptors and genes involved in regulating glucocorticoid-signaling, as well as CREB-signaling. These changes in gene expression underlie adaptations in cell metabolism, neuronal plasticity and synaptic transmission<sup>46,47</sup>.

As we discussed in the previous sections, most of the changes in gene expression and neuronal plasticity induced by acute and chronic stress in adult life are largely reversible. However, stress during early life is a risk factor for the development of long-lasting stress-related diseases such as depression and post-traumatic stress disorder (PTSD)<sup>2</sup>. An elegant study describes the significance of an altered glucocorticoid exposure in early life, and links it to lasting consequences in later life and even to transgenerational effects on the offspring<sup>48</sup>. These authors observed that prenatal exposure to synthetic GCs alters hippocampal GR levels and subsequently hippocampal GC feedback systems, ultimately resulting in an altered HPA axis function in the second offspring generation. Whether these observations are a result of a change in maternal behavior, metabolic state or lasting (epigenetic) changes in neuronal plasticity in the offspring will need to be further elucidated. Pup handling is one of the best documented paradigms used to study the effects of early life experience on the HPA axis, because handling of rat pups during their postnatal development permanently alters the function of their HPA axis. Experiments in the past demonstrated that adult rats handled

during infancy show a reduced response to stress in the hippocampus and a significant increase in GR expression, rendering the animals more sensitive to negative GC feedback<sup>49,50</sup>. Furthermore, prenatal stress induces high anxiety in adulthood, which is prevented by pup handling, and correlates with HPA activation levels and stress-induced corticosterone secretion<sup>51</sup>. Despite the consistency of these observations, how the long-term effects of early experience on neuronal plasticity are brought about is yet not fully understood. In terms of neuronal plasticity, and considering the persistence of the effects that early-life experience has on it, epigenetic mechanisms are strong candidates to explain how life events like stress, induce persistent changes in the brain<sup>52,53</sup>.

### microRNAs in epigenetic regulation and neuropsychiatric disorders.

The definition of epigenetics is broad but in molecular terms epigenetic changes can be understood as a group of molecular events, external to the genetic code itself, modulating gene expression over time. These alterations include DNA methylation, microRNAs (miRs) and other small non-coding RNAs, and covalent modifications of histones<sup>54</sup>. Under this definition, the hippocampus presents one of the best examples of epigenetic regulation, because differences in early life environment result in lasting changes in behavior that are preserved across generations. These changes are imprinted through histone modification and DNA methylation at the GR gene promoter, resulting in alterations in hippocampal GR expression that persists across generations, producing significant differences in stress responsiveness and affective behaviors<sup>55,56</sup>.

Small non-coding RNAs are involved in a variety of gene expression regulatory mechanisms in the cell, such as alternative splicing, ribosomal RNA modifications, and repression of messenger RNA (mRNA) expression by RNA interference (RNAi), a regulatory mechanism mediated by RNA-RNA interactions first observed in *C. elegans*<sup>57,58</sup>. They can be classified into several major classes, i.e., small nucleolar RNAs (snoRNAs), endogenous small interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), miRs, transfer RNAs (tRNAs), rRNAs, spliceosomal RNAs and RNase P/MRP genes<sup>59</sup>.

miRs are approximately 22 nucleotide (nt)-long small non-coding RNAs. miR biogenesis is initiated via transcription by RNA polymerase II<sup>60-64</sup>, generating primary transcripts known as pri-miRs. Pri-miRs are cropped by the ribonuclease III Drosha and its cofactor, DiGeorge syndrome critical region gene 8 (DGCR8) to generate approximately 65 nt-long hairpin-shaped precursors known as pre-miRs<sup>65-68</sup>. Drosha and DGCR8 form a protein complex called microprocessor, crucial for initial miR biogenesis. Pre-miRs are then exported by the nuclear transport factor exportin-5 (Exp5) to the cytoplasm<sup>69-71</sup>. Once there, the RNase III Dicer generates ~21 nt-long miR duplexes<sup>72-74</sup>. The sense strand of the duplex (passenger strand) is discarded and the antisense strand (guide strand) becomes a mature miR and is assembled into the RNAi effector complex called RNA-induced silencing complex (RISC)<sup>75,76</sup>. Once loaded into the RISC, mature miRs act through RNAi by imperfect match recognition of target sites in the 3'UTRs of mRNAs, resulting in repression of target mRNA expression<sup>68</sup>.

Since their first discovery almost two decades ago, hundreds of miRs have been identified in a wide range of organisms, making them the best characterized members of the small non-coding RNA family. They play important roles in virtually all biological processes studied, from development to cell death and metabolic control and over 60% of all mammalian mRNAs seem to be under the control of miRs, adding an extra layer of control to the already complex regulatory mechanism of gene expression<sup>77-79</sup>. Direct evidence that miRs regulate epigenetic states comes from studies performed in mouse embryonic stem cells (ES). Dicer-null mouse ES express significantly lower levels of the DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b, resulting in defects in DNA methylation and affecting ES differentiation<sup>80</sup>. Similarly, DGCR8 is essential for miR biogenesis and controls mouse ES self-renewal and differentiation<sup>81</sup>. Recent studies have demonstrated that epigenetic mechanisms, including DNA methylation and histone modification, not only regulate the expression of protein-encoding genes, but also miRs, such as let-7a, miR-9, miR-34a, miR-124, miR-137, miR-148, and miR-203<sup>82</sup>. Therefore, it is conceivable that a complex balance between miR expression and DNA methylation status controls the necessary genes required for adequate epigenetic regulation of gene expression.

