Mettl3-dependent m6A modification attenuates the brain stress response in *Drosophila*

Natasha E. Perlegos1, Emily J. Shields2,3,4, Hui Shen5, Kathy Fange Liu5,6 & Nancy M. Bonini1,2

Received: 16 July 2021
Accepted: 1 September 2022
Published online: 14 September 2022

*N*-methyladenosine (m6A), the most prevalent internal modification on eukaryotic mRNA, plays an essential role in various stress responses. The brain is uniquely vulnerable to cellular stress, thus defining how m6A sculpts the brain's susceptibility may provide insight to brain aging and disease-related stress. Here we investigate the impact of m6A mRNA methylation in the adult *Drosophila* brain with stress. We show that m6A is enriched in the adult brain and increases with heat stress. Through m6A-immunoprecipitation sequencing, we show 5'UTR Mettl3-dependent m6A is enriched in transcripts of neuronal processes and signaling pathways that increase upon stress. Mettl3 knockdown results in increased levels of m6A targets and confers resilience to stress. We find loss of Mettl3 results in decreased levels of nuclear m6A reader Ythdc1, and knockdown of Ythdc1 also leads to stress resilience. Overall, our data suggest that m6A modification in *Drosophila* dampens the brain's biological response to stress.

The cellular stress response is among the most ancient pathways, critical for all cells to combat insults. Despite this, prolonged exposure to cellular stress is detrimental, in particular for the maintenance of brain integrity and function1-4. During cellular stress, transcription and protein synthesis is globally repressed to reduce cell burden5. Concurrently, a subset of mRNAs undergoes selective translation to prioritize synthesizing proteins vital to stress recovery and disease suppression6. Neurons employ altered mechanisms to adapt and survive stress. These include delayed activation of HSF-1 in motor neurons7 and activation of pathways that promote axon regeneration, neuronal apoptosis, and brain longevity8-11. As neurons are postmitotic and cannot dilute potentially harmful species through cell division, they must adapt conserved pathways such as heat shock (HS) chaperones, mTOR signaling, and Hippo signaling to selectively maintain synaptic and dendritic homeostasis12-14. Given the striking susceptibility of the brain to neurodegenerative disease, understanding the effectors of the adult brain stress response in vivo may be critical to understanding brain disease and healthful brain aging.

Epigenetic modification of DNA and histone proteins are crucial in neuronal responses to cellular stress and disease15-17. However, recent techniques have provided increasing evidence that RNA modifications confer a critical layer of regulation18-20. m6A is the most abundant internal modification of mRNA in eukaryotic cells and is deposited co-transcriptionally by a methyltransferase, Mettl3, with the help of additional complex components21. m6A-modified transcripts are recognized by selective reader proteins that dictate downstream processing, such as splicing, decay, promotion and inhibition of translation22-25. In vitro work in mammalian cells has shown that m6A is dynamically regulated during stress, including upon UV DNA damage and heat-shock26-29. m6A-marked mRNAs are enriched in stress granules and RNA-protein complexes that form during cellular stress, and m6A YTH reader proteins are fundamental for stress granule

---

1Neuroscience Graduate Group, University of Pennsylvania, Philadelphia, PA 19104, USA. 2Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, USA. 3Epigenetics Institute and Department of Cell and Developmental Biology, University of Pennsylvania Perelman School of Medicine, Philadelphia, PA, USA. 4Department of Urology and Institute of Neuropathology, Medical Center-University of Freiburg, Faculty of Medicine, University of Freiburg, Freiburg, Germany. 5Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA. 6Graduate Group in Biochemistry and Molecular Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA. e-mail: nbonini@sas.upenn.edu
enzymes are enriched in the central nervous system. The data upon the brain heat shock (HS) response in vivo. We global m6A with heat stress in head tissue, which is brain-enriched. (Supplementary Fig. 1b). Conserved with mammals, including brain development and function. Unexpected stress resilience. This work highlights that knockdown increases protein and RNA levels of its targets and confers unexpeced stress resilience. Here we investigated mA regulation of the heat stress response of the adult fly brain. We find that mA is enriched in the S'UTR, and S'UTR modification is regulated by the methyltransferase Mettl3. Mettl3 knockdown increases protein and RNA levels of its targets and confers unexpeced stress resilience. This work highlights that Mettl3-dependent mA modification normally serves to dampen the brain's acute heat stress response by selective regulation of RNA levels and translation of critical signaling and cellular stress transcripts.

Results

mA levels are enriched in the brain and are modulated with heat stress

The stress response is critical to the brain's ability to endure the challenges of age and disease, and mA modulation of transcripts is associated with a range of stress responses in mammalian cells. Molecular chaperones crucial to the heat shock response are instrumental in understanding neurological disease, and preconditioning heat stress has been noted to suppress disease toxicity and increase longevity. Therefore, we examined if there were changes in mA upon the brain heat shock (HS) response in vivo. We first profiled global mA with heat stress in head tissue, which is brain-enriched. Intriguingly, the levels of mA were increased on polyadenylated RNA (polyA + RNA) after HS. The mA signal was robust after 10, 30, and 60 min of HS and returned to baseline by 24 h of recovery, indicating that mA levels were dynamically changed with HS (Fig. 1a, b).

To investigate this response further, we focused on the 30 min HS timepoint, as it showed the most significant increase in mA levels. Animals lacking eye tissue (a large fraction of the head tissue) showed a similar robust increase in mA levels with HS (Supplementary Fig. 1a), suggesting that the signal was largely from brain tissue and not the eyes. By liquid chromatography-tandem mass spectrometry (LC-MS/MS), we confirmed an increase in mA with HS from dissected brains (Fig. 1c). The HS-related change in mA levels was not detected in polyA- RNA isolated from whole fly tissue (Fig. 1d). Using LC-MS/MS, mA levels were 2.2 × higher in the brain compared to the whole fly (Fig. 1e), consistent with previous studies showing that fly mA enzymes are enriched in the central nervous system. These data indicate that mA is enriched in Drosophila brain tissue, and changes in mA with stress are primarily in the brain.

To define the extent to which the mA change was due to methyltransferase Mettl3 activity, we knocked down Mettl3 by RNAi (Supplementary Fig. 1b–d). This reduced mA levels on polyA+ RNA and reduced the increase seen with HS (Fig. 1b), indicating that the dynamic change of mA on polyA+ RNA with stress is largely dependent on Mettl3 gene function. Curiously, when we examined total RNA rather than polyA+ RNA, we found that mA levels in total RNA were decreased upon HS, with levels returning to normal after 24 h (Supplementary Fig. 1e). This decrease was not impacted by Mettl3 RNAi, indicating that Mettl3 methyltransferase activity does not affect total RNA (consisting mostly of rRNA) mA methylation (Supplementary Fig. 1f). Overall, these findings signify that mA increases on polyA+ RNA in the brain upon HS in a manner dependent on Mettl3 gene function.

The brain has a distinct stress response

Given these data showing that the brain has a unique mA response to HS, we investigated mRNA and protein changes of selected molecular chaperones known to increase with stress. Whole fly and whole head tissue undergo a typical Drosophila HS response, with rapid upregulation of Hsp70 protein by 30 min of HS that persists through a 6 h recovery (Supplementary Fig. 2a). However, the brain (dissected from the head capsule and eyes) showed a delayed upregulation of Hsp70 protein compared to whole fly and whole head (Supplementary Fig. 2a). Additionally, the protein levels of Hsp70 upon heat stress were lower in the brain relative to other tissues, specifically whole heads, and outer head capsules minus brain (Supplementary Fig. 2b, c). We investigated two additional fly chaperones, DnaJ-1 (homolog of mammalian HSP40; Supplementary Fig. 2d) and stv (homolog of mammalian BAG3; Supplementary Fig. 2e), as these two proteins function to stimulate heat shock protein chaperone function. Again, surprisingly, their protein-level upregulation was markedly dampened in the brain, with no significant increase in protein by Western immunoblot upon HS. By contrast, there was a similar strong transcriptional upregulation of all of these chaperones in all tissues examined (Supplementary Fig. 2f–h). These findings indicate a striking disconnect between RNA and protein levels of key chaperones upon HS in the brain and indicate that the brain has a dampened response to stress at the protein level compared to other tissues.

