SYNAPTIC MICRORNA EXPRESSION AND FUNCTION IN THE CENTRAL NERVOUS SYSTEM

by

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Introduction

Regulated gene expression is essential to the ability of cells in the body to perform specialized functions. In 2001, the Human Genome project published an almost complete sequencing of the human genome, and came to the surprising discovery that only 1.5% of it encode proteins while over 90% was believed to be 'junk DNA' that is never transcribed (Lander *et al.* 2001). Since then, novel technologies such as microarray profiling have demonstrated that the majority of the non-coding sequence is actively and dynamically transcribed into regulatory RNA sequences (Vreugdenhil and Berezikov 2010). Among the multiple families of non-coding RNA that have been discovered, one of the most important identified thus far is microRNA. The first microRNA (miR) to be described was lin-4 in the model organism Caenorhabditis elegans (*C. elegans*) in 1993, and since then hundreds of miRs have been identified in almost all organisms, including humans (Lee *et al.* 1993, Vreugdenhil and Berezikov 2010). Since then, all human chromosomes with the exception of the Y sex chromosome has been shown to contain microRNAs (Kim and Nam 2006). MicroRNAs act as local post-transcriptional regulators of gene expression, and it has been hypothesized that up to 60% of mRNA transcripts in the body are under microRNA control (Cao *et al.* 2006, Shafi *et al.* 2010). MicroRNAs have been identified in almost all cell types, and shown to regulate a wide variety of cellular functions (Fiore *et al.* 2008). In particular, numerous miRs have been found in the central nervous system (CNS), some exclusively so, with a list of described functions which continues to expand (He 2007). Here, I will review the current literature describing microRNAs in the CNS.

Biogenesis

MicroRNAs are a family of ~22-nucleotide (nt) single-stranded RNA sequences. The mature 22 nt form is the result of multiple levels of processing (Davis-Dusenbery and Hata 2010). Initially, miRs are transcribed by RNA polymerase II or III, and the resulting primary transcript (pri-microRNA) is processed in the nucleus into a 70-100 nt hairpin precursor structure (pre-microRNA) by the double-stranded RNA-specific RNase III enzyme Drosha (Kim 2005). After export into the cytoplasm, a second RNase III enzyme, Dicer, further cleaves the pre-microRNA into a ~22 nt double stranded transcript, which contains the mature miR strand as well as a passenger strand (Davis-Dusenbery and Hata 2010). This double stranded sequence then associates with the large multi-subunit RNA-induced silencing complex (RISC), which degrades the passenger strand. This results in a mature single-stranded microRNA, which stays in association with the RISC complex, goes on to recruit proteins within the Argonaute (Ago) family to ultimately alter mRNA levels (Fiore *et al.* 2008).

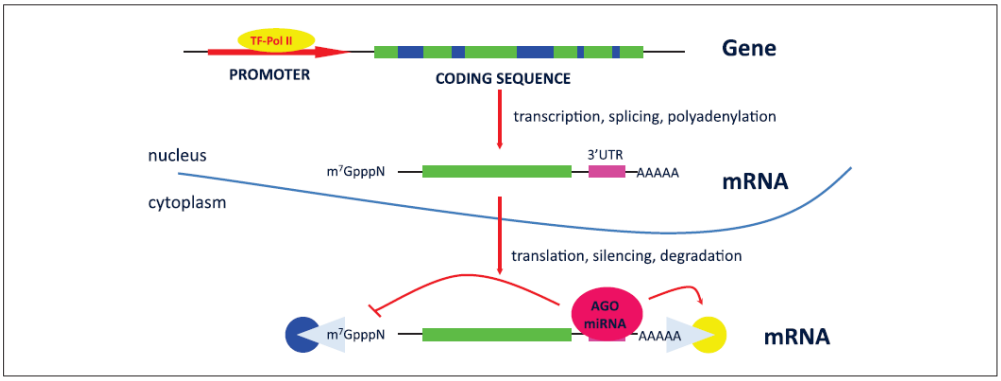

Fig 1. Role of microRNAs as a guide to 3′ UTR of target mRNA (Konopka *et al.* 2011).

mRNA processing

MicroRNAs regulate the levels of their target mRNAs by complementarily binding to the mRNA's 3' untranslated region (3'UTR) (Fig 1). While plant microRNAs require perfect

Watson and Crick base pairing with their target mRNAs, this is not the case with most mammalian miRs. Mammalian microRNAs contain a "seed region" at the 5' end that must exactly match the target, while variability in the 3' sequence is accepted. As partial complementarity is sufficient for mRNA targeting, a single microRNA has the potential to affect a large number of mRNA transcripts (Cao *et al.* 2006).

In general, microRNA binding causes a decrease in mRNA expression through a variety of mechanisms. One method is through deadenylation of the mRNAs, which targets them for degradation. This is accomplished when miRs are in the presence of processing bodies (P-bodies), which are discrete cytoplasmic domains where proteins which modulate and degrades mRNAs are localized (Hengst and Jaffrey 2007). Another method of degradation is through association with high molecular weight ribonucleoprotein particles (nRNPs) that are involved in translational control of mRNAs (Fiore *et al.* 2008). Through this interaction, the translation of the mRNA transcript is down regulated. Recently, some microRNAs have been found to activate mRNA translation, though the mechanism behind this has yet to be fully elucidated (Fabian *et al.* 2010).

