**miR-7 Buffers Differentiation in the Developing Drosophila Visual System**

**Highlights**
- miR-7 promotes neuroblast formation during optic lobe development
- miR-7 targets the Notch pathway
- miR-7 buffers the effects of environmental stress
- Without miR-7, timely neuroblast production is disrupted

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**In Brief**
Caygill and Brand show that miR-7 buffers optic lobe neural stem cell production to ensure that a precise and stereotypical pattern is maintained, even under conditions of environmental stress. These results echo the role that miR-7 plays in the eye imaginal disc, emphasizing the importance of robust visual system development.

Caygill & Brand, 2017, Cell Reports 20, 1255–1261
August 8, 2017 © 2017 The Authors.
http://dx.doi.org/10.1016/j.celrep.2017.07.047
miR-7 Buffers Differentiation in the Developing Drosophila Visual System

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http://dx.doi.org/10.1016/j.celrep.2017.07.047

SUMMARY

The 40,000 neurons of the medulla, the largest visual processing center of the Drosophila brain, derive from a sheet of neuroepithelial cells. During larval development, a wave of differentiation sweeps across the neuroepithelium, converting neuroepithelial cells into neuroblasts that sequentially express transcription factors specifying different neuronal cell fates. The switch from neuroepithelial cells to neuroblasts is controlled by a complex gene regulatory network and is marked by the expression of the proneural gene /sc. We discovered that microRNA miR-7 is expressed at the transition between neuroepithelial cells and neuroblasts. We showed that miR-7 promotes neuroepithelial cell-to-neuroblast transition by targeting downstream Notch effectors to limit Notch signaling. miR-7 acts as a buffer to ensure that a precise and stereotypical pattern of transition is maintained, even under conditions of environmental stress, echoing the role that miR-7 plays in the eye imaginal disc. This common mechanism reflects the importance of robust visual system development.

INTRODUCTION

Drosophila vision requires the accurate specification of over 80 different types of optic lobe neurons and the establishment of precise visual circuits between the neurons of the optic lobe and the photoreceptors of the eye. The medulla is the largest visual ganglion of the brain. Medulla neurons play roles in motion detection, through input from the R1–R6 photoreceptors via the lamina, and in the perception of color, via direct input from the R7 and R8 photoreceptors (Morante and Desplan, 2008). The 40,000 medulla neurons originate from a pseudostratified neuroepithelium (Egger et al., 2011; Sato et al., 2013). During early development, symmetric division expands the stem cell pool. As development progresses, the medial edge of the neuroepithelium is progressively converted into asymmetrically dividing neuroblasts (Figure 1A) (Egger et al., 2010; Yasugi et al., 2010). Medulla neuroblasts sequentially express a series of transcription factors that specify the differentiation of the medulla neurons (Li et al., 2013; Suzuki et al., 2013).

RESULTS

miR-7 Is Expressed at the Transition Zone in the Optic Lobe

Previously we used Targeted DamID to profile gene expression in neuroepithelial cells and neuroblasts of the developing larval optic lobe (Southall et al., 2013). A comparison of the genes that were highly enriched in either neuroepithelial cells or neuroblasts identified genes that may play roles in regulating the transition from neuroepithelium to neuroblasts. We found that hnRNP-K/miR-7 expression was enriched in neuroepithelial cells (Figure 1C).

The highly conserved microRNA miR-7 is produced from the primary transcript of heterogeneous nuclear ribonucleoprotein K (hnRNP-K) in both Drosophila (Li and Carthew, 2005) and humans (Choudhury et al., 2013). miR-7 is predicted to target multiple members of the Notch pathway. The ability of miR-7 to regulate Notch signaling was of interest, as Notch signaling is known to be essential to maintain neuroepithelial cells, and...
downregulation of Notch activity is required for transition to neuroblasts (Egger et al., 2010; Ngo et al., 2010; Orihara-Ono et al., 2011; Wang et al., 2011; Yasugi et al., 2010). We hypothesized that miR-7 plays a role in regulating Notch activity at the transition zone.

