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RESEARCH PAPER

Noncanonical cytoplasmic poly(A) polymerases regulate RNA levels, alternative RNA processing, and synaptic plasticity but not hippocampal-dependent behaviours

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ABSTRACT

Noncanonical poly(A) polymerases are frequently tethered to mRNA 3′ untranslated regions and regulate poly(A) tail length and resulting translation. In the brain, one such poly(A) polymerase is Gld2, which is anchored to mRNA by the RNA-binding protein CPEB1 to control local translation at postsynaptic regions. Depletion of CPEB1 or Gld2 from the mouse hippocampus results in a deficit in long-term potentiation (LTP), but only depletion of CPEB1 alters animal behaviour. To test whether a related enzyme, Gld4, compensates for the lack of Gld2, we separately or simultaneously depleted both proteins from hippocampal area CA1 and again found little change in animal behaviour, but observed a deficit in LTP as well as an increase in long-term depression (LTD), two forms of protein synthesis-dependent synaptic plasticity. RNA-seq data from Gld2, Gld4, and Gld2/Gld4-depleted hippocampus show widespread changes in steady state RNA levels, alternative splicing, and alternative poly(A) site selection. Many of the RNAs subject to these alterations encode proteins that mediate synaptic function, suggesting a molecular foundation for impaired synaptic plasticity.

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Introduction

In the mammalian brain, experience-induced changes in protein synthesis control the strength of synaptic connections, which in turn underlie learning and memory [1–3]. These activity-dependent alterations in mRNA translation can occur in dendritic shafts and spines and promote synaptic plasticity [4,5]. At Schaffer-collateral CA1 synapses in the hippocampus, translation is required for multiple forms of synaptic plasticity including late-phase long-term potentiation (L-LTP), metabotropic glutamate receptor-mediated long-term depression (mGluR-LTD), and N-methyl-D-aspartate receptor (NMDAR)-regulated LTD [6,7]. Translation of specific mRNAs is induced by chemical or electrophysiological stimulation as well as by learning [8–10] and can be regulated by cytoplasmic polyadenylation. Dormant mRNAs have short poly(A) tails, which when elongated following synaptic stimulation, promote translation [11,12]. A key factor controlling polyadenylation is cytoplasmic polyadenylation binding protein 1 (CPEB1), an RNA-binding protein with a strong avidity for the cis-acting cytoplasmic polyadenylation element (CPE) that resides in 3′ untranslated regions (UTRs) of target mRNAs. CPEB1 regulates poly(A) tail length interacting with deadenylating enzymes as well as noncanonical poly(A) polymerases; whether CPEB1 binds a deadenylating enzyme, favouring short poly(A) tails and translational dormancy, or noncanonical poly(A) polymerases, favouring elongated poly(A) tails and translation, depends on its phosphorylation on T171 [13,14]. Generally, synaptic stimulation promotes this phosphorylation event, which in turn stimulates polyadenylation and translation [12,15].

Immunocytochemistry has shown that key components of the cytoplasmic polyadenylation machinery reside at postsynaptic sites of hippocampal neurons; these include CPEB1, the scaffold protein symplekin, the deadenylase poly(A) ribonuclease (PARN), the noncanonical poly(A) polymerase germ line defective 2 (Gld2) (also called TENT2, TUTase2, TUT2, PAPD4), and the CPEB1-interacting factor neuroguidin (Ngd); this latter protein is a director repressor of translation [15–17]. These factors control the polyadenylation and translation of such RNAs as the *N*-methyl-D-aspartate receptor (NMDAR) GluN2A, which mediates certain forms of synaptic plasticity [15,17]. Based on these and other data, it is not surprising that ablation of *Cpeb1* results in a deficit in theta burst stimulation (TBS) of LTP [6,18] as well as impaired hippocampal-dependent memories [19]. Depletion of Gld2 from the dentate gyrus of the hippocampus also results in a deficit in TBS-LTP [15], which would be expected if this enzyme promoted translation. Indeed, this impaired plasticity is nearly identical to that observed when anisomycin, a protein synthesis inhibitor, is applied to hippocampal preparations [15]. Conversely, depletion of Ngd results in elevated LTP, which again would be expected if translation is elevated by removal of a repressor protein [15]. Depletion of

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Supplemental data for this article can be accessed here.