Lifespan of miRs

Once processed into their mature form, some microRNAs have been shown to persist for hours to days depending on the cell type (Bhattacharyya *et al.* 2006, Hwang *et al.* 2007 and Gatfield *et al.* 2009). There is evidence for the slow turnover of certain microRNAs in the nervous system as well. Time course studies have been performed utilizing inducible Dicer deletion in neurons of mice, which eliminates new mature microRNA from being produced. MicroRNA levels were shown to decrease very gradually with time, with expression still

detectable up to nine weeks after Dicer deletion (Konopka *et al.* 2010). However, the fast processes needed for successful and dynamic neuronal responses would indicate the need for tighter regulation and turnover of microRNAs. A report from Krol *et al.* (2010) found that the turnover of a select group of microRNAs in murine neuronal cells of the retina underwent rapid turnover within tens of minutes. This was much faster than the 9 weeks previously reported, and faster than that found in non-neuronal cells as well. The authors went on to test cells within the hippocampus and cortex and found similar results (Krol *et al.* 2010). Together this could indicate two functional populations of microRNAs, one which acts to regulate the steady state functions of the neuron while the other acts dynamically to modulate neuronal responsiveness to different cues. Very little is known of the catabolism of microRNAs, and the mechanism by which neurons are able to regulate specific microRNA levels is currently unknown.

Distribution in the CNS

The importance of miRs in the nervous system is highlighted by their tightly controlled distribution throughout the body. The basis for this spatial specificity could be due to the location of miR genes within the genome. The genes for miRs-218-1 and -218-2 have been found to be located within the introns of Slit 2 and 3, and also expressed at significantly elevated levels within the hippocampus (Su *et al.* 2004, Bak *et al.* 2008). Slit 2 and 3 are proteins enriched within the hippocampus, and have been shown to be necessary for formation of the commissural axonal pathway in Drosophila as well as for neurogenesis within the mammalian CNS (Liang *et al.* 1999). Thus, perhaps in this case hippocampal enrichment can be partially attributed to co-transcription with the genes they reside within. It has also been shown that the distribution of some miRs can be regulated by post-transcriptional mechanisms. Mature miR-138 in the mouse

has been shown to be restricted to areas such as the hippocampus and cerebellum, while the pre-miR-138 is ubiquitously expressed throughout the brain (Obernosterer *et al.* 2006). As discussed below, miR expression in the nervous system can drastically change depending on the time point, area of the brain, and the subcellular location under investigation.

Development

During development, there are waves of specific miR expression. In the murine nervous system at different developmental stages, 34 out of the 66 miRs analyzed by microarray changed more than twofold between the ages E12.5 and E17.5. Twelve of these miRs had peak expression in adult tissue while nine peaked at the neonatal age of P4 (Miska *et al.* 2004, Vreugdenhil and Berezikov 2010).

Brain region

MiR profiles also change within different structures. In the adult mouse, Bak *et al.* (2008) compared the miR expression profile of thirteen distinct areas within the brain: the spinal cord, cerebellum, medulla oblongata, pons, mesencephalon, thalamus, hypothalamus, hippocampus, amygdala, neocortex, olfactory bulb, eye, and pituitary gland. They found that there is spatial specificity to miR expression, and some miRs are only found in certain regions of the brain. Others have expression throughout all of the regions studied but are enriched in certain areas. Forty-four miRs were found to have more than three-fold increased expression level from their average in one brain region. For example, miRs-10b, -7 and -7b were shown to have levels significantly elevated by more than threefold within the hypothalamus compared to the cerebellum (Bak *et al.* 2008).

Cell type

Within these different regions of the brain, cell type also dictates expression. MiRs-124 and -128 have been shown to be present specifically in neurons, while miRs-23, -26, and -29 are only found in astrocytes (Smirnova *et al.* 2005, Vreugdenhil and Berezikov 2010). This specificity could be partially due to the action of microRNAs themselves. miR-124 expression in HEK cells has been shown to down-regulate the expression of non-neuronal genes by inhibiting the transcription factor neuron restrictive silence factor (NRSF) pathway which acts to inhibit the expression of neuronally-associated genes to promote a non-neuronal identity (Visvanathan *et al.* 2007). Thus, expression of miR-124 in neuronal cells contributes to promoting the specific differentiation of neurons.

Subcellular localization

Within neurons of a given region of the brain, clusters of miRs are trafficked away from the soma, presumably in order to locally modulate mRNA levels. Using in situ hybridization and fractionation techniques, dendrites and axon terminals within the forebrain and hippocampus have been shown to contain miR profiles that are distinct from soma as well as from each other (Kye *et al.* 2007, Lugli *et al.* 2008, Olde Loohuis *et al.* 2012). In addition, a considerable fraction of miRs detected were associated specifically with ribosomes (Nelson *et al.* 2004). This raises the question of how these transcripts are recognized and subsequently targeted to a new location. One possibility to explore is mature miR binding to a target mRNA, which has a subcellular localization signal, and then trafficking with that target (Lugli *et al.* 2005). Another possibility is that specific miRNAs themselves contain sequences which target them to specific areas. This has been shown to be the case with miR-29b; this miR contains a short terminal motif which was shown to localize it to the nucleus (Hwang *et al.* 2007). It stands to reason that a similar

mechanism may be in place for the subcellular localization of miRs in neurites as well. The presence of miR processing machinery and pre-miRs in synaptodendritic fractions of neurons further supports the possibility that the trafficked molecule could be the pre- form, which would restrict the site of action for the subsequently produced mature miR and promote strict spatial control (Lugli *et al.* 2005). It is likely that more than one mechanism is in place, and the sequence of miRs and their targets will determine how trafficking is achieved (Bicker and Schratt 2008).

MicroRNAs have classically been studied in the cell body of neurons in the context of regulating neurogenesis and fate determination (Fiore *et al.* 2008). Recent studies have highlighted the important role played by miRs in axons and dendrites, and the remainder of this review will focus on literature describing the different expression and functions of microRNAs at the synapse.