A substantial amount of evidence suggests that several individual miRs contribute to the risk of neuropsychiatric disorders, including Huntington disease, Parkinson disease, and Tourette's syndrome<sup>83-87</sup>. Similarly, some of the cellular factors that participate in miR biogenesis, and in particular members of the microprocessor complex, have been associated with human pathologies that affect the brain. This is important because the levels of DGCR8 adjust to those of its substrates, the pri-miRs, probably through autoregulatory feedback loops<sup>88</sup>. This may ensure that levels of the microprocessor components are kept at optimal range required for biological activity, balancing efficiency and specificity of miR biogenesis and suggests that even subtle alterations in the expression levels of the microprocessor components may result in broad changes in miR expression profiles.

Interestingly, DGCR8 is disrupted by the 22q11.2 microdeletion, a genetic alteration associated with cognitive and behavioral impairments and the highest known genetic risk for developing schizophrenia. The deficiency in DGCR8 expression associated with 22q11.2 microdeletion results in decreased miR biosynthesis and altered short-term plasticity in the prefrontal cortex<sup>89</sup>. On the contrary, other studies have found that schizophrenia is associated with an increase in cortical miR biogenesis and increased DGCR8 expression. However, the resulting miR upregulation impacted on several genes involved in synaptic plasticity<sup>90,91</sup>. Therefore, although conflicting in the characterization of the mechanisms linking schizophrenia to DGCR8 expression, these observations overall suggest that DGCR8 is a key regulator of synaptic plasticity in the cortex. Furthermore, genetic variations in DGCR8 and other genes involved in miR biogenesis are associated with susceptibility to depression, suicidal tendency, and response to antidepressants<sup>92</sup>. This is conceptually consistent with a recent study showing that miR expression is downregulated in prefrontal cortex of depressed suicide subjects<sup>93</sup>. However, in this study DGCR8 and Dicer levels were unchanged between depressed and control groups, suggesting that other mechanisms are involved in the global miR downregulation observed in the prefrontal cortex of depressed suicide subjects. Moreover, DNMT3b was strongly up-regulated in the depressed suicide group,

pointing again towards a potential interaction between miRNAs and other epigenetic mechanisms. Finally, the levels of Dicer were significantly lower in temporal lobe epilepsy (TLE) patients with hippocampal sclerosis (HS) and in the hippocampus of mice subject to experimentally-induced epilepsy, resulting in a large-scale reduction of miR expression, with 51% of all detected miRNAs expressed at lower levels, suggesting that loss of Dicer and failure of mature miR expression may be a feature of the pathophysiology of HS in patients with TLE<sup>94</sup>.

Recently, several groups have reported the possibility that epigenetic mechanisms, including changes in miR expression, are implicated in mediating the persistent effects of early-life experience on gene expression in the brain (recently reviewed by Korosi *et al.*<sup>52</sup>). In particular, The expression levels of several miRNAs (i.e. miR-124, miR-9, miR-132) with known functions in the regulation of neurogenesis and other forms of structural plasticity and was altered in the prefrontal cortex of maternally separated rats<sup>95-97</sup>. These observations will be discussed in detail in the following section.

### miRNAs in the context of the brain's adaptation to stress

miRNAs have the ability to fine-tune gene expression. This ability is important to control gene expression patterns that ensure dynamic stability under external or internal perturbations or favor the organism's adaptation to the environment. miRNAs can generate rapid and reversible responses and, in this way, are ideally positioned to optimize stress responses<sup>98,99</sup>. miRNAs are abundantly expressed in the nervous system and a relation between miRNAs and neuronal responses to stress has been demonstrated in different model systems. Indeed, a recent study in *C. elegans* showed that miR-71 functions in neurons to promote resistance to physiological stress and germline-mediated longevity<sup>100</sup>. Furthermore, miRNAs are involved in the epigenetic changes triggered by systemic stress induced by alcohol abuse<sup>101</sup>. Under these stress circumstances, neuronal adaptation correlates with a rapid increase in miR-9 expression. This observation suggests that miR-9 plays an important role in neuronal plasticity during adaptation to stress induced by alcohol abuse<sup>102</sup>. Other strong stressors, such as hypoxia, promote a

sharp upregulation of the miR-130 in primary rat hippocampal neurons<sup>103</sup>. Interestingly, hypoxia-induced changes in miR expression seem to be cell-specific, indicating that neurons and astrocytes can utilize different miR sets to respond to certain physiological stressors<sup>104</sup>. Together, these studies suggest that miRNAs are involved in neuronal adaptation to acute stress.

A recent study has further suggested that gene expression and epigenomic responses to chronic stress in the brain may involve miR-mediated re-programming in a region-specific fashion<sup>105</sup>. In this study, exposure of rats to two weeks of mild restraint stress altered miR expression in the cerebellum. There, two miRNAs, miR-186 and miR-381, were upregulated, while miR-709 was downregulated. Interestingly, the downregulation in miR-709 induced in the cerebellum was resistant to 2-week long recovery from stress, suggesting this miR-709 downregulation may be involved in long-lasting adaptation to chronic stress specifically in this brain region. The chronic stress/recovery paradigm used in this study also induced changes in miR-709 expression in the hippocampus and prefrontal cortex. In these brain regions miR-709 was unchanged after 2 weeks of chronic stress but was upregulated after two weeks recovery, reinforcing the conclusion that miR-709 regulation by stress was brain region-specific<sup>105</sup>. Another study found that stress induces brain region-specific alterations in miR expression in mice<sup>106</sup>. In this study, the expression levels of let-7a, miR-9 and miR-26-a/b in the frontal cortex were increased after 1 day acute restraint stress, while only minor changes were observed after repeated restraint stress (5 consecutive days). No differences in the expression of these 3 miRNAs were observed in the hippocampus under any stress paradigm tested<sup>105</sup>, reinforcing the idea that miRNAs may be involved in the mechanism by which stressful events regulate gene expression in a brain region-specific manner. With respect to the amygdala, another brain region crucially affected by stress-induced changes in neuronal plasticity, a recent study has demonstrated a physiological role for miR-34c in regulating the stress response in that region<sup>107</sup>. In this study, acute stress induced a differential expression profile of miRNAs in the amygdala. In particular, miR-34c was found upregulated after acute and chronic stressful challenges in the amygdala specifically.