Mettl3 knockdown animals are resilient to heat stress

Based on the enrichment of mA in the brain, mA modulation upon stress, and the diminished chaperone protein response of the brain, we reasoned that mA reduction may impact stress resilience. We performed an animal HS survival assay by subjecting control and Mettl3 RNAi male flies to severe HS (1.5 h at 38.5 °C), then scoring fly survival at 24 h (Fig. 2a). Approximately 50–60% of control animals survived 24 h after HS (Fig. 2b). Upon reduction of Mettl3, however, the animals were notably more resilient, showing a 10–20% increase in survival compared to controls treated in parallel (Fig. 2b). Additionally, knockdown of Mettl3's heterodimeric partner Mettl4 also conferred stress resilience (Supplementary Fig. 3a). By contrast, upregulation of Mettl3 selectively in neurons dramatically impaired HS survival, with nearly none of the animals surviving 24 h post-HS (Fig. 2c). The Mettl3 ΔCat (catalytic domain deletion) mutant in trans to a Mettl3 deficiency was also stress resilient (Fig. 2d and Supplementary Fig. 3b). We examined protein levels of Mettl3 upon HS and found no significant changes (Supplementary Fig. 3c). The resilience of the Mettl3 ΔCat and Mettl4 knockdown animals indicates this HS pathway is dependent on Mettl3 mA methyltransferase activity. Overall, these data indicate that mA modification in the brain is associated with reduced HS survival.

mA is enriched in the 5'UTR of Drosophila transcripts

Given the change in mA levels in polyA+ RNA and resilience of Mettl3 knockdown animals upon HS, we sought to define which transcripts are marked with mA upon HS in vivo. mA-Immunoprecipitation (IP) sequencing was performed from polyA+ RNA extracted from control and Mettl3 knockdown heads, in basal and 30 min HS conditions (Supplementary Fig. 4a). Using brains alone was challenging for mA-IP sequencing due to low RNA yield, and consequently we used head tissue, given our data showing mA in the head is associated with brain tissue (see Fig. 1b). To confirm robustness of the IP and targets, mA-modified RNA was assessed using mA antibodies produced by two different sources: New England Biolabs (NEB) and Synaptic Systems (SYS). The data were highly consistent between antibodies, with high overlap (80%) of mA-enriched targets (Supplementary Fig. 4b).
We found that m^6^A, which is dependent on Mettl3, was greatly enriched in the 5\'UTR, with less density along coding sequences (CDS) and 3\'UTR (Fig. 3a). The enrichment of m^6^A in the 5\'UTR may be unique to Drosophila and is consistent with other recent works\(^ {34,40} \), in contrast to mammalian m^6^A that is mostly in the 5\'UTR with modest 5\'UTR increase during stress\(^ {34,40} \). De novo motif analysis highlighted similar m^6^A motifs from both antibody m^6^AIP-seq (Supplementary Fig. 4c and Supplementary Data 1) that were consistent with motifs defined from other Drosophila m^6^AIP-seq studies\(^ {33,39,40} \). We used the SYS antibody for further analyses for ease of comparison to other sequencing studies using SYS. We used RADAR\(^ {37} \) differential methylation analysis to call Mettl3-dependent m^6^A peak changes between control and Mettl3 knockdown conditions; these targets will be referred to as m^6^A genes from here on (Supplementary Data 2). Our global analysis of m^6^A marked transcripts showed a slight enrichment of 5\'UTR m^6^A upon heat stress, consistent with our LC-MS/MS and dot blot analyses (Fig. 3a, b and Supplementary Fig. 4d).

Knockdown of Mettl3 showed 94% of Mettl3-dependent m^6^A was located in the 5\'UTR (Fig. 3b-d). The m^6^A targets affected by Mettl3 knockdown, defined here by m^6^A-IP-seq, showed considerable overlap (79%) with Drosophila head m^6^A miCLIP-seq targets\(^ {33} \) (Supplementary Fig. 4e). There was less overlap (47%) with S2R+ cell culture m^6^A miCLIP-seq targets\(^ {33} \) (Supplementary Fig. 4f). These data underscore the enrichment of m^6^A in the 5\'UTR in Drosophila transcripts and highlight that the identity of m^6^A-marked genes depends on the genes expressed in vivo vs cell culture conditions. Overall, our sequencing analysis showed consistency between replicates (Supplementary Fig. 4g).

m^6^A transcripts are enriched in neuronal and signaling pathways

To define and understand the types of genes regulated by m^6^A modification, we compared genes with m^6^A (a majority of which are in the 5\'UTR), to all other genes expressed in the brain. This comparison indicated that only 14.1% of all genes expressed in the brain are marked by m^6^A.

Unbiased GO term and KEGG pathway analyses highlighted that m^6^A transcripts were distinct from non-modified brain genes. Non-
modified brain genes were enriched for “metabolic process” and “oxidative phosphorylation” terms (Supplementary Fig. 5a, b and Supplementary Data 3), while genes with m6A were enriched in GO terms associated with “neurodevelopment” and “neurogenesis” (Fig. 3e, left). KEGG pathway analysis indicated that m6A genes were enriched for critical signaling pathways, including MAPK, WNT, TGF-beta, and Notch, among others (Fig. 3e, right). Intriguingly, neurons often employ these signaling pathways to deal with stressful situations. We visualized m6A enrichment across the gene body by calculating the m6A-IP reads divided by the background input reads for genes annotated with select KEGG pathway terms (Fig. 3f). These data emphasized that m6A genes involved in signaling pathways are enriched for m6A in the 5′UTR, consistent with the global analysis.

To further investigate the impact of m6A modification on brain gene expression, we examined protein levels of m6A-modified genes with available antibodies. We chose three genes: fti2/wtapl, a component of the m6A writer complex, futsch (MAP1A/B, a microtubule-associated protein that regulates axonal growth), and draper (a receptor that is involved in response to stimulation and phagocytosis by glia) (Supplementary Fig. 5c). Protein levels of all three genes increased in brain tissue upon Mettl3 knockdown compared to controls in basal and in HS conditions (Supplementary Fig. 5d–f). Brain transcript and protein levels of the non-modified gene Hsf1, the major transcription factor for the in vivo response, showed no significant difference with Mettl3 RNAi (Supplementary Fig. 5g). These data suggest that depletion of Mettl3 is associated with increased protein levels of m6A-target genes in the brain.

Comparing the transcriptome of epithelial S2 cells, which previous studies have focused on refs. 38, 48, to brain tissue, we found that m6A-marked genes are more highly expressed in the brain (Supplementary Fig. 5h and Supplementary Data 4). These data underscore that m6A genes are enriched in brain-specific pathways, indicated also from GO and KEGG analysis, and highlight the importance of defining the in vivo response.

m6A transcripts are increased with HS and upon Mettl3 knockdown
To further investigate the stress resilience of Mettl3 knockdown animals and increased m6A observed with HS, we performed brain tissue RNA-seq upon Mettl3 knockdown and with HS (Supplementary Fig. 6a–d and Supplementary Data 5). We analyzed the global transcriptional response with an RNA-seq time-course on dissected brain tissue from animals exposed to 30 min HS and allowed to recover for 6 and 24 h. The brain showed a strong transcriptional response to HS at 30 min, with similar numbers of genes up- and downregulated (Supplementary Data 5). After 6 and 24 h of recovery, the transcriptional response to the initial heat shock was greatly attenuated, although 16–20% of the genes did not return completely to baseline levels.

We examined m6A targets to probe the transcriptional response of this gene set in the brain. Surprisingly, m6A genes were preferentially upregulated with HS (1016 genes up and 227 down), compared to all other genes expressed in the brain, which did not show a skew towards increased transcription levels (Fig. 4a and Supplementary Fig. 6e). Examining the transcriptional dynamics of specific groups of m6A genes (see Fig. 3e), neurogenesis and MAPK signaling genes containing m6A were more upregulated with HS (Fig. 4b). Globally, of the 3193 genes upregulated with HS in the brain, 31.8% had m6A modification (Fig. 4c). The skewed increase in expression upon HS was specific for m6A gene sets, as no similar trend was observed when considering non-modified gene sets (Fig. 4d). The m6A-tagged genes significantly upregulated with HS (47.9% of all m6A genes) could also account, in part, for the increased m6A levels detected by dot blot and mass spectrometry with HS (see Fig. 1).

In Mettl3 RNAi brains at basal conditions, m6A genes skewed towards upregulated (78.3% of differentially expressed m6A genes were up with Mettl3 RNAi (Fig. 4e–h and Supplementary Fig. 6f)). This upregulation is also seen in m6A gene sets enriched for neurogenesis and MAPK signaling pathways (Fig. 4f–h). These data indicate that m6A transcripts are likely to be increased upon both HS and Mettl3 knockdown in the brain. The increased basal levels of these genes may allow for preconditioning to stress in Mettl3 knockdown animals, thus conferring the stress resilience observed (see Fig. 2).