Neurite growth and extension

Synaptic plasticity is dependent on the ability of a neuron to locally synthesize and modulate protein levels (Hevroni *et al.* 1998). MicroRNAs can modulate axonal and dendritic growth independent of the process of neurogenesis, likely through local modulation of mRNAs in these structures. In cultured rat hippocampal neurons, *in situ* hybridization (ISH) analysis using locked nucleic acid (LNA) probes revealed expression of the brain specific miR-134 in the neurites as well as in the neuronal cell body. Significantly, miR-134 was present in distal growth cones, co-localizing within the actin-containing lamellipodia and filopodia (Schratt *et al.* 2006). Axonal expression of miR-134 is dynamic, with progressively increasing levels until maximum expression is reached at P13, the age at which hippocampal synaptic maturation is known to

begin. The presence of miR-134 at nerve terminals negatively regulates dendrite spine density, suggesting a role in the plastic response of the neuron (Schratt *et al.* 2006). MiR-134 can also negatively regulate translation of LIM kinase 1 (LIMK1). LIMK1 is thought to be critical in successful growth cone formation by activation of its downstream targets (Meng *et al.* 2002). Over expression of the miR-134 decreased the fluorescent signal of a luciferase reporter fused to the LIMK1 transcript's 3'UTR (Schratt *et al.* 2006).

In addition, miR-34a, a neuron specific microRNA with high expression in the brain, can negatively regulate neurite outgrowth. Inhibition of miR-34a in cultured murine cortical neurons significantly increased the number of branches and overall size of the dendritic tree, while over expression caused a significant reduction in total dendrite length and branch number. Similar results were found using cultured murine hippocampal neurons. Sholl analysis, a quantitative method of analyzing various morphological characteristics of neurons, revealed decreased complexity of the dendritic tree with over expression of miR-34a (Agostinia *et al.* 2011).

Other miRs to influence dendritic growth includes miR-541, which was shown to promote dendritic growth in the model line of neuronal development PC12 (Zhang *et al.* 2011). Also, miR-132 activates the Rac signaling pathway, which in turn increases LIMK levels and results increased dendrite length and complexity (Wayman *et al.* 2008).

Axon guidance

Further work has shown that miR-134 is essential for axonal guidance in response to signaling from the growth factor brain-derived neurotrophic factor (BDNF). Under normal conditions, neurites of cultured Xenopus spinal neurons will grow towards a source of BDNF. However, miR-134 inhibition by antisense probes completely blocked this guidance in the

presence of BDNF. This blockade was specific to BDNF signaling; a similar assay using bone morphogenic protein 7 (BMP7) as a growth cue, which in immature neurons is an attractive cue while in mature neurons acts as a repellant, showed that blockade of miR-134 had no effect on guidance (Liang *et al.* 2011).

There is also evidence that miRs can indirectly influence axon guidance by modulating the neurons responsiveness to stimuli within the environment. Supporting this that, in the retina, miR-124 is necessary for the timed response to the growth factor semaphorin 3A (sema3A) (Baudet *et al.* 2011). During development, retinal ganglion cells (RGCs) alter their responsiveness to growth cues; initially, RGC growth cones are attracted to netrin-1 and are unresponsive to the presence of sema3A, but as they mature they become responsive to sema3A preferentially and lose responsiveness to netrin-1 (Campbell *et al.* 2001, Shewan *et al.* 2002). In explanted embryonic Xenopus RGC cultures, this switch to sema3A sensitivity is seen between 24 and 32 hours after plating, with full growth cone collapse by 36 hours. A similar developmental change in responsiveness is seen in many different neuronal types, and ensures the correct navigation and targeting of the growing neurites. Both cues are continually presented to the target neurons, suggesting that there are factors intrinsic to the neurons whose expressions are altered over time, acting as molecular timers in order to switch the response from attraction to repulsion. In explant cultures, knockdown of miR-124 by morpholino constructs greatly delays growth cone collapse in the presence of sema3A, with no collapse evident at 36 hours, and complete collapse not seen until 68 hours after culturing (Baudet *et al.* 2011). This indicates that, rather than intrinsically triggering neurite growth or collapse, the presence of miR-124 acts as a developmental molecular timer to modulate the interpretation of extrinsic signals.

The requirement for microRNAs in normal neurite elongation and targeting is also observed *in vivo*. During Xenopus embryonic development, RGC axons reach the optic chiasm at stage 32, the tectum at stage 37 and by stage 40 have synapsed and formed functional connections (Holt *et al.* 1989). MiR-124 levels in these neurons increase by a factor of 1.33 between stages 24 and 32, and then further increase by a factor of 1.64 between the stages 32 and 40. *In vivo* knockout of miR-124 by electroporation in Xenopus embryos showed a failure to respond to endogenous sema3A. A significant number of RGC axons did not follow the correct path in the tectum, and instead traversed the ventral tectum border to grow through sema3A containing regions. The effect on guidance *in vivo*, however, is not as pronounced as that observed *in vitro*, indicating that although miR-124 may play a role in targeting RGCs there are likely other factors that have redundant or similar functions (Baudet *et al.* 2011).