Furthermore, local ablation of Dicer in the central amygdala of adult mice induced a robust increase in anxiety-like behavior, and local overexpression of miR-34c was able to (partially) revert this phenotype. Finally, the authors identified corticotropin releasing factor receptor type 1, a central component of the HPA-axis, as target of miR-34c. Another study has found changes in miR expression in both acute and chronic stress rats in the amygdala specifically. In this study, miR-134 and -183 were upregulated in the amygdala after acute stress but miR-134 was downregulated in the amygdala and also in the hippocampus after chronic stress, while miR-183 was unchanged under chronic stress conditions<sup>108</sup>. These authors linked the changes observed in miR expression to changes in alternative splicing, because miR-134 and -183 target the serine/arginine-rich splicing factor 2 (SC35), which was upregulated in response to stress, promoting the alternative splicing of acetylcholinesterase and affecting the local regulation of cholinergic neurotransmission. Indeed, other miRs such as the brain specific miR-124 exert their broad biological actions at least partially by regulating alternative splicing, another key regulator of gene expression in the brain<sup>109</sup>.

Very few studies have assessed the reversibility or persistence of the changes in miR expression observed in the brain and their relationship with stress vulnerability later in life. One study by Uchida *et al.* has tackled this relevant question by showing that early life stress in the form of maternal separation, a well characterized HPA axis programming factor, affects the expression of neuronal plasticity-related miRs later in life<sup>110</sup>. In this study miR-132, -124, -9, and -29a levels were upregulated in the mPFC of 14-day old rats exposed to early life stress. In adult rats (approximately 60 day-old) exposed to early life stress, the levels of miR-132, -124, and -212 were still upregulated, suggesting that long-lasting upregulation of miR-132 and -124 are part of the persistent epigenetic changes induced by early life stress on neuronal plasticity in the mPFC. Indeed several studies have demonstrated that miR-132 and -124 are key mediators of activity-dependent and independent neuronal plasticity and target the stress pathways in the brain, GR activation suppresses miR-132 expression and results in a decrease in BDNF and glutamate receptors and miR-124 targets the GR<sup>111,112</sup>.

Outside the brain, there is evidence that GCs can regulate the expression of DGCR8, Dicer and specific miR sets<sup>113,114</sup>. Conversely, studies have demonstrated that miRs regulate the expression of the two GC receptor types expressed in the brain. More specifically, in the Paraventricular Nucleus (PVN) miR-18 regulates the expression of the GR in chronically stressed rats<sup>110</sup>, and miR-124 affects GR-mediated gene transcription and decreases GR protein levels<sup>111</sup>. Further, miR-124 and miR-135a may regulate the expression of the mineralocorticoid receptor<sup>115</sup>, and GC treatment induces expression of miR-124<sup>116</sup>, suggesting the possible engagement of the brain-specific miR-124 in feedback regulatory loops that could be involved in the coordination of steroid-mediated effects on neuronal plasticity<sup>117</sup>.

Other possible example of a regulatory feedback loop involving the GR and miRs involves the miR-17~92 cluster, which includes miR-18<sup>118</sup>. The GR is a key regulator of structural plasticity and neurogenesis in the hippocampus<sup>12</sup> and we have demonstrated before that miR-18 regulates GR protein expression levels and is expressed at high levels in the hippocampus during early postnatal development, when neurogenesis is still strong<sup>111</sup>. These results suggest that miR-18 could regulate early postnatal neurogenesis in the hippocampus by repressing GR expression. Interestingly, miR-18 forms a regulatory feedback loop with the estrogen-receptor  $\alpha$  (ER $\alpha$ )<sup>119</sup>. This implies that sex differences in adult neurogenesis levels observed after early life stress<sup>120,121</sup> could involve long-lasting changes in miR-18 expression. Although (sex)steroids may play a role in establishing sex differences in the effects of stress on hippocampal plasticity during early life, significant differences in hippocampal structural plasticity (i.e. neurogenesis) exist between control males and female rodents even before initiation of the oestrous cycle<sup>52</sup>.

#### **miRs and the regulation of neuronal synaptic and structural plasticity.**

Synaptic development and plasticity are important for fine-tuning brain circuits during embryonic development and for high-order brain functions such as learning, memory and cognition<sup>122</sup>. Multiple lines of evidence suggest that altered synaptic plasticity and

morphology as seen in neurodevelopmental and stress-based disorders may, in part, result from a common post-transcriptional process that is under tight regulation by miRs<sup>96,123-125</sup>. Various miRs have been shown to regulate the expression of proteins involved in the actin cytoskeleton<sup>96,126</sup>, mRNA transport<sup>127</sup>, as well as many other proteins associated with changes in spine morphogenesis. Notably, one significant difference between the various mechanisms of regulation of gene expression involving miRs at the synapse seems to be reversibility. While mRNA decay is an irreversible process, miR-dependent inhibition of mRNA translation to protein is often reversible and mRNA translation is resumed following elimination of miR-dependent repression. The inhibition of translation is an essential feature for involvement in activity-dependent synapse plasticity, since it serves as a basis for the dynamic regulation of the speed of global regulation of expression at the synapse and for miR function in response to synaptic stimuli. However, miR's reversible action is not universal for all miR:target interactions, since some miR binding results in significant target mRNA degradation<sup>128</sup> (reviewed by Valenci-Sanchez *et al.*<sup>129</sup>).