PARN had no effect on LTP, which may be due to compensation by another deadenylating enzyme in the hippocampus [20].

Because Gld2 is essential for normal synaptic efficacy at least in the dentate gyrus of the hippocampus, we surmised that this enzyme would also be necessary for hippocampal-dependent behaviours, yet its ablation had no demonstrable effect on higher cognitive function [21]. However, a second related enzyme, Gld4 (also called TENT4B, PAPD5, TUTase3, TUT3) also associates with CPEB1 and promotes polyadenylation-induced translation in cultured cells and thus might work similarly in the brain to thereby compensate for the absence of Gld2 in the brain [22,23]. To assess this possibility, we depleted Gld2, Gld4, or both Gld2 and Gld4 by injecting adeno-associated viruses (AAVs) expressing shRNAs targeting these mRNAs bilaterally into hippocampi of mice, which was followed by assays for several behavioural responses. In only one case, which was when both Gld2 and Gld4 were depleted, was there a mild effect on behaviour, yet this double Gld2/Gld4 depletion resulted in a strong deficit in TBS-LTP and an enhancement of NMDAR-dependent LTD, two protein synthesis-dependent forms of synaptic plasticity. These data indicate an uncoupling of behaviour from synaptic efficacy. We further performed RNA-seq from Gld2, Gld4, and Gld2/Gld4 depleted hippocampus and found heretofore unknown activities of these enzymes; they regulate steady state RNA levels, alternative splicing, and alternative poly(A) site selection. The preponderance of RNAs that are altered by depletion of these enzymes encode proteins with neural functions especially those related to synaptic function. Our results indicate underlying molecular deficits that account for impaired synaptic function.

Results

Gld2 and Gld4 depletion have negligible effects on hippocampal-dependent behaviours

Despite the observation that Gld2 depletion from the dentate gyrus of the hippocampus causes a deficit in TBS-LTP [15], we were unable to detect behavioural abnormalities in global Gld2 KO mice (assays were marble burying, elevated plus maze, open field test, T maze, novel object recognition, Morris water maze, and passive avoidance memory test) [21]. We surmised that a second noncanonical poly(A) polymerase, Gld4, might compensate for the lack of Gld2. To test this possibility, we injected AAVs expressing shRNAs for a non-specific sequence (i.e., scrambled control), Gld2, Gld4, or both Gld2 and Gld4 bilaterally into the hippocampal region CA1 of mice, which was followed 3 weeks later by a battery of behavioural assays including elevated plus maze (anxiety), open field test (anxiety, locomotion), novel object recognition (memory), marble burying (anxiety, obsessive-compulsive, and/or repetitive-like behaviours), three-chamber sociability test (social interactions), T maze (working memory), and passive avoidance (emotional learning and memory). A double depletion of Gld2 and Gld4 lowered marble-burying efficacy, suggesting a reduction in repetitive behaviours (Fig. 1). With this exception, however, there was no statistical difference in any other behaviour relative to the scrambled control. Therefore, we surmise that neither Gld2 nor Gld4 depletion in hippocampal area CA1 has a measurable effect on these behaviours in the hippocampus. However, changes in behaviours that are dependent on Gld2 and/or Gld4 expression throughout the hippocampus and not solely in area CA1 may not have been revealed in this analysis.