A useful *in vivo* model for the vertebrate nervous system is *C. elegans*, due to extensive and precise knowledge of its connectome and timing of growth (de Bono and Maricq 2005). The hermaphrodite specific neurons (HSNs) of *C. elegans* upregulate the UNC-40/DCC Netrin receptor at the L1 larval stage, but wait to extend neurites toward the ventral UNC-6/Netrin source until L4 (Adler *et al.* 2006). The hypothesis that a growth-independent molecular timer was the cause of this delay was further supported by the finding that *C. elegans* lacking UNC-6/Netrin or UNC-40/DCC both extend neurites at the correct stage (Adler *et al.* 2006). The miR lin-4 was identified as a candidate timer due to the growth retardant phenotype observed in loss of function studies. Sporadic neurite outgrowth was seen after L4, indicating lin-4's role in the timing rather than initiation of growth. Lin-4 has known non neuronal-specific effects through inhibition of lin-14 and lin-28 (Lee *et al.* 1993, Wightman *et al.* 1993, Martinez *et al.* 2008). The identification of lin-4 binding sites on both lin-14 and 28 indicates that this inhibition is caused

by direct binding of lin-4 (Stefani and Slack 2008). In animals mutated in either lin-14 or lin-28, neurite extension was prematurely seen at the L3 stage, without any observable effect on the subsequent steering of the axons to appropriate targets (Olsson-Carter and Slack 2010). Both lin-28 and lin-4 are conserved in vertebrates (mammalian homolog miR-125) and are widely expressed in the nervous system, suggesting this pathway might function in higher order animals as well as in *C. elegans* (Miska *et al.* 2004).

In vivo studies have also been performed using higher organisms, such as mice. MiR-124 is completely conserved from the level of worms all the way up to humans, and has been estimated to account for up to 48% of total brain miRNAs (Lagos-Quintana *et al.* 2002). Sanuki *et al.* (2011) used a knockout mouse model in order to elucidate the role of miR-124a in the brain. In the hippocampal CA3 region, knockout of miR-124a produced aberrant innervation by mossy fibers from dentate granule cell axons, producing connections which are not seen in wild-type animals. The axons from the dentate neurons in knockout mice were also significantly longer than control axons (Sanuki *et al.* 2011). MiR-124 was found to be expressed only in post-mitotic neurons, which supports the finding that normal neurogenesis is seen in the miR-124a knockout animals. MiR-124a seems to function in neurite growth and targeting rather than in fate determination.

Synapse strength

Evidence from Agostini *et al.* (2011) indicates that miRs are able to modulate the release of synaptic vesicles. When murine embryonic cortical neurons were transfected with pre-miR-34a, decreased mRNA and protein levels were found for the synaptic proteins synaptotagmin I and syntaxin IA. Synaptotagmins are calcium-binding proteins which act as calcium sensors in

the regulation of initiating synaptic vesicle release, while syntaxins are involved with the docking of vesicles at the plasma membrane for subsequent fusion (deWit *et al.* 2009). Bioinformatic analysis revealed predicted binding sites for miR-34a on the 3' UTRs of both synaptotagmin I and syntaxin IA. Similarly, over expression of miR-34a increased the protein levels of both synaptic proteins (Agostini *et al.* 2011).

Functionally, miR-34a was shown to be important for the response of neurons to stimulation. Electrophysiological analysis of cortical neurons was performed, where neurons in brain slices were voltage clamped and analyzed to see the amount of miniature excitatory post-synaptic current (mEPSCs). Over expression of miR-34a significantly reduced the frequency of detected events in the post-synaptic neuron. This data indicates a decrease in synaptic input and a loss of functional synapses as a result of over expression of miR-34a (Agostini *et al.* 2011).

This role for miR-34a was also confirmed *in vivo*. Laser-capture micro dissection was performed to remove the cerebellum, and subsequent real-time PCR analyses were used to determine the synaptic protein and miR-34a expression in each of the three distinct cerebellar layers. In layers where miR-34a was highly expressed, such as in the Purkinje layer, synaptotagmin I was significantly lower, whereas expression of synaptotagmin I was elevated in the molecular layer where decreased levels of miR-34a were detected (Agostini *et al.* 2011). Taken together, these data indicate a significant role of microRNAs synapse formation and transmission.

Activity dependent expression

Stimulation of neurons is known to alter the expression of many genes, including those which affect synaptic function (Lyons and West 2011). These changes in gene expression seem

to be specific to the type of stimulus applied; for example, stimulation with glutamate will induce an expression profile distinct from that of glycine in the post-synaptic neuron (Bading *et al.* 1993, Hardingham *et al.* 2002). The first identified activity-regulated gene was Fos, initially discovered in fibroblasts, and then extensively studied in neurons to find that it plays an important role in neurite outgrowth and plasticity (Bartel *et al.* 1989, Maze *et al.* 2010).

Recently, microRNAs have been shown to play a role in mediating the activity-dependent expression of mRNA. One of the first papers to demonstrate activity-dependent induction of miRs used cultured hippocampal neurons, and increased synaptic activity of the cultures was achieved by bath application of bicuculline, which blocks inhibitory neurotransmission. This decrease in inhibition caused an increase in dendrite outgrowth compared to control. It also caused the rapid increase in expression of miR-132. Knockout of miR-132 greatly reduced activity-induced growth, indicating that miR-132 is necessary for its occurrence. Blockade of NMDA receptors blocked the increase in miR-132 expression, further demonstrating activity dependence (Wayman *et al.* 2008).

Specific identification of mRNA transcripts whose levels are rapidly reduced due to post-transcriptional mechanisms can be accomplished in cultured neurons by stimulating synaptic activity in the presence of the transcriptional blocker actinomycin D (Cohen *et al.* 2011, Papadia *et al.* 2008). The levels of several mRNA transcripts were found to be rapidly decreased (within five minutes) in hippocampal neurons. In addition, following stimulation the brain-specific miR-485 was up regulated 1.3-fold. Subsequent motif based sequence analysis of the altered mRNA transcripts revealed that a significant number contained putative miR binding sites on the 3' UTRs of these transcripts (Cohen *et al.* 2011).