Evidence for a synaptic role of miRs was provided by molecular characterization of several promoters. Various upstream cis-acting elements of neural miR promoters can be occupied by typical activity-regulated transcription factors, including CREB and MEF2. These transcription factors couple Ca<sup>2+</sup>-regulated signaling cascades to the transcriptional machinery in cortical and hippocampal neurons<sup>127,130</sup>. In addition, a number of post-transcriptional mechanisms, such as miR 3' end modifications and miR 2'-O-methylation<sup>131-133</sup>, influence the expression and activity of mature miRs, and the regulation of proteins associated with these miRs has a significant impact on regulation of target gene expression at the synapse<sup>134-136</sup>.

Initial findings raised the possibility that miRs are formed via the processing of pre-miRs locally within dendritic spines<sup>137,138</sup>. More recent studies have provided further experimental evidence supporting the notion that miR biogenesis takes place locally in the vicinity of synapses. In particular, the microprocessor components Drosha and DGCR8 and their substrate the pri-miRs

are especially enriched in postsynaptic densities<sup>139</sup>. Thus, miRs maybe ideally positioned to quickly regulate translation in response to synaptic activity. In support of this hypothesis, Lugli *et al.*, demonstrated that Dicer and eIF2c (also know as Argonaute 1), both rate-limiting enzymes in mature miR production, are highly enriched at post-synaptic densities, and their levels are modulated through neuronal activity<sup>140</sup>. Moreover, synaptic stimulation can lead to activation or loss of the RISC complex component Armitage, suggesting a dynamic regulation of target gene expression at individual synapses by global changes in the miR biogenesis pathway<sup>134</sup>. On the same line, proteasome-dependent degradation of the RISC component MOV10 locally regulates the expression RISC-associated synaptic mRNAs such as CaMKII, lysophospholipase 1 (Lypla1) and LIM-domain-containing protein kinase 1 (Limk1) in hippocampal neuron<sup>135</sup>. In this study, MOV10 was degraded at the synapse upon N-methyl-D-aspartate receptor (NMDAR) activation, allowing activity-dependent local translation of synaptic mRNAs important for synaptic plasticity<sup>135</sup>. The outcome of these studies collectively suggested that both miRs and their associated biogenesis proteins have a central role in activity-regulated signaling networks, controlling adaptive processes such as dendritic spine maintenance or synaptic plasticity.

Previous studies have shown that long-term potentiation (LTP)-inducing stimuli cause an increase in the size of spines, whereas long-term depression (LTD)-inducing stimuli cause a reduction in the size of spines<sup>141</sup>. Based on this report and data showing that changes in miR levels regulate dendritic spine size in cortical and hippocampal neurons<sup>96</sup>, it is tempting to speculate that various miRs enriched at the synapse would contribute to regulation of local protein translation associated with the dendritic spine remodeling induced by LTP and LTD. Furthermore, synaptic activity can influence miR expression, as previous reports indicated that miR levels are altered in hippocampal neurons induced to display LTD or LTP<sup>142</sup>. These findings suggest that different levels of miR expression could affect translation of synaptic proteins during LTP or LTD establishment. Notably, a recent study investigated the roles of miRs after LTP in the rat hippocampus. Microarray analysis

identified that the levels of numerous miRs were altered in rat hippocampal slices after LTP, with miR-188 exhibiting the largest upregulation. This result suggests that miR-188 counteracted the decrease in miniature excitatory postsynaptic current (mEPSC) frequency induced by regulating Neuropilin-2 (Nrp-2) expression, fine-tuning synaptic plasticity in hippocampal neurons<sup>143</sup>.

Co-expression of neural miRs with their target has been shown to be common at synapses, suggesting that miRs may participate in regulatory mechanisms connecting the control of local protein synthesis at the dendrites with global regulation of gene expression<sup>144</sup>. Supporting this hypothesis, the compartmentalized Campenot culture chamber system<sup>145</sup> was used to identify miRs present in axons and pre-synaptic compartments of primary sympathetic neurons<sup>146</sup>. This study identified 130 miRs highly abundant in distal axons as compared to the soma. These axon-enriched miRs might be important regulators of local maintenance of synaptic structure and function and neuronal growth and development as well.

A number of individual miRs have central roles in synaptic plasticity. Schratt *et al.* demonstrated that miR-134 regulates dendritic spine morphology<sup>96</sup>. Overexpression of miR-134 caused a significant reduction in dendritic spine size, while its inhibition by 2'-O-methyl antisense oligonucleotide led to an increase in spine volume and width in hippocampal neurons. Here, the actin filament regulator Limk1 was also identified as a miR-134 target. Notably, repression of Limk1 translation by miR-134 is decreased by brain-derived neurotrophic factor (BDNF)-dependent stimulation of synaptic activity, suggesting a downstream role for miR-134 in BDNF mediated synaptic plasticity. Since the original study by Schratt *et al.*, other miRs have been shown to regulate translation of mRNAs involved in dendritic spine morphology in hippocampal neurons, such as miR-138, that regulates dendritic spine size through its target APT1 mRNA<sup>126</sup>, as well as miR-132 that acts as a suppressor of p250GAP expression in an activity dependent manner<sup>147,148</sup>. Another recent study demonstrated that miR-125a targeting PSD-95 mRNA allows for reversible inhibition of translation and

regulation by synaptic mGluR signaling<sup>149</sup>. mGluR signaling of translation requires FMRP dephosphorylation, thereby providing a reversible switch for miR-125a to selectively regulate PSD-95 translation at synapses. Interestingly, this bidirectional control of PSD-95 expression depends on the phosphorylation status of FMRP, a critical synaptic protein previously shown to associate with miR-125a<sup>124</sup>. Other studies have shown that miR-125a and -b target other mRNAs important in synaptic function such as the NMDA receptor subunit NR2A and EphA4, involved in synaptic scaling<sup>150-152</sup>.