To investigate miR-7 function at the transition zone, we determined the precise expression pattern of hnRNP-K/miR-7 in the neuroepithelium. Examination of the miR-7 enhancer driving GFP (miR-7E-GFP) (Li et al., 2009), revealed expression in the transition zone that overlapped precisely L'sc expression (Figures 1D and 1D0), suggesting that miR-7 is upregulated in the transition zone. We confirmed this expression pattern using single-molecule fluorescence in situ hybridization (smFISH) against the hnRNP-K/miR-7 transcript (Figures 1E and 1E0). We observed a band of increased hnRNP-K/miR-7 transcription that overlaps the transition zone, marked by the downregulation of E(spl)m-gamma-GFP (Almeida and Bray, 2005; Egger et al., 2011). Similar to the expression of (miR-7E)E-GFP in the eye imaginal disc (Li et al., 2009), expression of hnRNP-K/miR-7 in the optic lobe was positively regulated by EGF signaling (Figure S1). This highly specific upregulation of hnRNP-K/miR-7 transcript at the transition zone suggested a role in regulation of the transition of neuroepithelial cells to neuroblasts.

miR-7 Is Sufficient to Promote the Transition from Neuroepithelial Cells to Neuroblasts and Is Necessary for Robust Transition

To investigate the role of miR-7 at the transition zone, we generated a UAS-miR-7 construct and drove its expression in the neuroepithelium using c855a-GAL4 (Manseau et al., 1997). Misexpression of miR-7 in neuroepithelial cells resulted in disruption of the neuroepithelium and ectopic neuroblast formation (Figures 2A and 2B), similar to what was seen when Notch activity was downregulated throughout the neuroepithelium (Egger et al., 2010). To assess more precisely the effects of miR-7 misexpression, we generated clones of cells expressing miR-7. Clones that spanned the transition zone showed premature neuroblast formation (Figures 2C–2C0). Therefore, miR-7 is sufficient to convert neuroepithelial cells into neuroblasts.

The available miR-7 allele is a 6.8-kb deletion generated by P-element excision that partially deletes both hnRNP-K
and the downstream gene Hillarian (Li and Carthew, 2005). To investigate the effect of loss of miR-7 without disrupting hnRNP-K or Hillarian, we generated a new miR-7 CRISPR allele (Figure S2). miR-7CRISPR1 is a 13-bp deletion that removes the 5’ 12 nt of the 23-nt miR-7 (Figure 2D) without disrupting neighboring genes. miR-7CRISPR1 homozygous mutants were both viable and fertile. Interestingly, homozygous mutants did not display defects in wing development that had previously been attributed to loss of miR-7 function (Aparicio et al., 2014). Therefore, it is likely that the reduced wing size observed was due to the disruption of hnRNP-K, which has been reported to display reduced cell division and increased apoptosis in imaginal discs, resulting in small adult appendages (Charroux et al., 1999). These results highlight the necessity of generating small targeted deletions when investigating the biological functions of microRNAs and, in particular, the advantage of using CRISPR for miRNA mutation.

To assess whether miR-7 was necessary for the neuroepithelial-to-neuroblast transition, we generated miR-7CRISPR1 mutant clones that cross the transition zone (Figures 2E–2E’). 30% of mutant clones showed a delay in transition and an autonomous increase in the width of the L’sc-positive cells (n = 36). In control clones, only 2.85% (n = 35) showed any cell-autonomous effects on transition. Interestingly, clones that expressed a constitutively active form of Notch also displayed a delay in the onset of neuroblast formation and prolonged expression of L’sc (Yasugi et al., 2010). These results show that miR-7 plays a role in regulating the timing of the proneural wave and suggest that it may act by negatively regulating Notch signaling.