Under extended periods of high-frequency stimulation, enhancement of synaptic strength and signaling can be seen in the post-synaptic neurons long after the cessation of the increased stimulation. This phenomenon is known as long-term potentiation (LTP). At the CA3-CA1 synapse in the hippocampus, LTP involves an increase in the synthesis and membrane insertion of AMPA receptors, the fast-acting receptors for the excitatory neurotransmitter glutamate. Under normal conditions, increasing synaptic activity for an extended period of time will cause an increase in dendritic spine formation and an increase in AMPA density (Kessels and Malinow 2009). In these neurons, miR-485 over expression significantly reduced AMPA receptor trafficking to the cell surface, visualized by transfecting hippocampal neurons with the AMPA receptor subunit GluR2 fused to a pH-sensitive analog of green fluorescent protein (GFP). When hippocampal neurons were stimulated following knockout of miR-485, there was a significant reduction in AMPA receptor density as well as in dendritic spines number compared to control (Cohen *et al.* 2011). It is possible that the upregulation of miR-485 normally seen after stimulation functions to downregulate transcripts which would inhibit synaptic plasticity.

A potential mechanism for activity-induced changes in microRNA expression is due to changes in the local processing of pre-miRs into mature forms dependent upon the level of stimulation. Dicer has been shown to be present in the synaptodendritic fraction of neurons where it likely acts to control local synthesis of mature miRs. Lugli *et al.* (2005) demonstrated that, in addition to functional Dicer, murine neurons also contained a larger and enzymatically inactive form of Dicer. Significantly, this form was seen in high levels in dendritic spines. Upon synaptic stimulation, the inactive form of Dicer underwent cleavage into an enzymatically active form in a calcium-dependent manner, thus promoting the synthesis of mature miRs in post-synaptic densities (Lugli *et al.* 2005).

Specific induction of LTP also alters miR expression. Generation of LTP in the CA1 region of hippocampal slices was achieved by application of ACSF with 0 mM $MgSO_4$, 5 mM $CaCl_2$, and 25 mM tetraethylammonium (TEA). Microarray analysis showed that most microRNA levels were increased after LTP induction (Park and Tang 2009). In addition, the authors investigated long-term depression (LTD) in these neurons. LTD is the process by which low-frequency stimulation is able to cause a sustained reduction in output from the post-synaptic cell. Induction of LTD by bath application of ACSF with 100 μM (S)-3,5-dihydroxyphenylglycine (S-DHPG) demonstrated that while almost all of the microRNAs involved in LTP are also involved in LTD, the induced expression profile is distinct between the two (Park and Tang 2009).

Very limited evidence showing activity-dependent microRNA expression *in vivo* is available. One of the first to look at this investigated LTP in the hippocampus (Park and Tang 2008). In rats, high frequency electrical stimulation was used to induce LTP in the dentate gyrus (Wibrand *et al.* 2010). Two hours after induction, microarray profiling and subsequent validation by rt-PCR confirmed a 50-fold increase in miR-132 and -212 precursors within dentate neurons. This up regulation was a transcription-dependent increase rather than a decrease in degradation. However, the significance for such an increase *in vivo* was not explored (Wibrand *et al.* 2010).

Levels of different microRNAs can either increase or decrease in response to the same stimuli. In the retina it was found that pharmacological activation of glutamate receptors induced the upregulation of miRs-124, -128, -134 and -138, while miR-132 levels were decreased (Krol *et al.* 2010). Important to note is that the presence of a microRNA at the synapse does not necessarily imply that its transcription is altered depending on outside stimulation.

Functional Memory

The modification of synaptic strength and structure is a basic mechanism for encoding and storing memory. The processes of LTP and LTD are thought to be critical to learning, where important connections are stabilized while unimportant ones are weakened (Bailey *et al.* 2004). The pronounced effect of microRNAs on mRNA regulation, protein synthesis, and synaptic plasticity summarized here raises the question of whether microRNAs play a role in higher order behavioral functions such as learning and memory.

The first study to draw a direct connection between microRNAs and memory formation utilized an inducible disruption of the Dicer gene specifically within the forebrain of mice, and asked whether this decrease in local regulation of mRNA transcripts influenced the functional consequences of synaptic plasticity. Mice deficient in Dicer performed significantly better than control mice in place learning tasks, and displayed increased levels of fear conditioning, a process that is known to be hippocampus-dependent (Crestani *et al.* 2002, Konopka *et al.* 2011) In addition, post-tetanic potentiation, a transient increase in a neuron's synaptic strength following strong stimulation, was enhanced in the knockout. Increased number and surface area of dendritic spines is also a hallmark in memory formation, and these hippocampal neurons displayed abnormally long spines on their dendrites (Konopka *et al.* 2011, Bredy *et al.* 2011).

Multiple individual miRs have since been investigated in the context of learning and memory. For example, the activity dependent induction of miR-132 causes a subsequent increase in incorporation of the highly glutamate-sensitive NR2B subunit into the NMDA receptor, as well as decrease the presence of the in the less glutamate-sensitive NMDA subunit NR2A. This process has been shown in other studies to be critical for learning (Edbauer *et al.* 2010, Hansen *et al.* 2010). On the other hand, over expression of miR-132 *in vivo,* while increasing spine

density, hinders rather than increases the formation of novel recognition memories (Hansen *et al.* 2010). This apparent contradiction highlights the complex balance that must be achieved to encourage learning, in addition to the need for a more thorough characterization of the mechanism of microRNA action in the nervous system (Bredy *et al.* 2011).

Addiction

A large number of microRNA studies focused on investigating functional learning have been performed using drug addiction as a model. Addiction to drugs is characterized by the formation of novel synapses and changes in network connectivity. Tolerance to a specific drug is the result of decreased circuit activity after repeated exposure to a stimulus, resulting in activation of other molecular pathways to signify withdrawal (Picciotto 2010). These pronounced alterations in the brain and the resulting drug-seeking behavior represents a powerful form of learning.