In conclusion, the unique feature of miRs to locally and on activity demand fine-tune entire gene circuitries makes them key regulators of synaptic plasticity and attractive candidates for future therapeutic interventions for mental disorders. Ultimately, the characterization of the roles in synaptic plasticity of individual miRs or global changes in miR expression induced by regulation of the miR biosynthesis pathway may generate original insights that could advance our understanding of these small regulatory RNAs in synaptic function, and may provide with significant potential to generate new molecular-based therapies to treat nervous system disorders.

#### **miRs as novel biomarkers for detection of stress-related neuropathologies.**

Serum and cerebrospinal fluid (CSF) biomarker levels have been shown to be a consistent source to evaluate the level of neurological and neurodegenerative disorders, as well as the efficacy of potential therapies<sup>153</sup>. Because many protein-based, validated markers are unstable and easily affected by body stress and metabolic turnovers, more reliable biomarkers are needed to assess the progression of disease states or the extent of therapeutic treatments. Recent studies suggested that miRs are promising and highly reliable biomarkers in a number of disorders, including cancer, heart failure, and neurodegenerative disorders<sup>154-158</sup>. Circulating miRs are released into serum and CSF from cells through an endocytotic pathway, and it has been suggested that the RNAs are packed into exosomes that protect them from endoribonuclease dependent degradation in bodily fluids<sup>159</sup>. Previous studies have shown that secreted miRs contained in exosomes

and other microvesicles potentially influence cellular microenvironments affecting immune, endothelial and fibroblast cells<sup>160-162</sup>.

In healthy individuals the levels of cell-free miRs are stable and the plasma miR profile is similar to that of circulating blood cells<sup>163</sup>. Thus alterations of serum miR levels may indicate physiological and pathological changes. While serum miRs cannot cross the blood-brain barrier (BBB), previous studies suggested that naturally occurring exosomes are capable of crossing the BBB<sup>164,165</sup>. Moreover, accumulating experimental and clinical evidence indicate that a number of neurological diseases are associated with BBB dysfunctions<sup>166</sup>, resulting in elevated barrier permeability, enabling leakage of larger molecules such miRs into and out of the brain.

Currently, major depression is best characterized as a behavioral endpoint, and increased levels of plasma and urinary free cortisol<sup>167</sup>. Due to the broad phenotype associated with this disorder, it seems rather challenging to identify a single biomarker reflecting the activity of a pathway (or pathways) involved in depression symptoms. However, identifying and analyzing miR profiles in peripheral blood or CSF of depressed patients may identify a subset of individuals at risk of depression and ultimately result in a more personalized approach to treatment for depression<sup>166</sup>. Notably, a recent high throughput miR expression study indicated that the levels of 30 miRs in the blood of patients with major depression were modulated by the antidepressant treatment<sup>168</sup>. From this work, it has been concluded that many of these miRs may be relevant for antidepressant-induced regulation of gene expression in the brain. Moreover, miR-144 and miR-16 were altered in the blood of healthy individuals subjected to naturalistic stress situation such as academic examinations<sup>169</sup>. Interestingly, miR-16 is known to be involved in the serotonergic pathway by targeting the SERT transporter at the synapse, suggesting a role for miR-16 in serotonergic regulation<sup>170</sup>. In addition, two recent studies revealed the potential of bodily fluid miRs to serve as practicable clinical biomarkers for diverse physiological and pathological conditions. However, very little is known yet about the use of circulating miRs as reliable biomarkers of depression and other stress-related neuropathologies.

### Molecular mechanisms of target specificity in neurons.

Despite all the evidence gathered on the role of miRs in the regulation of important targets, we do not yet have complete knowledge of the factors determining which mRNAs are targeted by their corresponding targeting miRs or the molecular mechanism through which individual mRNA silencing is accomplished (translation repression or mRNA destabilization). Despite earlier reports, recent large-scale studies in animal cells have indicated that in many cases, a reduction in protein synthesis can be explained by direct downregulation of target mRNA levels<sup>171,172</sup>. It is now widely accepted that the primary determinant for miR binding is perfect consecutive Watson-Crick base-pairing between the target mRNA and the miR at position 2-7 or 2-8 of the 5' end of the mature miR, often denoted as the 'seed region'. Nevertheless, a 'seed' is neither necessary nor sufficient for miR downregulation. For instance, miR target sites can tolerate G:U wobble base pairs within the seed region<sup>173,174</sup> and extensive base pairing at the 3' end of the miR may offset missing complementarity at the seed region<sup>175</sup>. Further, even sites with extensive 5' complementarity can be inactive when tested in reporter constructs<sup>176</sup>.

In recent years, considerable progress has been made to identify additional features that could help to predict target regulation accurately (Figure 1). Grimson *et al.*, have reported that local sequence context, such AU-rich nucleotide composition near the site, proximity to sites for co-expressed miRs, proximity to residues pairing to miR nucleotides 13-16, positioning within the 3'UTR at least 15nt away from the stop codon, and positioning away from the center of long UTRs can all promote efficient miR efficacy<sup>177</sup>. In this respect, other studies have confirmed that miR sites in the same 3'UTR can potentiate the degree of translational repression. Two reports have shown that miR cooperativity on target downregulation is optimal when two miR-binding sites are closely positioned, usually between 13-35 nucleotides apart<sup>178,179</sup> and when seed regions are weak<sup>180</sup>.