Noncanonical poly(A) polymerase regulation of TBS-LTP and Glycine-LTD

Gld2 depletion by lentivirus-expressing shRNA resulted in a deficit in TBS-LTP in the hippocampal dentate gyrus [15]. To determine whether a double Gld2/Gld4 knockdown alters synaptic plasticity, we injected hippocampal area CA1 with AAVs expressing shRNAs for both enzymes and compared the synaptic response to animals injected with shRNA for a scrambled control. Fig. 2A shows that simultaneous depletion of both enzymes did not affect basal synaptic transmission but resulted in impaired TBS-LTP (Fig. 2B), similar to what was observed with Gld2 depletion alone or when wild type hippocampal slices were treated with anisomycin, a protein synthesis inhibitor [15]. Therefore, depletion of Gld2 and Gld4 alters TBS-LTP in a manner similar to Gld2 depletion alone or by inhibiting protein synthesis. The deficit in LTP could be consequence of a deficient induction mechanism, as demonstrated by a decreased envelope of TBS-induced depolarization (Fig. 2B insert). We also tested glycine-induced LTD (Fig. 2C) and found an enhancement in double Gld2/Gld4 knockdown mice. Glycine modulation requires activation of post-synaptic N-methyl-D-aspartate receptors (NMDAR) and metabotropic glutamate receptors (mGluR) as well as the subsequent activation of extracellular signal-regulated kinase (ERK) 1/2 [24,25]. Unlike glycine-induced LTP, glycine-induced LTD recruits – in addition to NMDAR – extrasynaptic glycine receptors (GlyR) and causes NMDAR endocytosis [26]. Our findings suggest that depletion of Gld2 and Gld4 could impair synaptic potentiation and facilitate synaptic depression via modulation of NMDAR expression. Notably, NMDAR-dependent LTD, but not mGluR-dependent LTD, was enhanced in the CPEB1 KO mouse [6].

Gld2 and Gld4 depletion alter RNA steady-state levels

We performed RNA-seq to determine whether Gld2 and/or Gld4 depletion alters RNA levels in the hippocampus. The volcano plots in Fig. 3A show that Gld2 depletion results in over 1000 RNAs with a statistically significant increase and hundreds with a significant decrease relative to a scrambled (non-specific, NS) shRNA control depletion (Gld2 vs. NS) (fold change >1.5, padj<0.05). However, Gld4 depletion had no observable effect (Gld4 vs. NS). A double depletion of Gld2 and Gld4 compared to NS (Gld2_4 vs NS) altered RNA expression similar to Gld2 depletion alone (Gld2_4 vs Gld2). Gld2/Gld4 depletion compared to Gld4 depletion (Gld2/4 vs Gld4) was similar to Gld2 depletion vs NS. Finally, Gld4 vs Gld2 showed that >1000 RNAs were down-regulated (i.e., the reverse image of Gld2 vs NS). These data show that Gld2 depletion alters RNA levels but that Gld4 depletion has little effect. Simultaneous Gld2/Gld4 depletion has little effect beyond