MicroRNAs are known to alter the learned behaviors associated with cocaine addiction. Knockout of the Argonaute 2 (Ago2) subunit of the RISC complex significantly reduces motivation for cocaine self-administration in animals with intermittent access, indicating the need for microRNAs in the process of learning that cocaine causes pleasure (Schaefer *et al.* 2010). Specific miRs have also been investigated. For example, miRs-324 and -369 regulate the expression of MEF2 and FosB, respectively (Pulipparacharuvil *et al..*, 2008). MEF2 and FosB are known to be necessary for learning cocaine-seeking behavior in mice (Renthal *et al.* 2009). In addition, microRNAs miR-124, let7d and miR-181 are upregulated in the nucleus accumbens in response to cocaine. Control animals given cocaine in a specific location within a chamber learn to associate the drug with that location and preferentially reside there. Knockdown of each

of these three miRs resulted in animals that were deficient in conditioned place preference (Chandrasekar and Dreyer 2011).

MicroRNAs have also been found to inhibit the process of addiction, and thus inhibit plastic memory formation. For example, increasing miR-212 expression reduces cocaine intake when animals have extended, continuous access to the drug. The authors go on to show that miR-212 expression results in an upregulation in the activity of the small GTPase Raf1, which stimulates the cAMP-CREB pathway to limit cocaine intake (Hollander *et al.* 2010). CREB is also downstream of other microRNAs, such as miR-134, to mediate plasticity and increase functional memory formation of non-drug associated events *in vivo*. Over expression of miR-134 leads to a down regulation of CREB which restricts new memory formation (Gao *et al.* 2010). The differential activity of the CREB pathway in both promotion and suppression of memories cautions against generalizing the mechanisms of microRNA-mediated learning that are obtained from drug studies.

Diseases

Many diseases of the CNS are characterized by location-specific synaptic dysfunction and circuit remodeling (Schratt 2009). MicroRNA involvement has been hypothesized due to their ability to modulate many targets within cells, and may also prove to be useful as prognostic biomarkers (Kim and Nam 2006, Fiore *et al.* 2008). Some of the first evidence of microRNA involvement in neurodegenerative disorders was the observation that Dicer knockout in the striatum of mice in early development caused a slow progression of neurodegeneration and apoptosis (Kim *et al.* 2007, Cuellar *et al.* 2008). Since then, microRNAs have been investigated

and implicated in numerous spectrum disorders affecting brain connectivity and cognitive function.

Schizophrenia

Schizophrenia is a multifaceted illness which affects a wide variety of cognitive functions, including perception, memory, and personality (Glausier and Lewis 2012). It is characterized by global changes in brain connectivity and synaptic remodeling. The cause(s) of schizophrenia are currently unknown, although some evidence suggests a genetic predisposition; micro-deletion of chromosome 22q11.1 has been correlated with a significantly higher risk of developing schizophrenia. (Stark *et al.* 2008) However, it is also clear that there is a developmental switch that is necessary for its induction (Glausier and Lewis 2012). Currently, the pharmacological treatment for schizophrenia is antipsychotic drugs such as haloperidol and clozapine. However, it is clear that these medicines do not target the cause of the disease, and only aid in alleviating a handful of the more severe symptoms (Beveridge and Cairns 2012).

Post-mortem studies of brains from schizophrenic individuals have shown altered levels of many miRNAs in the prefrontal cortex (Perkins *et al.* 2007). One of the first studies of miRs in schizophrenia identified a significant correlation between the presence polymorphisms within the miRs-198 and -206 and the disease (Hansen *et al.* 2007). However, the causal link between the two has not been established. Other studies have made the connection more clearly between microRNA dysregulation and behavioral manifestations indicative of schizophrenia. Glutamate receptor subunit gene GRID1, which is down regulated in schizophrenia, contains miR-346 within one of its introns (Zhu *et al.* 2009). The genes involved with the 22q11.2 locus were investigated, and it was found to encode the Drosha interacting protein Dgcr8. Dgcr8 has been shown to increase Drosha activity by 8-fold, and impairment of this protein impairs the

conversion of pri-microRNAs to pre-microRNAs almost as effectively as Drosha knockdown (Loohuis *et al.* 2012). Mice heterozygous for Dgcr8 were not only deficient in miR processing but also displayed marked reduction in dendritic tree complexity and a reduction the size of dendritic spines. Behavioral studies went on to reveal functional cognitive defects in these animals such as hyperactivity. In addition, the NMDA-activity inducible miR-219 caused altered behavioral responses when knocked out in the prefrontal cortex, and these behavioral deficits are preventable by treatment with antipsychotics (Kocerha *et al.* 2009).

Depression

Depression is a spectrum mood disorder which affects over 121 million people worldwide, and like schizophrenia is thought to be caused by a combination of both genetic and environmental factors (Moulleit-Richard *et al.* 2012). Many genes have been found to be dysregulated during depression, including the gene for BDNF and the gene encoding the serotonin transporter SERT (Levinson 2006). A common treatment for depression is the administration of drugs such as Prozac to elevate mood by inhibiting SERT, thus increasing the amount of time serotonin is allowed to act on the post-synaptic cell. MiR-16 functions normally as a repressor of SERT *in vivo* (Mouillet-Richard *et al.* 2012). Administration of chronic levels of Prozac in mice was shown to increase the level of miR-16 in the raphe nucleus while simultaneously decreasing SERT levels, thus increasing serotonin levels (Aqeilan *et al.* 2010). In addition, multiple miR targeting sites have been identified on the 3'UTR of BDNF's mRNA sequence. Human post-mortem tissue displays an inverse relationship between miRs-30a and-195 and BDNF protein levels. Follow-up experiments found that these miRs are able to directly bind to and reduce the levels of BDNF transcripts (Mellios *et al.* 2008).