However, target sites spaced in substantially longer distances may still cooperate to lower the expression of neuronal proteins<sup>181,182</sup>.

In this context, miR cooperativity is defined as the positive interaction of two or more individual miRs, or one individual miR acting on multiple seed regions on the same 3'UTR, on target repression. In addition, miR seed density in synaptic mRNAs is higher than in non-synaptic mRNAs, indicating that they may be under stronger miR cooperative control. Furthermore, approximately 50% of synaptic mRNAs are predicted to have more than 5 miR binding sites<sup>182</sup>. Therefore, miR cooperativity could be a relevant mechanism in the regulation of synaptic mRNAs. For instance, a recent elegant study has shown that the plasticity-related miRs miR-9 and miR-132 cooperate in the embryonic mouse neocortex in the regulation of *Foxp2*, a transcription factor associated with speech and language development<sup>183</sup>.

Another important determinant of efficient silencing is mRNA folding, with several reports indicating that miR seeds are preferentially positioned in highly accessible regions at the start and end of 3'UTRs<sup>184-186</sup>. Indeed, target sites in the middle of 3'UTRs are less efficient in inducing regulation by RNA interference<sup>187</sup> while those positioned near both ends of 3'UTRs were associated with higher repression<sup>178</sup>. This has been confirmed for synaptic mRNAs where an over 2-fold increase in the number of sites near both ends of 3'UTR has been observed<sup>188</sup>.

Another important determinant is the length of the 3'UTR. Genes with short or intermediate 3'UTRs (0-1000 nt) are significantly more repressed than genes with long 3'UTR<sup>188</sup>. This is, likely, because long 3'UTRs encode complex regulatory environments in which other factors could bind, affecting the overall repression of the transcript. Such factors could be other miRs or RNA binding proteins (RBPs). Indeed, it has been shown that destabilization mediated by a transfected miR is generally attenuated by the presence of destabilizing AU-rich motifs and augmented by stabilizing U-motifs; these motifs are the targets of tenths of RNA-binding proteins<sup>189,190</sup>. Perhaps not surprisingly, brain mRNAs that require more elaborate regulation, have 3'UTRs significantly longer than average<sup>191</sup>. With respect to synaptic mRNAs, there is considerable variability among the alternatively spliced 3'UTRs. The longest 3'UTR sequence of

presynaptic mRNAs is in average 1800 nt-long. This is significantly longer than the average postsynaptic 3'UTR (1400 nt) and that of other protein-coding genes (1100 nt). On the other hand, the shortest presynaptic mRNA 3'UTRs is in average 270 nt-long, while postsynaptic and other mRNAs 3'UTRs are in average 130 nt and 260 nt-long, respectively<sup>182</sup>. These data indicate that mostly postsynaptic but also presynaptic mRNAs display a broader spectrum of 3'UTR lengths than the average protein coding genes, possibly allowing both low and high complexity regulation.

Currently, we know little about what determines 3'UTR length variation in neurons, but one report indicated that both short and long forms coexist with the longer form determining localization in dendrites<sup>192</sup>. Another report indicated that longer 3'UTR forms appear with aging as a result of weakened mRNA polyadenylation activity<sup>193</sup>. Given that multiple elements act simultaneously to regulate individual mRNAs, variable miR responses may thus be explained partly by these variations in mRNA turnover dynamics. It remains to be seen which of the two mechanisms, destabilization or stabilization (mostly mediated by RBPs), prevails in the nervous system during aging, synaptic activity and stress. This could be important for the postulated actions of miRs in regulating neuronal plasticity during aging<sup>194</sup>.

miR silencing efficiency is also regulated by the cellular concentrations or stoichiometrical relationships between: a) the target mRNA, b) the miR and c) the RISC complex. miRs that have multiple targets and are not highly expressed are expected to downregulate individual target genes to a lesser extent than those with a lower number of targets. Similarly, highly abundant target transcripts that may act as decoys, dilute the effect of miRs under specific conditions<sup>195-197</sup>. This effect is more pronounced when the miR is capable of perfect base pairing with its target<sup>198</sup>. Along these lines, lower levels of a miR may fail to regulate target mRNA, but retain the ability to promote inhibition in conjunction with another miR, indicating that cooperative silencing requires lower miR concentrations<sup>182</sup>. Therefore, miR cooperativity may be relevant for miRs

with lower expression levels or may require smaller changes in miR concentrations. Further, we could predict that imbalances in the relative concentrations of miRs and gene targets might exaggerate or compensate for sequence mismatches between miR-mRNA pairs and, thus, contribute to the heterogeneity of miR regulation observed experimentally.