**Mettl3 knockdown increases HS chaperone protein levels and decreases their rate of RNA decay.**
To understand mechanistically how m6A influences the brain HS response, we analyzed differential m6A methylation in basal vs HS

---

*Fig. 2 | Mettl3 knockdown animals are resilient to heat stress. a | Schematic of HS stress assay. Flies are 6-day-old. Flies are put onto RU486 food if needed to turn on the expression of the RNAi transgene for 6 days. Flies are HS at 38.5 °C and allowed to recover overnight in normal food or RU486 food at 25 °C. Percent survival in each vial of 20 animals are scored after 24 h. b | Ubiquitous knockdown of Mettl3 (DaGal4, DaGS), Neuron-specific knockdown (ElavGS), and corresponding controls (mCherry RNAi) were HE for 1.5 at 38.5 °C and scored for survival after 24 h recovery. n = 7 biological replicates, each data point represents percent survival in a vial of 20 flies per replicate. Data presented as mean ± SD, **p < 0.01, ****p < 0.0001. Student’s two-tailed t-test, p = 0.0001. c | Neuron-specific (ElavGS) upregulation of Mettl3 and control were HS for 1 h at 38.5 °C and scored for survival after 24 h recovery. UAS-Mettl3 flies were severely stress-sensitive, and HS time was reduced to 1 h to have at least 5–10% of the flies surviving. Each data point represents percent survival in vials of 20 flies per replicate. Data presented as mean ± SD, **p < 0.01, ****p < 0.0001, One-way ANOVA with Tukey’s test, p < 0.0001, \( p < 0.0001, p = 0.0014 \). Source data and statistical analysis are provided as a Source Data file.*
conditions. Although we saw a global shift toward increased m6A methylation upon HS (Fig. 3a, b), few genes exhibited statistically significant thresholds of differential methylation upon stress (Supplementary Data 2), consistent with previous m6A stress studies in mammals34,35.

To further understand the impact of m6A on HS chaperones, we utilized the FlyBase49 curated list of HS chaperones, plus 20 of the most significantly differentially expressed genes from our brain HS RNA-seq analysis (Supplementary Data 5 and Supplementary Data 6). We were surprised to find only a few HS chaperones with m6A upon HS (red
Fig. 3 | m6A transcripts are enriched in neuronal and signaling pathways.

a Normalized read coverage plot of m6A-IP/input on polyA+ transcripts in the 5′ UTR, CDS, and 3′UTR of m6A genes (Mettl3-dependent m6A). m6A-IP sequencing in basal and HS conditions from control fly (DaGal4 > mCherry) RNAi heads.

b Scatterplot of normalized read coverage of m6A-IP/input from the 5′UTR of m6A genes in basal versus HS conditions. n = 2 biological replicates for each condition, 200 fly heads per replicate per condition. Violin plot showing indicating mean 5′ UTR mRNA coverage in each condition and statistical significance between each group. Data presented as mean ± SD, ***p < 0.0001, Paired t-test and two-tailed. p < 0.0001, p < 0.0001, p < 0.0001. c Normalized read coverage plot of m6A-IP/input in basal and HS conditions from DaGal4>Mettl3 RNAi vs DaGal4>mCherry RNAi samples show a loss of m6A primarily in the 5′UTR of transcripts upon knockdown of Mettl3.

d Percent location of Mettl3-dependent m6A. Mettl3-dependent m6A peaks are enriched in the 5′UTR in Drosophila. e GO term enrichment and KEGG pathway enrichment of m6A genes. The −log10(p val) enrichment of genes in each category is shown, as well as the number of genes that fall into each category (left). Full GO term, KEGG term, and p value lists are shown in Supplementary Data 3. Heat map of m6A enrichment on KEGG Pathway: MAPK signaling and hippo signaling genes with Mettl3-dependent m6A. Shown is mCherry RNAi (control) and Mettl3 RNAi m6A enrichment in basal conditions. m6A enrichment is shown as a log (m6A/IP divided by the input control). Heat map displays z-score values, which is scaled by row; each gene is relative to itself across all six boxes. Segmented into 5′UTR, CDS, and 3′UTR. Source data and statistical analysis are provided as a Source Data file.

asterisks, Fig. 5a and Supplementary Fig. 7a–c). Perplexingly, the inducible Hsp70 chaperone genes did not show increased 5′UTR m6A upon HS, as in mammalian cell HS studies29. Closer examination, however, revealed that several Hsp70/Hsp68 isoforms showed m6A enrichment in the 5′UTR at baseline that was lost with HS and with Mettl3 RNAi (Fig. 5b and Supplementary Fig. 7a–c). These findings are consistent with both m6A-IP antibodies (Supplementary Fig. 7b). We also observed a significant elevation of Hsp70 transcript levels in Mettl3 RNAi brains at basal conditions (Supplementary Fig. 7d and Supplementary Data 5). Although Hsp70 did not pass our conservative threshold above baseline, we observed a trend of higher Hsp70 mRNA in Mettl3 RNAi brains (Fig. 5d). By contrast, upregulation of Hsp70/Hsp68 in Mettl3 knockdown brains maintained higher pur-romycin incorporation in the brain upon Mettl3 upregulation (Fig. 5a, right). Upon HS, Mettl3 knockdown brains maintained higher pur-romycin incorporation than brains with Mettl3 knockdown or HS exposure alone (Fig. 5b). Mettl3 ACat mutant/Mettl3 deficiency brains, and Mettl4 RNAi brains similarly showed an increase in pur-romycin levels compared to controls (Supplementary Fig. 9b–c). Treatment of puromycin plus actinomycin D (a transcription inhibitor) consistently showed increased puromycin incorporation in Mettl3 knockdown brains (Supplementary Fig. 9d). Together, these results indicate that Mettl3 gene function modulates global protein translation in the brain in vivo.

To probe the possible downstream processing of m6A transcripts, we focused on understanding the impact of the two YTH m6A reader proteins: cytoplasmic Ythdf1 and nuclear Ythdc1. Knockdown of either Ythdf1 or Ythdc1 showed increased puromycin incorporation in the brain (Fig. 6c), and upregulation yielded the reverse (Fig. 6d). These results support Ythdf1 and Ythdc1 as key regulators of translation of m6A targets in the brain.

Ythdc1 knockdown animals are resilient to heat stress

To determine the YTH m6A reader protein involved in the brain’s stress response downstream of Mettl3, we examined whether HS or Mettl3 loss influenced the transcript levels of Ythdf1 or Ythdc1. Brain RNA-Seq data indicated that Ythdc1 transcript levels were increased in control brains upon HS, in an effect that lasted 6 h post-HS (Fig. 7a and Supplementary Data 5).Surprisingly, Mettl3 knockdown attenuated this stress-induced feature of Ythdc1, and Mettl3 RNAi brains had reduced Ythdc1 levels compared to control in basal and heat shock conditions (Fig. 7a, b). The protein levels of Ythdc1 were similarly impacted by Mettl3 knockdown and by HS (Fig. 7b and Supplementary Fig. 9e). By contrast, Ythdf1 transcript levels were minimally impacted in control, HS, or with Mettl3 knockdown (Supplementary Data 5 and Supplementary Data 8). Due to the changes in Ythdc1 transcript and protein levels induced with HS and with Mettl3 knockdown in the brain, we focused on the biological effects of Ythdc1 perturbation.

We first assessed whether the knockdown of Ythdc1 influenced the animals’ heat stress resilience. Ythdc1 knockdown conferred heat...
stress resilience (Fig. 7c), similar to the effect of Mettl3 knockdown, while upregulation resulted in increased sensitivity to heat stress (Fig. 7d). We then performed RNA-Seq from control and Ythdc1 RNAi brains in basal and HS conditions. We examined the genes that were differentially expressed upon Ythdc1 RNAi (Supplementary Data 5). Sixty-five genes upregulated in Ythdc1 knockdown brains at baseline overlapped genes upregulated with Mettl3 knockdown (Fig. 7e). Many of these genes were key HS chaperones and marked by m6A and upregulated by Mettl3 knockdown (Hsp70, DnaJ-1, Hsp68) (Fig. 7e). GO term analysis of all upregulated transcripts in Ythdc1 RNAi brains showed enrichment in heat shock protein chaperone pathways (Fig. 7f). GO term analysis of genes marked by m6A and upregulated...
with Ythdc1 RNAi showed enrichment in signaling and neurogenesis terms (Fig. 7f), pathways we observed being increased with HS (Fig. 4). We confirmed that Ythdc1 RNAi, like Mettl3 RNAi, showed increased levels of Hsp70 protein at baseline and after HS compared to control brains (Fig. 7g). Thus, Ythdc1 knockdown in the brain resulted in both increased levels of heat stress chaperone genes and animal stress resilience, both of which are similar to Mettl3 knockdown, suggesting that Ythdc1 plays a role in the m6A HS response of the brain.