**miR-7 Targets E(spl)m-gamma at the Transition Zone**

Predicted miR-7 binding sites can be found in the 3’ UTRs of many genes encoding downstream effectors and modulators of Notch pathway activity, including members of the Enhancer of split complex (E[spl]-C) and Bearded complex (Brd-C) (Kheradpour et al., 2007; Lai et al., 2005; Robins et al., 2005; Ruby et al., 2007). One of these, E(spl)m-gamma, is known to be expressed in the neuroepithelium (Egger et al., 2011), intriguingly, in a pattern reciprocal to that of hnRNP-K/miR-7 expression (Figure 1E’). To examine whether miR-7 targets E(spl)m-gamma in vivo, we took advantage of the E(spl)m-gamma-GFP reporter, a genomic fragment containing GFP cloned in frame, 19 amino acids (aas) from the end of E(spl)m-gamma.
Expression of UAS-miR-7 in clones was able to autonomously downregulate E(spl)m-gamma-GFP at the transition zone (Figures 3A and 3A'). Loss of miR-7 in miR-7CRISPR1 mutant clones resulted in a disruption of the normal E(spl)m-gamma-GFP pattern in a subset of clones, consistent with the observed delay in transition (Figures 3B and 3B'). Together, these results confirm that E(spl)m-gamma is a target of miR-7 at the transition zone and that miR-7 represses a downstream effector of Notch signaling at the transition zone.

**mir-7 Regulates Notch Effectors to Ensure Timely Transition**

We have shown that miR-7 is able to repress E(spl)m-gamma at the transition zone (Figure 3). miR-7 is predicted to target a number of other basic-helix-loop-helix (bHLH) transcription factors and Brd genes of the E(spl) complex and Brd complex (Kheradpour et al., 2007; Lai et al., 2005; Ruby et al., 2007). To determine which predicted targets miR-7 regulates at the transition zone, we performed genetic suppression experiments. We reasoned that, in the absence of miR-7, the levels of its target gene(s) should increase and that this increase could be suppressed by reducing the gene dose of the target gene(s). We examined proneural wave progression in the absence of miR-7 in a background heterozygous for deficiencies that delete large parts of either the E(spl) or the Brd complex (Chanet et al., 2009). While loss of one copy of Df(3)Brd-C1 did not change the severity of proneural wave disruption relative to loss of miR-7 alone (p = 0.50, Fisher’s exact test) (Figures 4C and 4H), loss of one copy of the Df(3)E(spl) control and miR-7<sup>CRISPR1</sup> mutant larvae to temperature stress, shifting developing larvae between 18°C and 31°C every 2 hr for 2 days. Under normal laboratory conditions, the absence of miR-7 resulted in the sporadic disruption of the progression of the proneural wave. L’sc-positive cells, which are normally restricted to the transition zone, could be observed trailing behind the wave, either still adjacent to the wave (interpreted as a weak phenotype) or completely isolated from the wave (interpreted as a strong phenotype) (Figures 4B and 4C). Temperature stress increased the severity of the disruption of proneural wave progression in miR-7<sup>CRISPR1</sup> mutant brains (p = 0.049, Fisher’s exact test) but had no effect on control brains (p = 1, Fisher’s exact test) (Figures 4A–4C). This increase in severity of phenotype observed under temperature stress demonstrates that miR-7 buffers the neuroepithelial-cell-to-neuroblast transition.