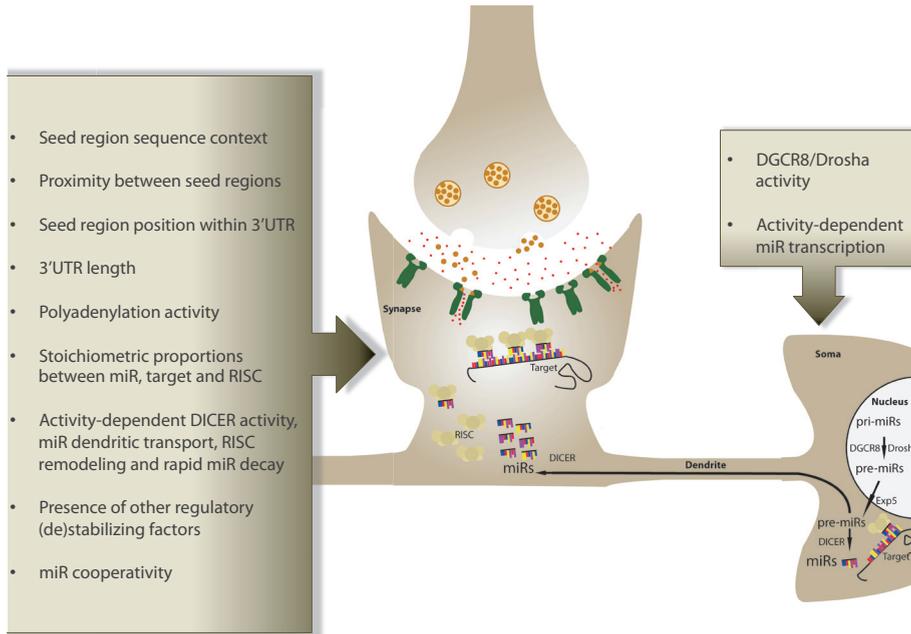
Rapid miR turnover in response to external stimuli, such as growth factors and neuronal activity, or cellular conditions that affect miR stability, such as cell cycle progression, add to the complexity of miR expression regulation. Recently, active degradation of mature miRs has been identified as another mechanism that is important for miR homeostasis<sup>199</sup>. This active and rapid mature miR degradation seems to be a characteristic of neurons<sup>200</sup>. Supporting this hypothesis, neuronal miRs display rapid decay and this effect appears to be subject to activity dependent-regulation. Interestingly, miR rapid decay was not observed in other cell types<sup>200-202</sup>. The presence of this rapid neuronal miR decay may represent a serious problem for the characterization of miR expression in postmortem human brain, where the half-lives of several miRs were not longer than 3.5h<sup>201</sup>. In summary, although neuronal activity has been identified as a key regulator of rapid miR decay, the molecular mechanisms involved are not completely understood. However, it is fair to speculate that activity-dependent rapid miR decay is associated with a need for rapid fine-tuning of synaptic mRNA expression. Within the framework of rapid changes in synaptic plasticity induced by acute stress and GC<sup>203</sup>, it is tempting to speculate that rapid miR decay, and other epigenetic changes, could be a relevant mechanisms involved in fine tuning gene expression at the synapse. Indeed very recent observations suggest that epigenetic regulation of the Rho GTPase Rac1 is crucial for synaptic remodeling mechanisms involved in adaptation to chronic stress<sup>204</sup>. Interestingly, epigenetic methylation of miR-124 genes (miR-124-1, miR-124-2 and miR-124-3) control the expression of miR-124, which directly targets Rac1<sup>205</sup>. This example emphasizes once more the complex interplay between epigenetic mechanisms, including miRs, involved in the regulation of neuronal plasticity<sup>194</sup>.

As discussed before, functional seed regions are generally located in the 3'UTR of mRNAs. However, both coding regions and to a lesser extent 5'UTRs can confer miR regulation, albeit at lower levels than 3'UTRs<sup>171,172</sup>. Furthermore, it has been shown that miR 'seeds' in coding regions potentiate the effect of 3'UTRs<sup>206</sup>. Accordingly, it was later reported that about a third of predicted miR-3'UTR interactions at the synapse also involved at least one binding site in the coding region<sup>182</sup>.

Finally, it has recently emerged that Drosha cleaves precursor pri-miR hairpins with selectivity towards conserved and highly expressed miRs, possibly explaining why the tenths of thousands of hairpin RNAi elements predicted by mining algorithms are never or rarely expressed/detected<sup>207</sup>. To add to this complexity, it has been shown that individual miRs may also display distinct mRNA targeting rules. For instance, neuronal miR-124 targets tend to have seed regions in the 3'UTR, while miR-107 targets tend to have seed region in the mRNA coding regions. Further, mRNA targets of neuronal miR-128 and miR-320 are less enriched in 6-mer seed sequences than miR-124 and miR-107 targets<sup>208</sup>. The reason for these differences is, currently, unknown but evidently they enrich the heterogeneity of miR-mediated silencing (Figure 1).

**How to predict biological function based on miR expression.**

Determining the role of miRs in cellular regulatory processes remains a major challenge. Currently, the great majority of miRs have not been characterized and for those studied in greater depth using knockout or knockin studies we cannot be certain of their true molecular function. This is in part, because the large number of redundant miRs could compensate for their role, their function may only be context specific or because simply the manipulation of their levels may modify other miRs activity through altered availability to RISC complex<sup>209</sup>. Moreover, the few individual targets that have been analyzed and often claimed to mediate miR functions are often misleading or out of context in an effort by the researchers to attribute miR roles based on single target regulation.



**Figure 1 - Mechanisms conveying specificity to target regulation by miRNAs in neurons.**

The left arrowed box shows the molecular mechanisms described in the main text, acting locally at the synaptic level and affecting synaptic plasticity. These mechanisms could also be active at the soma, where they could be involved in regulating targets at the whole-cell level. Additionally, the right arrowed box shows two other molecular mechanisms that could affect miR expression levels and thus target regulation: the activity of the microprocessor components DGCR8 and Drosha and miR transcription regulated by neuronal activity. These two mechanisms only take place at the nuclear level. Therefore, the final target expression level, and its effect on neuronal plasticity, will be a complex balance resulting from local and whole-cell regulation by miRNAs. Stress and other environmental, genetic and epigenetic factors have the potential to affect this delicate balance in a brain region-specific manner.