**Discussion**

m6A regulates diverse critical physiological processes, including neurodevelopment, immune responses, cancer metastasis, learning,
Fig. 5 | Mettl3 knockdown increases select HS chaperone protein levels and decreases their rate of RNA decay. a Heat map of m’A enrichment of HS Chaperone Genes (Supplementary Data 6). Shown is control and Mettl3 RNAi m’A enrichment in HS conditions. m’A enrichment is shown as m’A/IP divided by the input. Heat map displays z-score values, which are scaled by row, each gene is relative to itself and relative across all six boxes. Segmented into 5’UTR, CDS, and 3’UTR. Example tracks from genome browser of m’A locations for HS chaperone transcripts Hsp70a/Ab and Hsp68 in basal and HS 30 min, from control and Mettl3 RNAi brains. Tracks shown as log(m’A-IP divided by input). Separate IP and Input tracks are shown in Supplementary Data 7c. Example tracks from genome browser of m’A locations for m’A marked HS co-chaperone genes DnaJ-1 and stv in basal and HS from control and Mettl3 RNAi brains. d Hsp70, DnaJ-1, and stv protein levels from DaGal4 > Mettl3 RNAi vs DaGal4 > mCherry RNAi brains dissected in basal, HS 30 min at 38.5 °C, 6 h recovery post-HS, or 24 h recovery post-HS. Right is the quantification of three biological replicate immunoblots, 15 brains per replicate.

Fig. 6 | Mettl3 attenuates protein translation in the brain. a Control, Mettl3 RNAi, and UAS-Mettl3 upregulation flies were fed 600 μM puromycin for 24 h. (Genotypes: DaGal4 > mCherry RNAi vs DaGal4 > Mettl3 RNAi, ElavGS > UAS-mCherry vs ElavGS > UAS-Mettl3).Brains were dissected from the heads, and protein immunoblots were probed for puromycin incorporation in each sample. Ponceau S staining shows total protein levels. n = 3 biological replicates, 15 brains per replicate. Data presented as mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001, two-way ANOVA with Sidak’s test. b Control and Mettl3 RNAi flies were HS for 30 min and then fed 600 μM puromycin for 4 h or control and fed puromycin for 4 h. (Genotypes: DaGal4 > mCherry RNAi vs DaGal4 > Mettl3 RNAi). Brains were dissected after 4 h, and protein immunoblots were probed for puromycin levels in each sample. Ponceau S staining shows total protein levels. n = 3 biological replicates, 15 brains per replicate. Data presented as mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001, two-way ANOVA with Sidak’s test. c ElavGS > mCherry RNAi vs Ythdc1 RNAi, and ElavGS > mCherry vs UAS-Ythdc1 upregulation flies brain puromycin assay. n = 3 biological replicates, 15 brains per replicate. Data presented as mean ± SD, *p < 0.01, Student’s two-tailed t-test. d ElavGS > mCherry RNAi vs Ythdc1 RNAi, and ElavGS > mCherry vs UAS-Ythdc1 upregulation flies brain puromycin assay. n = 3 biological replicates, 15 brains per replicate. Data presented as mean ± SD, *p < 0.01, Student’s two-tailed t-test. ns not significant.
**Fig. 7 | m6A reader protein Ythdc1 knockdown animals are resilient to heat stress.** a Normalized read counts of Ythdc1 from brain RNA-sequencing in baseline, HS, Control, and Mettl3 RNAi conditions (DaGal4 > mCherry RNAi vs Mettl3 RNAi). Statistical analysis from RNA-seq differential expression analysis, provided in Supplementary Data 5. ****p < 0.0001 From left to right p = 1.53E-11, p = 5.59E-07, p = 1.71E 38, ns not significant. b Ythdc1 protein levels from DaGal4 > mCherry RNAi vs Mettl3 RNAi brains dissected in basal or HS 30 min at 38.5 °C. n = 4, 15 brains per replicate. Quantification of four biological immunoblots show decreased expression of Ythdc1 protein in Mettl3 RNAi fly brain and increase of Ythdc1 protein with HS. Data presented as mean ± SD, **p < 0.01, ***p < 0.001, ****p < 0.0001. Student’s two-tailed t-test, p = 0.0078, p = 0.0001, p = 0.0013, p = 0.0003. c Ubiquitous knockdown of Ythdc1 or Mettl3 (DaGal4), and neuron-specific knockdown (ElavGS), and corresponding controls (mCherry RNAi) were HS for 1.5 h at 38.5 °C and scored for survival after 24 h recovery. n = 6 biological replicates for DaGal4, n = 4 biological replicates for ElavGS. Each data point represents percent survival in a vial of 20 flies per replicate. Data presented as mean ± SD, **p < 0.01, ***p < 0.001, ****p < 0.0001. One-way ANOVA, Student’s two-tailed t-test, p = 0.0002, p = 0.0001, ns not significant, p = 0.0085. d Neuron-specific (ElavGS) upregulation of Ythdc1 in control and control were HSed for 1 h at 38.5 °C and scored for survival after 24 h recovery. n = 4 biological replicates. Each data point represents percent survival in a vial of 20 flies per replicate. Data presented as mean ± SD, ***p < 0.0001, Student’s two-tailed t-test, p = 0.0002. e Overlap of genes upregulated upon DaGal4 > Mettl3 RNAi and Ythdc1 RNAi (baseline) and overlap of m6A genes and genes upregulated with Ythdc1 RNAi. Differential expression analysis is provided in Supplementary Data 5. Hypergeometric test, one-sided, p < 3.742e-15. f GO term enrichment of genes upregulated upon Ythdc1 RNAi (baseline). Go terms of m6A genes upregulated upon Ythdc1 RNAi. The -log10(p-val) enrichment of genes in each category is shown. Supplementary Data 3 for full GO term list and p values. g Hsp70 protein levels from DaGal4 > mCherry RNAi and Ythdc1 RNAi brains dissected in basal or HS 30 min at 38.5 °C. n = 3, 15 brains per replicate. Quantification of three biological immunoblots, 15 brains per replicate, show increased expression of Hsp70 protein in Ythdc1 RNAi fly brain. Data presented as mean ± SD, *p < 0.05, Student’s two-tailed t-test, p = 0.0237, p = 0.0126. Source data and statistical analysis are provided as a Source Data file.
memory, and stress. Here, we provide insight into the role of m6A in the adult Drosophila brain in vivo in an acute stress situation: heat shock. m6A modulation of polyA+ RNA shows a dynamic increase in the brain with acute heat stress. Levels of m6A modified gene transcripts that increase with heat stress are enriched for neurogenesis and dynamic signaling pathways. Mettl3 knockdown decreases the level of m6A in the 5'UTR of its target transcripts and biologically results in increased heat stress resilience of the animal, and a brain-specific increase in protein and RNA levels of m6A targets, with decreased RNA decay. Mettl3 knockdown also leads to a decrease in transcript and protein levels of Ythdc1. Ythdc1 knockdown is associated with an increase in key HS chaperone transcripts and protein levels, and stress resilience.

The impact of m6A on the brain stress response

m6A likely contributes to the surprisingly distinct response of the brain to acute HS in Drosophila. While the brain and head both have a robust increase in Hsp70 transcription following HS, the brain, Hsp70 protein levels are only mildly upregulated compared to heads (see Supplementary Fig. 2c). DnaJ-1 and stv, which promote Hsp70 function, also had a robust transcriptional response to HS in brains, heads, and whole fly tissue, but strikingly their protein levels were not well upregulated with HS in brain tissue. Mettl3 knockdown increased stress-induced protein levels of Hsp70, DnaJ-1, and stv, suggesting that 5'UTR m6A dampens the levels of key stress chaperones associated with the acute HS response in the Drosophila brain. Furthermore, Mettl3 and Ythdc1 knockdown elevated the levels of these key HS chaperones, mimicking mild preconditioning stress; preconditioning is known to be crucial for an organism’s response to severe stress. Conversely, upregulation of Mettl3 or Ythdc1 increased stress sensitivity.

In mammalian cells, m6A has been shown to be critical to the cap-independent translation of Hsp70 with HS. However, in mammalian cells Mettl3 (along with microprocessor Dgcr8) has also been shown to co-transcriptionally mark Hsp70, leading to RNA degradation. In our in vivo Drosophila brain data, stress-induced Hsp70 transcripts were not m6A modified in the 5’UTR upon HS. By contrast, we observed basal m6A enrichment in the 5’UTR of Hsp70 and Hsp68 in control flies that was lost upon HS and with Mettl3 knockdown (see Fig. 3b, c and Supplementary Fig. 7b). Brain RNA-seq analysis showed that both Hsp70 and Hsp68 RNA levels were elevated at
resilience. m6A genes are enriched for many critical signaling pathways, including MAPK, Hippo, WNT, mTOR, and Notch signaling, and our data indicate transcripts for these pathways increase with HS in the brain (Fig. 3c). These signaling pathways have not been previously implicated in the canonical HS response, although they are employed by neurons to deal with various types of stressful situations, such as proteostasis and aging. By dot blot, mass spec and m6A-IP, our data indicate an increased level of m6A with HS. We underscore that our RNA-seq data are from dissected adult fly brain tissue vs heads or cultured cells. In the brain, m6A targets are upregulated upon both HS and Mettl3 RNAi, and the increase with HS may account for the overall increase in m6A levels we observe upon HS. The increased transcript levels of m6A genes, and higher levels of critical chaperones Hsp70 and DnaJ, may contribute to the increased stress resilience of the animals upon Mettl3 knockdown. We suggest that m6A modulation of genes normally leads to an attenuated acute stress response in the brain, given the dampened levels of these m6A-modified chaperone genes in the brain compared to other tissues. This may underlie, at least in part, the unique susceptibility of the brain to severe stresses like protein misfolding diseases and dementia with aging.