Bipolar disorder

Bipolar disorder is characterized by intense mood swings which vary in intensity between individuals. It is defined by the presence of manic events, or episodes of elevated energy and mood, followed by depressive episodes. During severe manic episodes symptoms also include impaired judgment and decreased attention span, and can result is psychotic episodes such as hallucinations and delusions. This severe instability, if not treated, can endanger both affected individuals as well as others around them. Additionally, individuals who go untreated show a negative correlation between number of manic episodes and cognitive function (Schneider *et al.* 2012). MicroRNA profiling has shown altered expression of miRs whose predicted targets are known players in the pathology of bipolar disorder, such as BDNF (Chen *et al.* 2009).The most frequently prescribed medication for bipolar disorder is lithium which affects many processes throughout the body, involving effects on extracellular signal regulated kinase (ERK), BDNF, and glycogen synthase kinase 3 (GSK-3β). The miR profile is also altered following lithium treatment, and also following another mood stabilizer valproate. From this screen, elevated miR-34a was specifically shown to negatively regulate metabotropic glutamate receptor 7 (GRM7) following lithium treatment (Zhou *et al.* 2009). Also, plasma miR-134 was elevated during episodes of mania, and the authors hypothesize that miR-134 may be involved in the induction of these extreme changes in mood (Rong *et al.* 2011). MiR-132 levels were reduced in the prefrontal cortex of post-mortem tissue taken from bipolar patients (Miller and Wahlestedt 2010). MiR-132 in known to be able to influence BDNF levels, which may alter the pathogenesis of the disorder (Mellios *et al.* 2008). However, a more causal relationship between the disease and miRs has yet to be established.

Stress component of mood disorders

Stress is an important component in triggering mood disorders. The response of the body to stress is mediated by many intermediates. One of the most important class of molecules is the glucocorticoids, which are steroid hormones secreted from the adrenal cortex and alters neural function and architecture (Hunsberger *et al.* 2009). In a model of chronic stress achieved by intermittent restraint, it was found that 13 microRNAs were upregulated while only three were downregulated in the hippocampus (Leung and Sharp 2007).

Tourette's syndrome

Tourette's syndrome is an inherited disorder diagnosed by the presence of various persistent motor and vocal tics (Robertson 2003). While Tourette's itself is not life threatening and is not associated with a decrease in intelligence, it often has comorbid conditions such as attention-deficit disorder and obsessive-compulsive disorder which further decreases societal functionality (Bicker and Schratt 2008). A candidate protein involved in the pathology of Tourette's is Slit and Trk-like 1 (SLITRK1), which has known functions for growth and guidance of neuronal processes (Aruga and Mikoshiba 2003). Decreased levels of SLITRK1 have been seen in the brains of post-mortem patients with Tourette's. Studies have shown that mutations in miR-189, which has been shown to bind to SLITRK1's 3'UTR, are significantly correlated with the appearance of Tourette's in humans. The base pair exchanges seen results in stronger binding between miR and SLITRK1, which would decrease levels of the protein (Abelson *et al.* 2005). The mutations seen in miR-189 within the affected group were not seen in any individuals in the control study group.

Epilepsy

Epilepsy is a disorder characterized by the episodic occurrence of seizures, resulting from the aberrant activation and coordinated firing of neurons in the brain. In children, severe seizure activity can cause long-term brain damage, and cause the development of aberrant synaptic connections in the brain (Holmes and Ben-Ari 2001). In humans, miR-124a-1 is located on the chromosome region 8p23, an area that contains genes implicated in neurological disorders including epilepsy (Tabarés-Seisdedos and Rubenstein 2009). As discussed earlier, miR-124a knockout in mice results in mossy fiber sprouting and abnormal innervations patterns in the CA3 region of the hippocampus (Sanuki *et al.* 2011). This type of mossy fiber sprouting is seen in models of temporal lobe epilepsy (Koryama and Ikegaya 2004). Taken together, this implicates miR-124a dysfunction as a potential mechanism for the occurrence of epileptiform activity.

Considerations

Methods of Detection

A basic need in any scientific study is the assurance that the method of biological detection used is both consistent and reliable. Ideally, an assay should be relatively inexpensive, simple to perform and require small amounts of sample to give dependable readouts. The current technology used to study microRNAs represents a huge barrier in interpreting current literature regarding their role and function (Takeda and Mano 2007). Multiple characteristics of the microRNA molecules make them intrinsically difficult to study. Their variable structure, which lacks a common sequence such as a poly-A tail, and extremely small size means measures that rely on primer binding can be relatively inefficient. There is a high degree of false-positive

detection, where one miR is mistakenly pulled out of a sample when assaying for another, due to the relatively small sequence differences (de Planell-Saguer and Rodicio 2010).