**mir-7 Buffers the Neuroepithelial-Cell-to-Neuroblast Transition**

Although expression of miR-7 was sufficient to promote the neuroepithelial-to-neuroblast transition, we only observed a delay in transition in 30% of miR-7<sup>CRISPR1</sup> mutant clones (Figures 2E–2E'), suggesting that transition can occur correctly in the absence of miR-7 and that only under certain circumstances does the loss of miR-7 affect timely neuroblast production. This raised the possibility that miR-7 could be acting as a biological buffer in the developing optic lobe, sufficient to promote transition but necessary only under conditions of physiological stress. miR-7 had been identified previously as a buffer in photoreceptor and proprioceptor determination (Li and Carthew, 2005; Li et al., 2003). To test whether miR-7 buffers the neuroepithelial-to-neuroblast transition, we subjected
Delta-6 was able to suppress the proneural wave disruption observed in miR-7\textsuperscript{CRISPR1} mutant brains under temperature stress (p = 0.002, Fisher’s exact test) (Figures 4C and 4G). This shows that deregulation of targets within the E(spl)-complex is responsible for the observed defect in proneural wave co-ordination seen in miR-7\textsuperscript{CRISPR1} mutants. Therefore, regulation of members of the E(spl) complex by miR-7 is necessary for buffering the transition from neuroepithelial cells to neuroblasts against environmental stress.

**DISCUSSION**

We have shown here that miR-7 targets members of the E(spl) family of bHLH transcription factors to define the boundaries of the transition zone, buffering the transition from neuroepithelial cell to neuroblast in the developing optic lobe. Downregulation of Notch signaling is essential for the neuroepithelial-to-neuroblast transition (Egger et al., 2010; Yasugi et al., 2010). L’sc provides one mechanism for Notch downregulation (Egger et al., 2010). L’sc, expressed in a pattern similar to that of L’sc at the transition zone, provides another, emphasizing the importance of Notch regulation at the transition zone.

The transition zone of the proneural wave mediates the specification of the neuroblasts of the medulla, the largest ganglion of the adult Drosophila visual system (Yasugi et al., 2008). The medulla receives information directly from the R7 and R8...
photoreceptors of the ommatidia. Similar to the specification of neuroblasts by the proneural wave in the optic lobe, photoreceptor differentiation is triggered by the movement of the morphogenetic furrow across the epithelium of the eye imaginal disc (Sato et al., 2013). As the furrow passes, expression of the proneural gene atonal (ato) is induced in a stripe that is later refined to the R6 cells by Notch-mediated lateral inhibition (Jarman et al., 1994).

Similar to our observations in the optic lobe, miR-7 has been shown to play a role in photoreceptor differentiation (Li et al., 2009). Misexpression of miR-7 results in an increase in Ato expression and R8 cell specification, while a loss of miR-7 results in a decrease in Ato expression under conditions of temperature stress. These results show that miR-7 acts to buffer the development of both the medulla and the eye, two tissues that will directly communicate in the adult brain.

miR-7 has been shown to target anterior open (aop; also known as yan) in the eye imaginal disc (Li and Carthew, 2005). Within the neuroepithelium, aop activity helps to repress the neuroepithelial-to-neuroblast transition (Wang et al., 2011). This raises the possibility that miR-7 targeting of aop could also contribute to its function in buffering the neuroepithelial-to-neuroblast transition and that aop could represent a common target during the progression of the proneural wave and the morphogenetic furrow.

In the adult brain, each ommatidium maps to a columnar unit within the lamina and medulla, providing a retinotopic map of the visual field. Signaling from innervating photoreceptors induces the differentiation of lamina neurons. This direct communication provides a strict control of the mapping of photoreceptor and lamina neuron numbers (Umetsu et al., 2006). In contrast, while final numbers of ommatidia and medulla neurons show some co-ordination based on nutrient availability (Lanet et al., 2013), there is no evidence for direct communication between the eye disc and the developing medulla. The presence of miR-7 in both the eye imaginal disc and the optic lobe represents an independent but conserved buffer that operates to coordinate appropriate developmental progression in each system, in spite of external environmental fluctuations. The presence of this common buffer provides robustness within each system that may contribute to ensuring the eventual connectivity required for retinotopic mapping of the visual system.