Due to their postmitotic state, neurons often utilize altered molecular mechanisms, and perhaps m6A is utilized in neuronal cells to tightly control neuronal signaling and chronic stress pathways, with the caveat of a dampened acute stress response. In our data, we find that m6A targets are more highly expressed in brain tissue compared to epithelial S2 cells (Supplementary Fig. 5h and Supplementary Data 4). This suggests that regulation of m6A is best examined in the tissue type of interest where the m6A targets are expressed. Landscape m6A methylation analysis in human and mouse tissues has recently shown that brain tissue methylomes are highly specific and correlate with the relative expression levels of m6A complex reader and writers.

The molecular role of m6A on transcript and protein levels

Some studies have shown m6A associated with stress translation initiation, although additional studies point towards m6A association with translation inhibition. m6A-modified transcripts are regulated through m6A binding proteins, and can be directed to specific compartments of the cell for stability and translation. RNA reader proteins may sequester RNAs during stress, such as YTHDF shunting of m6A-marked transcripts into P-bodies and stress granules. This may prevent the translation of m6A transcripts and promote their degradation. Furthermore, recent studies have uncovered additional m6A binding factors, such as Fmr1, which suppresses the translation of its targets. In Drosophila, most m6A sites reside in the 5′UTR (this study and ), which might suggest a role in translation regulation. Additional studies suggest that m6A is preferentially deposited on fly transcripts with lower translational efficiency. In the Drosophila brain, our data suggest that decreased Mettl3 promotes increased protein translation and increased RNA levels of targets. Knockdown of m6A reader proteins Yuhdc1 and Yuhdf both led to increased nascent protein synthesis in the brain. Thus, these two reader proteins appear to lead to repressed translation. Although Mettl3 has functions other than serving in the methyltransferase complex, including chromatin regulation and modifies RNAs beyond mRNAs (enhancer RNAs, circRNAs, and lncRNAs), we found that HS resilience and protein translation in the brain are dependent on both Mettl3 and Mettl4 (see Fig. 2d, Supplementary Fig. 3, Supplementary Fig. 9).

Previous studies in mammalian cells have shown that YTHDF2 is the m6A reader protein that increases upon heat stress. Our in vivo brain RNA-sequencing revealed that nuclear m6A reader Ythdc1 levels are increased upon heat stress in the fly brain and that knockdown of Mettl3 strikingly decreases levels of Ythdc1. Functional analyses revealed that knockdown of Ythdc1 was beneficial to the brain stress response in vivo, with the animals being more resilient to heat stress. The increase in Ythdc1 levels upon HS in wild-type brains may serve as insight into mechanisms the brain has in place that abrogate the acute stress response. Our data indicate that knockdown of Ythdc1 up-regulates m6A target chaperones such as Hsp68 and DnaJ, as well as signaling pathway genes, which may be critical to the HS recovery response. Given the enrichment of m6A-modified genes in signaling pathways, the role of m6A in the brain may be to allow greater control of gene players in chronic stress signaling, but with a compromise in response to acute stress.

Our work expands the understanding of the role of m6A in the brain and in the acute stress response. We suggest that m6A genes enriched in critical neuronal and signaling pathways may require exquisite dynamic control. m6A modification may be one mechanism that the brain uses uniquely to exert that control. m6A deposition thus modulates neuronal signaling and brain-enriched stress response pathways by fine-tuning RNA translation and decay in the brain. We speculate that m6A modification of these genes benefits the overall long-term maintenance of brain function by adding a greater level of dynamic regulation of those genes. However, this appears to come at the cost of decreased resilience of the brain to acute stress, and perhaps also contributes to the selective vulnerability of the brain to neurodegenerative disease.

Methods

Drosophila work and lines

A full list of Drosophila stocks used in this study are described in Supplementary Data 7. RNAi lines were generated by the Harvard Transgenic RNAi Project (TRiP), and stocks were obtained from the Bloomington Drosophila stock center, Indiana, USA. Crosses were performed at 25°C and grown on standard cornmeal molasses agar. Driver lines used: DaGal4, DaGS(GeneSwitch)-Gal4, elav(GeneSwitch)-Gal4 as indicated per experiment. For conditional expression using the Gal4-GS(GeneSwitch) system, flies were collected 1–2 days after eclosion and placed on food vials pre-coated with 100 ul of 4 mg/mL RU486 per vial for 6 days before stress experiments. Food is changed every second day. (Milenpristine, Sigma-Aldrich). For all experiments, male flies were used for consistency in the experiments and to avoid issues in RU486 food due to egg laying of females.

Brain dissections

Brain dissections were conducted as previously described in . Briefly, flies were anesthetized using CO2 and decapitated using forceps. The head was placed posterior side down and the proboscis was then removed using Dumont #5SF forceps (Fine Science Tools, 11254-20). The brain was then gently popped out through the proboscis cavity, cleaned in PBS, and transferred to an RNAse-free microfuge tube and boiled at 98 °C for 3 min and then centrifuged at 1500 rpm for 3 min at 10,000g. PBS was aspirated. Brains were then ground in Laemmli Buffer (50 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.1% bromophenol blue, and 1 mM PMSF (Sigma, P7626). About 5 ul of sample buffer is added per brain, 7.5 ul added per head, and 40 ul added per whole fly. Samples are boiled at 98°C for 3 min and then centrifuged at 1500 rpm for 3 min at room temperature. Sample was loaded onto 15 wells of 1.0 mm 4–12%
Bis-Tris NuPAGE gels (Thermo Fisher, WG1401) with a pre-stained protein ladder (Thermo Scientific, 22619). 1 brain, 1 head, or 8% of whole fly tissue is loaded on each lane per experiment. Gel electrophoresis was performed using Xcell Surelock Mini-Cell Electrophoresis System at 140 V and transferred overnight onto a nitrocellulose membrane 0.45 μM (Bio-rad, 1620115), using a Bio-rad mini transblot cell at 90 A for 16 h. Membranes were stained in Ponceau S (Sigma, P7170-1L), washed in DI water, and imaged with Amersham Imager 600. Ponceau S was washed off in 3 × 5 min in Tris-buffered saline with 0.1% Tween20 (TBST). The membrane was blocked in 5% non-fat dry milk (LabScientific, M08410) in TBST for 1 h, and incubated with primary antibodies with blocking buffer overnight at 4 °C. Following 3 × 5 min washed in TBST, membranes were incubated with HRP-conjugated secondary antibodies at 1:3000 for 1 h at room temperature in blocking solution. Membranes were washed 3 × 5 min in TBST and the signal was developed using ECL prime (Cytivia, RPN2232) and detected using an Amersham Imager 600. Primary antibodies used: anti-tubulin (1:5000, DHB5, AA4.3), 5/31/18–44 μg/ml), anti-Hsp70 (1:5000, Sigma, 7TB-SAB520024-100ug, 141002), anti-MetI (1:5000, Proteinintech, 15073-1-AP, Ag7110), anti-HSP40 (1:5000, Enzo Life Sciences, ADI-SPA-400-D, 40624121), anti-stv (1:5000, Proteinintech, 13913-1-AP, Ag4905, validation included in Source Data File), anti-Hsp60 (1:10, DSHB-9G2, 10/18/18–42 μg/ml), anti-futsch (1:62, Synaptic systems, 202003, 2 μg/ml), anti-Agr4905 (1:20,000, anti-rabbit, gift from John Lis60,61). Rabbit anti-Ythdc1 and anti-puromycin (1:1,000, Kerafast, EQ0001, 200517), anti-hsf 13913-1-AP, Ag4905, validation included in Source Data File), anti-phospho-Hsp70 (1:5000, DSHB-42C10, 10/10/19–53 ug/ml), anti-drpr (1:400, DSHB-5D14, 6/22/17–36 μg/ml), anti-tubulin (1:5000, DHSB, AA4.3, 5/31/18–44 μg/ml), anti-futsch (1:600, DSHB-22C10, 10/10/19–53 μg/ml), anti-drpr (1:400, DSHB-SD14, 6/22/17–36 μg/ml), anti-purumycin (1:1000, Kerafast, EQ0001, 200517), anti-hsf (1:20,000, anti-rabbit, gift from John Lis60,61). Rabbit anti-Ythdc1 (1:5000), affinity purified rabbit antibody created by Vivitide against 18 residues of Ythdc1 (157–173 “CRTKPSNSADGHSKSD”). Secondary Antibodies used: Goat anti-mouse (1:5000, Jackson ImmunoResearch, 115-035-144, 138306), Goat anti-Rabbit (1:5000, Jackson ImmunoResearch, 111-035-144, 138306), Goat anti-rat (1:5000, Thermo Fisher Scientific, A10549, 2273679).