Deciphering miR expression during development plus analyzing the properties of its many mRNA targets is, currently, the best approach to predict miR function. For this, one could transcriptomics (mRNA), microRNA-omics, and proteomics expression data from miR overexpression or knockdown experiments to identify deregulated genes. Alternatively, one could collect all predicted targets of a miR using available bioinformatics tools that include among others miRanda and TargetScan<sup>210,211</sup>. The gene lists obtained could then be analyzed for Gene Ontology, KEGG, and BioCarta enriched pathways using a number of available algorithms that include DAVID<sup>212</sup> and Ingenuity. If the targets of a specific miR are enriched for a particular biological process or pathway, then it is reasonable to infer that this miR is

involved in that particular biological process. However, there are some limitations to this methodology, as it does not take into account the degree of deregulated targets, whether they are up- or down-regulated or the relative importance of each target in the various processes. It should, also, be noted that there is considerable difference between analyzed data obtained from experiments and bioinformatics predictions and there is a need of careful interpretation.

Experimental data provide insights on miR function based on all deregulated genes (primary targets or not) reflecting the end-point of miR regulation in a particular cell type (neurons or cell-line) and state (developmental stage, split number, culture conditions). Bioinformatics data, on the other hand, provide summated information

of the properties of all primary targets without revealing the final outcome of their regulation. Further, the bioinformatics analysis is not context-specific as it assumes that all targets are co-expressed and it is not influenced by experimental caveats, tissue specificity or other regulatory factors such as RBPs, as we discussed in previous sections.

Importantly, the results obtained with these two approaches may be very different. A characteristic example comes from analyzing neuronal miR-124 and miR-128 experimental data. Both of these miRNAs are known (and predicted) to regulate important regulators of mRNA alternative splicing. miR-124 controls PTBP1 expression and thus the transition from non-NS to NS-specific alternative splicing<sup>109</sup> while miR-128 controls the expression of UPF1 and MLN51 which are key determinants of nonsense-mediated decay (NMD) and thus, the alternative splicing and maintenance of hundreds of neuronal mRNAs that are normally targeted for decay by NMD<sup>213</sup>. In both of the aforementioned cases, bioinformatics would fail to predict the outcome of miR-124 and miR-128 regulation, which is the switch into the expression of hundreds of proneural genes. To conclude, we find that using a combination of expression studies and experimental and bioinformatics analyses is currently the best route to gain insights into the function of individual miRNAs.

### **Conclusion and future perspectives**

In the past few years, miRNAs have emerged as an important class of small RNAs encoded in the genome. They act to control the expression of sets of genes and entire pathways and are thus thought of as master regulators of gene expression<sup>214</sup>. Some miRNAs are specifically expressed in the brain, suggesting unique regulatory roles in neuronal development and function<sup>215</sup>, and recent studies have suggested that they may be involved in the etiology of many neurodevelopmental and stress-related disorders<sup>216</sup>.

Although it was evident soon after their discovery that miRNAs play important roles in most biological processes, including neurodevelopmental timing, growth control, and differentiation and they function by

inhibiting protein production from their targeted mRNAs<sup>217</sup>, it is still unclear how miRNAs are integrated into broader cellular networks of gene expression control. Only recently we have started to understand their involvement and interplay with other components of the cellular epigenetic regulation machinery in coordinating the adaptation of gene expression profiles to environmental demands.

Particularly in neurons, this epigenetic control seems to be of crucial relevance, since neurons face the challenging task of converting complex environmental stimuli into high-order functions, using a vast repertoire of dynamic plasticity processes and long-lasting cellular responses<sup>218</sup>. In contrast to classical small molecules that act on specific cellular targets, the unique feature of miRNAs is to modulate complex physiological or disease phenotypes by regulating entire epigenetic circuitries. This characteristic may make miRNAs attractive and novel therapeutic targets and diagnostic molecules for the treatment and detection of complex mental or stress-related disorders. In this context, it is worthwhile to note that besides the pivotal role miRNAs could play in fine-tuning gene expression in the brain, miRNAs present in bodily fluids such as blood, saliva and CSF have recently been applied to the detection of various types of pathologies<sup>219-221</sup>. Although little is known about the function and origin of miRNAs in bodily fluids, it has been hypothesised that they are excreted in exosomes physiologically or in response to damage and stress<sup>222-224</sup>, suggesting they are interesting candidates as biomarkers for stress-related neuropathologies.

Here we have reviewed the literature demonstrating local changes in miR expression in brain regions known to be crucial for the brain's response to stress and to be critically affected by stress-induced changes in neuronal plasticity: the prefrontal cortex, the hippocampus and the amygdala. Furthermore, we discuss a variety of mechanisms by which changes in miR expression could result in local changes in protein expression regulating neuronal plasticity not only at the regional level, but also at the (intra)cellular level. Finally, we discuss molecular mechanisms of target specificity and degree of silencing by miRNAs. These mechanisms are determined by

numerous factors that include miR identity, target mRNA and RISC levels; 3'UTR splicing; other (de)stabilizing factors; Drosha or RISC preferences, selective rapid decay and miR cooperativity on specific targets (Figure 1). The complexity of these inputs indicates that multiple approaches, including developmental expression studies, bioinformatics and "omics" target identification strategies will be required for efficient characterization of miR function in the frame of stress-induced local changes in neuronal plasticity. Although until now most studies have focused on identifying miR functions on individual targets, in the near future new strategies may be able to integrate different approaches. This will enable the thorough identification of the specific role of miRs in gene expression regulatory networks controlling complex cellular functions such as neuronal plasticity under physiological and pathological conditions.

### Acknowledgements

This work was financed by The Netherlands Organization for Scientific Research (NWO) VIDI grant H64.09.016 to CPF. CPF is grateful to Dr. S.A. Fratantoni for useful comments on the manuscript. We apologize to all colleagues whose work has not been included in this review due to space constraints.

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