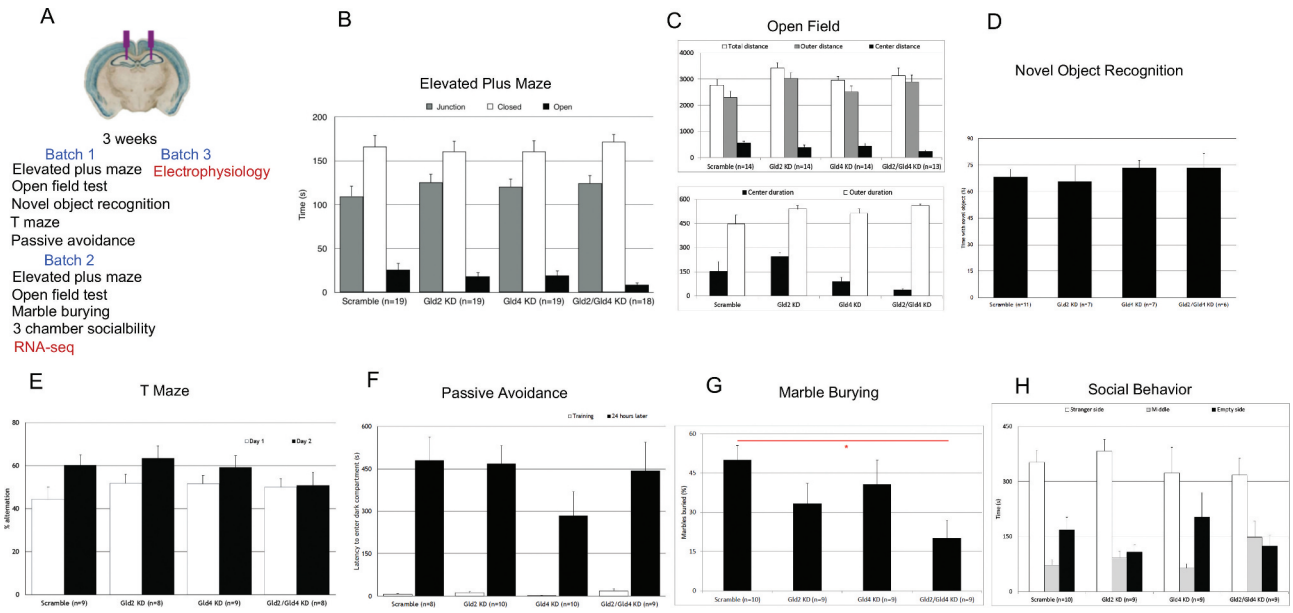

Figure 1. Effects of noncanonical poly(A) polymerase depletion from the hippocampus. A. AAV9 vectors expressing a scrambled (i.e., non-specific) control sequence or shRNAs for Gld2 or Gld4 in various combinations were injected bilaterally into the mouse hippocampus in 3 separate batches. Batch 1 animals were analysed for various behaviours as were batch 2 animals, but in this case, the animals were sacrificed and the hippocampus processed for RNA-seq. Batch 3 animals were processed for electrophysiology. B. Batch 1 and 2 animals injected with scrambled (n = 19), Gld2 shRNA (n = 19) (KD, or knockdown), Gld4 shRNA (n = 19) or a combination of Gld2 and Gld4 shRNAs (n = 18) were examined on an elevated plus maze for time spent at the closed or open arms or at the junction. C. Batch 1 and 2 animals were subjected to an open field test and assessed for total distance moved, outer distance, centre distance, duration in the centre or duration on the outer periphery (scrambled n = 14; Gld2 shRNA, n = 14; Gld4 shRNA n = 14; Gld2/4 shRNA n = 13). D. Batch 1 animals were assessed for novel object recognition (scrambled n = 11; Gld2 shRNA n = 7; Gld4 shRNA n = 7; Gld2/4 shRNA n = 5). E. Batch 1 animals were examined in a T maze for day 1 and 2 (scrambled n = 9; Gld2 shRNA n = 8; Gld4 shRNA n = 9; Gld2/4 shRNA n = 8). F. Batch 1 animals were assessed in a passive avoidance chamber during training and 24 hours later (scrambled n = 8; Gld2 shRNA n = 10; Gld4 shRNA n = 10; Gld2/4 shRNA n = 9). G. Batch 2 animals were examined for marble burying capacity (scrambled n = 10; Gld2 shRNA n = 9; Gld4 shRNA n = 9; Id2/4 shRNA n = 9). * $p < 0.05$, one-way non-parametric ANOVA. H. Batch 2 animas were assessed for social behaviour in a 3-chamber sociality task; time in the stranger side, the middle, or the empty side was determined (scrambled n = 10; Gld2 shRNA n = 9; Gld4 shRNA n = 9; Gld2/4 shRNA n = 9).

Gld2 depletion alone. The Venn diagrams in Fig. 3B demonstrate that generally, the levels of the same RNAs are altered by depletion of Gld2 and Gld2 plus Gld4. That mostly the same RNAs are affected by Gld2 and Gld2 plus Gld4 depletion is also illustrated in the heat map in Fig. 3C. However, it is evident that the combination of Gld2 and Gld4 depletion causes a greater decrease (cluster 3) and increase (cluster 1) of RNAs than Gld2 depletion alone. Therefore, although ablating Gld4 from hippocampal area CA1 does not substantially impact RNA levels, Gld2 depletion dramatically affects RNA expression. Depleting both Gld2 and Gld4 from this region further affects the magnitude of RNA level changes with little effect on the specificity of the RNAs involved,

Gene ontology (GO) analysis of the RNAs down-regulated by Gld2 or by Gld2 plus Gld4 depletion shows they control a spectrum of neural functions including regulation of synapse vesicle activity, ion transport, cognition, learning and memory, and many others (**Figure S1**). In addition, GO analysis specifically for RNAs in cluster 3 (cf heatmap in Fig. 3), which illustrates an enhanced effect of a Gld2 plus Gld4 depletion relative to Gld2 depletion alone, again shows enrichment for RNAs affecting learning and memory, synapse function, and other neural activities (**Figure S2**). These results complement the observation that depletion of Gld2 plus Gld4 cause impaired synaptic transmission, but surprisingly, not animal behaviour. A Kyoto Encyclopaedia of Genes and Genomes (KEGG) analysis, which indicates related cellular and molecular pathways, of cluster 3 also shows a preponderance of neural activities including calcium signalling, GABAergic synapse, among others (**Figure S2**). These data indicate that mostly Gld2, but Gld4 as well, mediate the expression of factors involved in neural function (e.g., regulation of neurotransmitter transport, synaptic vesicle localization, neuroactive ligand–receptor interaction, GABAergic synapse).