RNA extraction

Tissue was homogenized in 200 ul of Trizol (Thermo Fischer Scientific, 15596026) in RNase-free 1.5 ml microfuge tubes (Thermo Fischer Scientific, AM12400). About 800 ul of Trizol (Thermo Fischer Scientific, 15596026) was added to the tube and 200 ul of chloroform (Fisher, 15596026) in RNase-free 1.5 ml microfuge tubes (Thermo Fischer Scientific, 4368814). cDNA was then used for qPCR reactions set up with SYBR Green Fast Reagents, using 384-well plates on the Applied Biosystems ViiA7 machine. Primers used are in Supplementary Data 7. Mean fold-change was determined using the ΔΔCt method. Each experiment used technical triplicates as well as three biological replicates; Graphpad prism 8/9 software was used for statistics.

Stress sensitivity assay

Fly crosses were carried out at 25 °C. Adult flies were collected and aged to 6 days post eclosion or after 6 days on RU food. Flies were anesthetized and transferred to clear plastic 13 ml vials, and cotton was placed at the 4 ml mark on the vials to concentrate the flies near the bottom. Each vial contained 20 flies. Flies were allowed to recover for 30 min and then transferred to a water bath for mild non-lethal heat stress (30 min at 38.5 °C) or a longer heat shock (1–1.5 h at 38.5 °C) for a severe stressor to measure stress sensitivity survival. The flies were then transferred to normal food and allowed to recover overnight at 25 °C. After recovery, the percent of flies alive versus dead was recorded per vial.

LC-MS/MS analysis of m6A Levels

PolyA+ RNA was extracted from brains and heads using the NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB, E7490L). LC-MS/MS was conducted as previously described in ref. 62. All quantifications were performed by using the standard curve obtained from pure nucleoside standards running with the same group of samples. Then, the percentage ratio of m6A to A was used to compare the different modification levels.

Dot blot assay

Total RNA was collected from heads or whole flies using standard trizol chloroform extraction. n = 200 head per condition or n = 10 whole flies per condition. PolyA+ RNA was obtained using Dynabeads mRNA Direct Purification Kit (Ambion, 61011). Dots (total RNA or polyA+ RNA) were applied to an Amersham Hybond-N’ membrane (GE Healthcare, RN199B) in duplicate as 100 ng RNA per 1 ul dot. Dots were done on a Dry Membrane in a clean petri dish. The membrane was completely dried before RNA was crosslinked to the membrane using a UV Stratalinker 2400 by running the auto-crosslink program twice. The membrane was then washed in PBST three times 5 min each, blocked with 5% non-fat milk in PBST for 2 h. The membrane was incubated with primary anti-m6A antibody (1:1000, Synaptic Systems, 202003, 2–97) overnight at 4 °C. Then washed in PBST 3 × 5 min, incubated in HRP-conjugated anti-rabbit IgG secondary antibody (1:5000) for 2 h at room temperature, washed in PBST 3 × 5 min, and visualized using ECL prime. The membrane is washed in PBST and then incubated in methylene blue for 15 min, rinsed in PBST and imaged.

M6A-IP sequencing

Total RNA was extracted from 200 Drosophila heads per replicate using Trizol/chloroform extraction. PolyA+ mRNA was obtained using NEBNext Poly(A) mRNA Magnetic Isolation Module. PolyA+ RNA was fragmented using the NEB Next Magnesium Fragmentation Module (NEB, E6150S) for 4 min at 95 °C for a 250 ng sample of polyA+ RNA, and RNA was repurified using the Zymo RNA clean & concentrator –5 kit (Zymo, R1031). 10% of the fragmented polyA+ RNA was saved as input control for sequencing. m6A-immunoprecipitation was done using the EpiMark N6-Methyladenosine Enrichment kit protocol with some minor alterations described. About 30 ul of protein G-magnetic beads (NEB, #S1430) were washed and resuspended in IP buffer (150 mM NaCl, 10 mM Tris-HCl, 0.1% NP-40). About 1.4 ul of NEB m6A antibody (1:178, NEB, E1610S), or 4 ul of synaptic systems antibody (1:62, Synaptic systems, 202003, 2–97) was conjugated to protein G-magnetic beads (NEB, E161A, 10015190) for 2 h at 4 °C. Beads/antibody were washed twice in IP buffer. Approximately 1 μg PolyA+ RNA was incubated with beads/antibody in IP buffer supplemented with 0.1% SUPERase-In RNase Inhibitor (Thermo Fisher; AM2696) for 2 h at 4 °C. After incubation, RNA/beads/antibody are washed twice in IP buffer, twice in low salt IP buffer (50 mM NaCl, 10 mM Tris-HCl, 0.1%
NP-40), and twice in high salt IP buffer (500 mM NaCl, 10 mM Tris-HCL, 0.1% NP-40). RNA is eluted from beads with 25 μl of RLT buffer twice, and elution was pooled and concentrated using Zymo RNA clean and concentrator kit-5 (R1015). Libraries were made using SMARTer Stranded Total RNA-Seq Kit V2 without RNA depletion (Takara bio, 634411) for IPed and input RNA, and sequenced using Illumina HiSeq × series with 40 M paired-end reads (2 × 150 bp). Library preparation and sequencing was done by Admera Health. Three biological replicates per genotype and condition were done with NEB m6A antibody, and two biological replicates were done with Synaptic Systems m6A antibody.

M6A enrichment analysis

Regions of m6A enrichment were found for each condition using MetPeak (v.1.1) with default parameters, using the input and m6A pulldown bam files as input, and with the FlyBase FB2019.05 annotation provided. Peak locations (5′ UTR, CDS, or 3′ UTR) were defined from the regions indicated by MetPeak as having significant m6A enrichment. If a peak was not contained in one region (i.e., if the peak is partly in the CDS and partly in the 3′ UTR), it was assigned to the region where more of the peak resided.

Differential m6A peak analysis

Regions of differential methylation between two conditions (frequently called “Mettl3-dependent genes” or “m6A genes”) were found using RADAR (v.0.2.4) with input and m6A pulldown bam files as input, as well as the FlyBase FB2019.05 annotation. All replicates of SY5 antibody are used for differential peak calling. The minimum cutoff for bin filtering was 15, the cutoff was set as 0.05, and the Beta_cutoff was set as 0.5. Any region with an adjusted p value <0.05 was retained, and regions with a fold-change <−1 from the control (mCherry) to the knockout (Mettl3) at basal or heat shock (30 min) conditions were kept as dependent peaks. Non-Mettl3-dependent m6A were considered as all other genes expressed in the brain that did not have Mettl3-dependent m6A.

M6A metaplots, heatmaps, and genome browser visualization

Heatmaps and metagene plots showing the location of m6A enrichment on a specific set of genes were constructed with using heatmaps (v.1.0.12) and mrkITools (v.0.2.1). Specifically, the exons of all transcripts in each gene were collapsed using the GenomicRanges (v.1.44.0) function reduce. Genes with a 5′ UTR or 3′ UTR shorter than 30 base pairs, a CDS shorter than 100 bp or lacking a 5′ UTR (i.e., IncRNAs) were not considered in this analysis. For each gene, the 5′ UTR and 3′ UTR were tiled in 100 evenly spaced bins, and the CDS was tiled in 100 evenly spaced bins. The number of input and m6A reads overlapping each bin was calculated and this number was divided by the bin width and library size and a normalization factor of one million to produce a normalized read per million in each bin. The heatmaps show the enrichment of m6A above input in each bin by dividing the m6A coverage by the input coverage after adding replicates from the same condition and sample type together. For heat map normalization, reads per million are normalized by the size of the bin, total reads, and library size. For genome browser snapshots, tracks visualized as log2 (m6A/input) or separated input and m6A-IP tracks in supplementary figures. Tracks were made by first converting bam files to bigWig files using deepTools (v.3.5.1) bamCoverage using CPM normalization, then deepTools bigwigCompare with operation log2.

GO and pathway analysis

GO analysis for genes with non-Mettl3-dependent m6A or Mettl3-dependent m6A was conducted using FlyMine (v.53) with a max p value of 0.05. KEGG pathway analysis was done using the “enrichKEGG” function from ClusterProfiler (v.4.0.5) package in R. A list of all genes with detectable expression was used as background for both GO and pathway analysis.

Motif analysis

Motif enrichment for m6A obtained with pulldowns using either NEB or SYS antibodies was performed using HOMER (v.4.11) findMotifs.pl with the parameters len 5,6,7, FASTA files containing the sequence of the RNA base pairs under each peak were compared with FASTA comprising of background sequences, which were generated by taking random regions of expressed transcripts without an m6A peak that were length-matched to the peak sequences.