EXPERIMENTAL PROCEDURES

Fly Strains

The following strains were generated: (miR-7)E > GFP (Li et al., 2009), m-gamma-GFP (Almeida and Bray, 2005), c55a-GAL4 (Manseau et al., 1997), w; Df(E(spm)delta-m6 X. DfBrd-C14/Rb600084/TM6B (Chenet et al., 2009), w1118; Df(1)Brd-C1/TM6B, To (Chenet et al., 2009), UAS-miR-7 (this study), miR-7CRISPR (this study), and FRT42D miR-7CRISPR (this study). Clones were generated with the MARCM lines: y w hsFLP; FRT40A tub-GAL80/Cyo; tub-GAL4/TM6, y w hsFLP tub-GAL4 UAS-GFPnls/FM7; FRT42D tub-GAL80/Cyo, y w hsFLP; FRT42D tub-GAL80/Cyo; and tub-GAL4 UAS-myr-tetO/TM6B.

HnRNP-K/miR-7 Stellaris Probes

A set of 48 Stellaris probes was designed against an HnRNP-K-RC transcript and labeled with Quasar 670. Third-instar larval brains were fixed in 4% formaldehyde for 1 hr at room temperature and transferred to 70% ethanol over-night at 4°C. Brains were incubated with 125 μM probes in hybridization buffer (100 mg/mL dextran sulfate, 10% formamide, 2× saline sodium citrate [SSC]) overnight at 45°C and washed in wash buffer (10% formamide, 2× SSC).

Generation of UAS-miR-7

The 88-bp miR-7 hairpin was amplified using primers forward (fwd): 5’-caag aagagaactctgaatagggatttggaGAGTGCATTCCGTATGGAAAG-3’ and reverse (rev) 5’-aagataaaggtttcctcacaagatcctgcttagaATATGACCGCTTAAGAAG-3’ and cloned via Gibson assembly into pCfD3 (Port et al., 2014). This vector was injected into nos-phiC integrase; +; attP2 embryos to generate a stable line expressing the miR-7 gRNA under control of the U6:3 promoter.

Mutations in miR-7 were detected by sequencing of a 256-bp PCR product, amplified using primers that flank the gRNA cut site. 100% of F1 flies produced offspring with mutations. 58.6% (n = 46) of F2 showed alterations to the miR-7 locus, generating a total of 20 independent alleles.

Immunohistochemistry

Fixation and immunocytochemistry of larval brains was carried out as described previously (Gold and Brand, 2014). The following primary antibodies and dilutions were used: guinea pig anti-Dpn (1:10,000) and rat anti-L-ac (1:5,000) were generated by C.M. Davidson, E.E.C., and A.H.B. (this study) using constructs that were a kind gift of J. Skeath; chick anti-GFP (1:2,000) and rabbit anti-RFP (1:1,000) from Abcam; and fluorescently conjugated secondary antibodies Alexa 405, Alexa 488, Alexa 546, and Alexa 633 (all 1:200) from Life Technologies.

Statistics

To analyze the effect of temperature shift and genetic backgrounds on the severity of the transition zone defect, we assessed differences in the number of brains exhibiting the strong phenotype via pairwise comparisons (2 × 2 contingency tables) performed with the two-tailed Fisher’s exact test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.07.047.

AUTHOR CONTRIBUTIONS

E.E.C. and A.H.B. designed the experiments; E.E.C. carried out the experiments; E.E.C. and A.H.B. analyzed the data and wrote the manuscript.

ACKNOWLEDGMENTS

We thank Richard Carthew and Francois Schweiguth for Drosophila lines. We thank Catherine Davidson for help generating antisera, Robert Krautz for help with statistical analysis, and Seth Cheetham for comments on the manuscript. This work was funded by a Wellcome Trust Programme grant (092545), a Wellcome Trust Senior Investigator Award (103792), and a BBSRC Project Grant (BB/L007800/1) to A.H.B. A.H.B. acknowledges core funding to the Gurdon Institute from the Wellcome Trust (092096) and CRUK (C6946/A14942).

Received: December 6, 2016
Revised: May 23, 2017
Accepted: July 18, 2017
Published: August 8, 2017
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