The most common methods of detection for miRs include high-throughput sequencing methods (e.g., microarray analysis), Northern blot, PCR, and in situ hybridization (De Panell-Sauguer and Rodicio 2010). Northern blots, while the most cost effective, have the disadvantage of relatively low sensitivity, and detection requires microRNA samples within the nanomolar range (Chamnongpol *et al.* 2010). In general, microRNA concentration compared to total RNA amount within a sample is extremely low (De Panell-Sauguer and Rodicio 2011). Detection by *in situ* hybridization is much more sensitive, allowing the spatial localization as well as relative amount of microRNA within a cell to be determined. Like the Northern blot, however, due to inefficient probe binding this method can be challenging (Chamnongpol *et al.* 2010). The advent of locked nucleic acid (LNA) probes has considerably improved detection by both of these methods, as LNA probes are able to distinguish between single nucleotide differences, and their increased stability allows for more stringent binding conditions to be applied (Várallyay *et al.* 2008, de Planell-Saguer *et al.* 2010). Normal PCR methods represent perhaps the most reliable form of microRNA detection, though standard reverse-transcriptase or quantitative PCR must be used on the much longer pre-transcript in order to provide accurate readouts. Detection using quantitative and reverse-transcriptase PCR methods has a high degree of reproducibility when the same sample is run at different times (Chen *et al.* 2009). New forms of PCR utilizing SYBR Green and LNA-modified primers have been shown to be able to detect mature microRNA levels with relatively high precision (Raymond *et al.* 2005). Microarray technology is invaluable in its ability to screen a sample for a large number of microRNAs at one time. The lack of reliable quantification of expression inherent in the technique along with a relatively high degree of

false-positive readings requires the validation of microarray data with another technique such as quantitative PCR (qPCR) (De Panell-Sauguer and Rodicio 2011). Expectedly, qPCR was found to be more reliable than microarray analysis of miRs (Chen *et al.* 2009).

An additional barrier to interpreting microRNA studies is the fact that each of the above methods requires some degree of normalization. While numerous normalization techniques are described in microRNA literature they are inconsistent with each other. It is difficult to compare the results of one study with another if there is no consistent method of normalizing data (Meyer *et al.* 2010). Additionally, a consistent quantification method of these techniques in order to allow the determination of relative copy number of a microRNA molecule in comparison to total/target RNA is critical to the ability of researchers to determine the importance of a microRNA molecule in a system (Shafi *et al.* 2010).

Prediction of miR targets is one of the most important aspects of the field, and also the most inconsistent. Multiple bioinformatic programs have been developed to predict the potential mRNA transcripts to which miRs can bind. The results from different software do not agree with each other. Figure 2 depicts the miRs which are predicted to target the AMPA receptor subunit GluR1 using three different bioinformatic software systems. Startlingly, while many miRs was predicted by each program, none were predicted by all three (Vo *et al.* 2010).

Consistency within the field is necessary in order to move forward. While some of the predicted targets of miRs have been validated following microarray data, there are a large number that still have not been investigated. In addition, relatively few studies have looked into downstream effectors and consequences of miR expression. Greater effort needs to be taken to understand the significance, if there is any, of individual miRs within these systems.

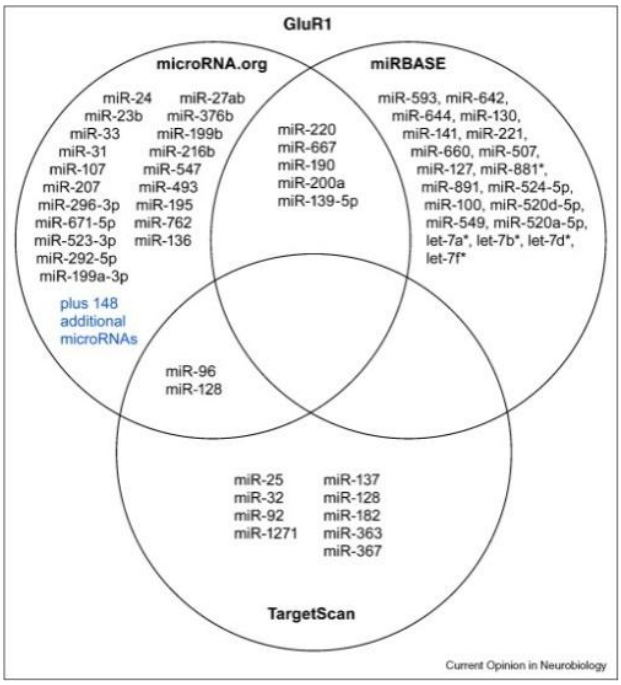

Fig 2. Schematic of microRNAs which are predicted to target GluR1 using three different bioinformatic screens (Vo *et al.* 2010).

Modeling microRNA function

There are only a handful of studies which explore the consequences of miRs at the synapse *in vivo*. Previously, this has been hampered by the difficulty and expense of producing knockout mice. Even after generation of knockout mice, it is likely that a compensatory mechanism may overshadow the phenotype and function of the microRNA under study. The knockout would also ideally be inducible and conditional, to tease out potentially confounding developmental and organ specific roles. New advances in microRNA inhibitors allow single microRNAs to be altered without the need of a specific genotype of mouse. For example, Hollander *et al.* (2010) successfully used direct injection of antisense LNA oligonucleotide probes targeting a specific miR into the striatum of mice. For over expression studies, they used

direct injection of lentiviral vector. Behavioral studies are essential to draw conclusions on the function of miRs in the brain.

When studies investigating microRNAs *in vivo* are performed, it is important to be aware of the animal used for modeling. One study found that in a model of restraint stress miR-18a down regulated the glucocorticoid receptor (GR) in Fischer 344 rats, but no effect was seen when Sprague-Dawley rats were used. Fischer 344 rats also exhibited more prominent anxiety following restraint stress than Sprague-Dawley rats (Ushida *et al.* 2008). Almost none of the studies in microRNAs in the brain have been validated using more than one rat or mouse strain in parallel, so it is essential to be aware that all animal models do not behave identically.

Conclusion

The importance of microRNAs in synaptic function is just beginning to be elucidated. Many studies show that microRNA profiles are not only present at pre- and post-synaptic densities but are also altered in disease states and normal environmental cues. Future work in this area will yield novel insights into how the central nervous system works.

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