RNA-sequencing

Total RNA was extracted from brains using trizol/chloroform and Zymo RNA clean and concentrator kit-5 (R1015). The RNA-seq libraries from brains were prepared using the True-seq stranded mRNA library prep. Library preparation and sequencing was done by Admera Health, and sequenced using Illumina NovaSeq S4 with 40 M paired-end reads (2 × 150 bp). Three biological replicates were done for each experimental timepoint, condition, and genotype. Technical repeats and at least n = 3–6 biological replicates of at least 15 brains per replicate were done as indicated in Supplementary Data 8.

RNA-seq analysis

Raw paired-end fastqs were processed with TrimGalore (v.0.6.6) (https://github.com/FelixKrueger/TrimGalore) with default settings to remove Illumina adapters and mapped using STAR 2.7.3a to the Drosophila melanogaster genome annotation dm6. Unmapped and improperly paired reads were filtered out of aligned bam files. Reads per gene in the Flybase release 2019.05 were computed using an R script using GenomicRanges (v.1.44.0) summarizeOverlaps that counts the number of reads overlapping with the exons of each gene in the default “union” mode. Differential expression analysis was performed using DESeq2 (v.1.32.0), with count files produced by summarizeOverlaps as input. PCA plots were made using the plotPCA function in DESeq2, with variance stabilized counts as the input. MA plots were constructed from the adjusted p values and baseMean values output from DESeq2, and volcano plots were constructed from adjusted p values and fold changes reported by DESeq2. Normalized counts produced by DESeq2 were used to show expression levels. Differentially expressed genes were considered to be any gene with p-adjusted values of <0.05.

Actinomycin D assay

Brains from u641 (BL5905), mCherry RNAi, and Mettl3 RNAI adults were dissected and incubated in 250 ul of Schneider’s Drosophila Medium that was pre-warmed to 25 °C in RNAse-free 1.5 ml microfuge tubes. Fifteen to 20 brains were dissected for each timepoint. About 169.5 ug/ml of actinomycin D (Sigma-Aldrich, A1410) was added to each sample. Samples were incubated at 25 °C, shaking at 300 rpm for indicated timepoint, and mixed by pipetting every 30 min. After the incubation time, samples were collected, spun at room temp, washed in cold RNAse-free PBS, and processed in Trizol. Brain total RNA was collected using trizol/chloroform and Zymo RNA clean and concentrator kit-5 (R1015). RNA was then used for RT-qPCR. About 400 ng of RNA is used per cDNA reaction. cDNA is also quantified by Qubit ssDNA Assay (Invitrogen, Q10212) before use in RT-qPCR experiments.

Puromycin assay

Food was made by mixing 600 μM puromycin (Sigma-Aldrich, P8833-100MG) with 2% agar + 5% sucrose. Adult flies were plated onto puromycin food for 24 h. Brains were dissected from flies after 24 h of feeding and samples were processed for western blot analysis. Protein loading was determined by the Pierce BCA Protein Assay Kit (Pierce: #23235) with a protein concentration of 480 μg/ml. Protein loading was determined by the Pierce BCA Protein Assay Kit (Pierce: #23235) with a protein concentration of 480 μg/ml.
Quantification and statistical analysis
Statistical tests used were performed on GraphPad Prism (v.9) and are indicated in the figure legend. *P* values of <0.05 were considered significant. Unpaired two-tailed *t*-tests were used when comparing two groups; one-way ANOVA was used when comparing multiple groups, followed by Tukey’s post-test when each group was compared against every other group. Sidak’s post-test when pre-defined groups were compared to each other, or Dunnett’s test when compared to a defined control sample. Two-way ANOVA was used when there were two factors in the analysis (usually RNA amount and HS condition). Fisher’s exact test was used when comparing m^6^A vs non-m^6^A genes (Fig. 4). One-sided Hypergeometric test was used to compare Venn diagram overlaps.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The Raw sequencing data generated in this study have been deposited in the Gene Expression Omnibus under accession code GSE178955. Full motif analysis are available in Supplementary Data 1. The m^6^A peak RADAR data used in this study are available in Supplementary Data 2. Go term and Kegg pathway analysis are available in Supplementary Data 3. The differential gene expression tables from RNA-seq analysis generated in this study are provided in Supplementary Data 4 and 5. HS gene lists are provided in Supplementary Data 6. Fly genotypes and primers used are provided in Supplementary Data 7. Mapping rates for all sequencing experiments are provided in Supplementary Data 8. Any additional inquiries can be directed to the corresponding author. Source data are provided with this paper.

References
1. Muchowski, P. J. & Wacker, J. L. Modulation of neurodegeneration by molecular chaperones. Nat. Rev. Neurosci. 6, 11–22 (2005).
2. Haigis, M. C. & Yankner, B. A. The aging stress response. Mol. Cell 40, 333–344 (2010).
3. Leak, R. K. Heat shock proteins in neurodegenerative disorders and aging. J. Cell Commun. Signal 8, 293–310 (2014).
4. McGurk, L., Berson, A. & Bonini, N. M. Drosophila as an in vivo model for human neurodegenerative disease. Genetics 201, 377–402 (2015).
5. Genuth, N. R. & Barna, M. Heterogeneity and specialized functions of translation machinery: from genes to organisms. Nat. Rev. Genet. 19, 431–452 (2018).
6. Vihervaara, A., Duarte, F. M. & Lis, J. T. Molecular mechanisms driving transcriptional stress responses. Nat. Rev. Genet. 19, 385–397 (2018).
7. Batulan, Z. et al. High threshold for induction of the stress response in motor neurons is associated with failure to activate HSF1. J. Neurosci. 23, 5789–5798 (2003).
8. Korn, A., Ackermann, B., Clement, A. M., Duerk, H. & Behl, C. HSF1-controlled and age-associated chaperone capacity in neurons and muscle cells of C. elegans. PLoS ONE 5, e8568 (2010).
9. Hammarlund, M., Nix, P., Hauth, L., Jorgensen, E. M. & Bastiani, M. Axon regeneration requires a conserved MAP kinase pathway. Science 323, 802–806 (2009).
10. Biteau, B., Karpac, J., Hwangbo, D. & Jasper, H. Regulation of Drosophila lifespan by JNK signaling. Exp. Gerontol. 46, 349–354 (2011).
11. Wang, L. et al. JNK modifies neuronal metabolism to promote proteostasis and longevity. Aging Cell 18, e12849 (2019).
12. Emoto, K., Parrish, J. Z., Jan, L. Y. & Jan, Y.-N. The tumour suppressor Hippo acts with the NDR kinases in dendritic tiling and maintenance. Nature 443, 210–213 (2006).
13. Penney, J. et al. TOR is required for the retrograde regulation of synaptic homeostasis at the Drosophila neuromuscular junction. Neuron 74, 166–178 (2012).
14. Tang, H.-W. et al. mTORC1-chaperonin CCT signaling regulates mRNA methylation to suppress autophagy. Proc. Natl Acad. Sci. USA 118, e2021945118 (2021).
15. Stankiewicz, A. M., Swijngel, A. H. & Lisowski, P. Epigenetics of stress adaptations in the brain. Brain Res. Bull. 96, 76–92 (2013).
16. Sen, P., Shah, P. P., Nativio, R. & Berger, S. L. Epigenetic mechanisms of longevity and aging. Cell 166, 822–839 (2016).
17. Nativio, R. et al. Dysregulation of the epigenetic landscape of normal aging in Alzheimer’s disease. Nat. Neurosci. 21, 497–505 (2018).
18. Fu, Y., Dominissini, D., Rechavi, G. & He, C. Gene expression regulation mediated through reversible m^6^A mRNA methylation. Nat. Rev. Genet. 18, 293–306 (2014).
19. Zhao, B. S., Roundtree, I. A. & He, C. Post-transcriptional gene regulation by mRNA modifications. Nat. Rev. Mol. Cell Biol. 18, 31–42 (2017).
20. Meyer, K. D. DART-seq: an antibody-free method for global m^6^A detection. Nat. Methods 16, 1275–1280 (2019).
21. Lence, T., Soller, M. & Roignant, J.-Y. A fly view on the roles and mechanisms of the m^6^A reader DmYTHDC1 and its players. RNA Biol. 14, 1232–1240 (2017).
22. Xiao, W. et al. Nuclear m^6^A reader YTHDC1 regulates mRNA splicing. Mol. Cell 61, 507–519 (2016).
23. Shi, H. et al. YTHDF3 facilitates translation and decay of N^6^-methyladenosine-modified mRNA. Cell Res. 27, 315–328 (2017).
24. Slobodin, B. et al. Transcription impacts the efficiency of mRNA translation via co-transcriptional N^6^-adenosine methylation. Cell 169, 326–337.e12 (2017).
25. Zaccara, S. & Jaffrey, S. R. A unified model for the function of YTHDF proteins in regulating m^6^A-modified mRNA. Cell 181, 1582–1595.e18 (2020).
26. Meyer, K. D. et al. 5’UTR m^6^A promotes cap-independent translation. Cell 163, 999–1010 (2015).
27. Zhou, J. et al. Dynamic m^6^A mRNA methylation directs translational control of heat shock response. Nature 526, 591–594 (2015).
28. Xiang, Y. et al. RNA m^6^A methylation regulates the ultraviolet-induced DNA damage response. Nature 543, 573–576 (2017).
29. Yoon, K.-J. et al. Temporal control of mammalian cortical neurogenesis by m^6^A methylation. Cell 171, 877–889.e17 (2017).
30. Wang, X. et al. N^6^-methyladenosine-dependent regulation of messenger RNA stability. Nature 505, 117–120 (2014).
31. Anders, M. et al. Dynamic m^6^A methylation facilitates mRNA triaging to stress granules. Life Sci Alliance 1, e201800113 (2018).
32. Ries, R. J. et al. m^6^A enhances the phase separation potential of mRNA. Nature 571, 424–428 (2019).
33. Fu, Y. & Zhuang, X. m^6^A-binding YTHDF proteins promote stress granule formation. Nat. Chem. Biol. 16, 955–963 (2020).
34. Engel, M. et al. The role of m^6^A/m-RNA methylation in stress response regulation. Neuron 99, 389–403.e9 (2018).
35. Weng, Y.-L. et al. Epitranscriptomic m^6^A regulation of axon regeneration in the adult mammalian nervous system. Neuron 97, 313–325.e6 (2018).
36. Yu, J. et al. Dynamic m^6^A modification regulates local translation of mRNA in axons. Nucleic Acids Res. 46, 1412–1423 (2018).
37. Haussmann, I. U. et al. m^6^A potentiates Sxl alternative pre-mRNA splicing for robust Drosophila sex determination. Nature 540, 301–304 (2016).
38. Lence, T. et al. m^6^A modulates neuronal functions and sex determination in Drosophila. Nature 540, 242–247 (2016).
39. Kan, L. et al. A neural m^6^A/Ythdf pathway is required for learning and memory in Drosophila. Nat. Commun. 12, 1458 (2021).
40. Worpenberg, L. et al. Yfh1 is a N6-methyladenosine reader that modulates Fmr1 target mRNA selection and restricts axonal growth in Drosophila. *EMBO J.* 40, e104975 (2021).

41. Lindquist, S. The heat-shock response. *Annu. Rev. Biochem.* 55, 1151–1191 (1986).

42. Tower, J. Heat shock proteins and Drosophila aging. *Exp. Gerontol.* 46, 352–362 (2011).

43. Qi, X.-B., Shao, Y.-M., Miao, S. & Wang, L. The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. *Cell. Mol. Life Sci.* 63, 2560–2570 (2006).

44. Carra, S., Seguin, S. J., Lambert, H. & Landry, J. Hsp88 chaperone activity toward poly(Q)-containing proteins depends on its association with Bag3, a stimulus of macroautophagy. *J. Biol. Chem.* 283, 1437–1444 (2008).

45. Meyer, K. D. et al. Comprehensive analysis of mRNA methylation reveals enrichment in 3' UTRs and near stop codons. *Cell* 149, 1635–1646 (2012).

46. Wang, Y. et al. Role of Hakai in m6A modification pathway in Drosophila. *Nat. Commun.* 12, 2159 (2021).

47. Zhang, Z. et al. RADAR: differential analysis of MeRIP-seq data with a random effect model. *Genome Biol.* 20, 294 (2019).

48. Duarte, F. M. et al. Transcription factors GAF and HSF act at distinct regulatory steps to modulate stress-induced gene activation. *Genes Dev.* 30, 1731–1746 (2016).

49. Larkin, A. et al. FlyBase: updates to the Drosophila melanogaster knowledge base. *Nucleic Acids Res.* 49, D899–D907 (2021).

50. Knuckles, P. et al. RNA fate determination through cotranscriptional adenosine methylation and microprocessor binding. *Nat. Struct. Mol. Biol.* 24, 561–569 (2017).

51. Zamurrad, S., Hatch, H. A. M., Drelon, C., Belalcazar, H. M. & Secombe, J. A. Drosophila model of intellectual disability caused by mutations in the histone demethylase KDM5. *Cell Rep.* 22, 2359–2369 (2018).

52. Parsell, D. A. & Lindquist, S. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* 27, 437–496 (1993).

53. Coots, R. A. et al. m6A facilitates eIF4F-independent mRNA translation. *Mol. Cell* 68, 504–514.e7 (2017).

54. Liu, J. et al. Landscape and regulation of m6A and m5Am methylome across human and mouse tissues. *Mol. Cell* 77, 426–440.e6 (2020).

55. Wang, Y. et al. N6-methyladenosine RNA modification regulates embryonic neural stem cell self-renewal through histone modifications. *Nat. Neurosci.* 21, 195–206 (2018).

56. Lee, J.-H. et al. Enhancer RNA m6A methylation facilitates transcriptional condensate formation and gene activation. *Mol. Cell* 81, 3368–3385.e9 (2021).

57. Liu, F. et al. m6A-independent genome-wide METTL3 and METTL14 redistribution drives the senescence-associated secretory phenotype. *Nat. Cell Biol.* 23, 355–365 (2021).

58. Perkins, L. A. et al. The transgenic RNAi project at Harvard Medical School: resources and validation. *Genetics* 201, 843–852 (2015).

59. Kennerdell, J. R., Liu, N. & Bonini, N. M. MiR-34 inhibits polycomb repressive complex 2 to modulate chaperone expression and promote healthy brain aging. *Nat. Commun.* 9, 4188 (2018).

60. Shopland, L. S., Hirayoshi, K., Fernandes, M. & Lis, J. T. HSF access to heat shock elements in vivo depends critically on promoter architecture defined by GAGA factor, TFIIID, and RNA polymerase II binding sites. *Genes Dev.* 9, 2756–2769 (1995).

61. Westwood, J. T., Clos, J. & Wu, C. Stress-induced oligomerization and chromosomal relocalization of heat-shock factor. *Nature* 353, 822–827 (1991).

62. Ontiveros, R. J. et al. Coordination of mRNA and tRNA methylations by TRMT10A. *Proc. Natl Acad. Sci. USA* 117, 7782–7791 (2020).

63. Cui, X., Meng, J., Zhang, S., Chen, Y. & Huang, Y. A novel algorithm for calling mRNA m6A peaks by modeling biological variances in MeRIP-seq data. *Bioinformatics* 32, i378–i385 (2016).

64. Zhang, Z. et al. Genetic analyses support the contribution of mRNA N6-methyladenosine (m6A) modification to human disease heritability. *Nat. Genet.* 52, 939–949 (2020).

65. Ramirez, F. et al. deepTools2: a next generation web server for deep-sequencing data analysis. *Nucleic Acids Res.* 44, W160–W165 (2016).

66. Smith, R. N. et al. InterMine: a flexible data warehouse system for the integration and analysis of heterogeneous biological data. *Bioinformatics* 28, 3163–3165 (2012).

67. Yu, G., Wang, L.-G., Han, Y. & He, Q.-Y. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* 16, 284–287 (2012).

68. Heinz, S. et al. Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. *Mol. Cell* 38, 576–589 (2010).

69. Dobin, A. et al. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* 29, 15–21 (2013).

70. Lawrence, M. et al. Software for computing and annotating genomic ranges. *PLoS Comput. Biol.* 9, e1003118 (2013).

71. Love, M. I., Huber, W. & Anders, S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15, 550 (2014).

**Acknowledgements**

We thank China N. Byrns and Ananth R. Srinivasan for critical manuscript feedback; M. Kayser, K. Jordan-Sciutto, G. Ming, and B. Gregory for valuable input. We thank the Drosophila Genomics Resource Center, supported by NIH grant 2P40OD010949, and the Bloomington Drosophila Stock Center (NIH P40OD018537) for fly lines. We thank the Transgenic RNAi Project (TRIP) at Harvard Medical School (NIH/NIGMS R01-GM084947) for developing transgenic RNAi fly stocks used in this study. This work was supported by T32-GM007229 and F31-AG063470 (to A.E.P.), R35-GM133721 (to K.F.L.), and R35-NS097275 (to N.M.B.).

**Author contributions**

A.E.P. conceived, designed and performed experiments, statistical analysis, bioinformatic analysis, and analyzed data. E.J.S. designed and performed bioinformatic analysis. H.S. performed mass spec experiments. K.F.L. supervised research and analyzed data. N.M.B. conceived, designed experiments, analyzed data, and supervised the research. E.J.S. and K.F.L. gave input and edited the manuscript. A.E.P. and N.M.B. wrote the manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

**Supplementary information** The online version contains supplementary material available at https://doi.org/10.1038/s41467-022-33085-3.

**Correspondence** and requests for materials should be addressed to Nancy M. Bonini.

**Peer review information** Nature Communications thanks the anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

**Reprints and permission information** is available at http://www.nature.com/reprints

**Publisher’s note** Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