Interestingly, the RNAs that are up-regulated following Gld2 alone or a combination of Gld2 plus Gld4 depletion encode factors involved in the immune response (**Figure S3**). This inflammatory response appears to be specific for Gld2 depletion because these RNAs were not identified when comparing Gld2 plus Gld4 depletion relative to Gld4 depletion alone. Although we do not know the cell type that is responsible for this inflammation, it is likely to be microglia and/or astrocytes. Brain inflammation is linked to a number of neurologic disorders including Alzheimer's Disease [27], Down's Syndrome [28], learning disabilities [29], as well as synaptic plasticity [30].

Gld2 and Gld4 control RNA processing

A further analysis of our RNA-seq results shows that Gld2 and Gld4 mediate alternative RNA processing, particularly exon skipping. Fig. 4 demonstrates that depletion of Gld2 especially but Gld4 as well results in significant exon exclusion (i.e., an exon is skipped more frequently when the enzymes are depleted), or inclusion (i.e., an exon is included more frequently when the enzymes are depleted, relative to

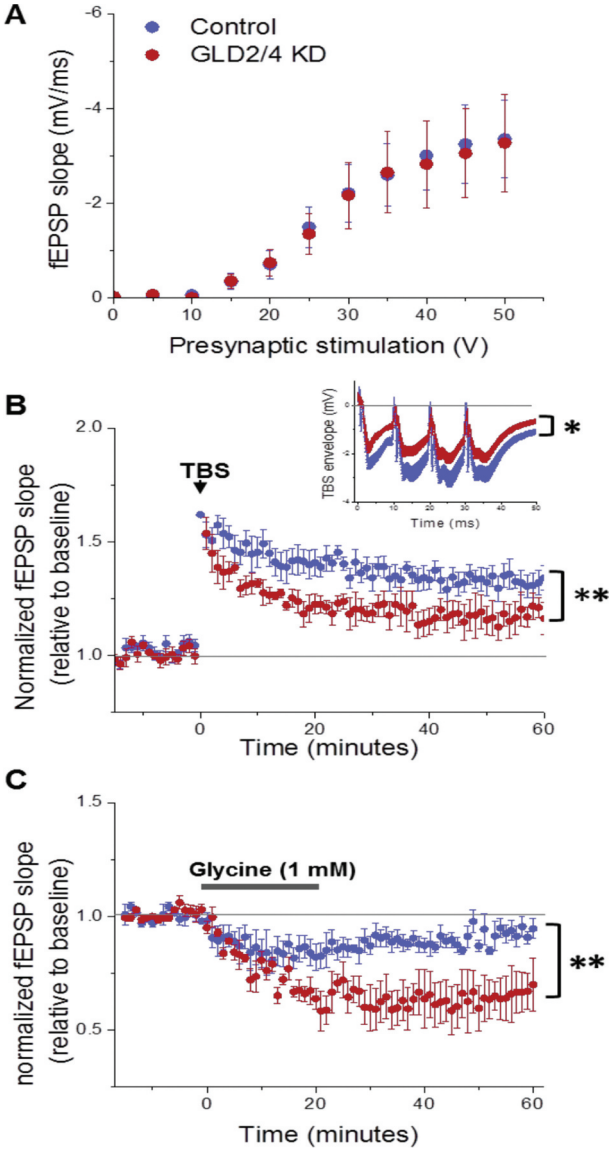

Figure 2. Effect of noncanonical poly(A) polymerase depletion on plasticity of hippocampal Schafer collateral CA1 synapses. AAV9 vectors expressing a scrambled control sequence or shRNAs for Gld2 and Gld4 were injected bilaterally into the mouse hippocampus. A. Stimulus–response relationship of Schaffer Collateral to CA1 (SC-CA1) synaptic responses from Control scrambled and Gld2/4 shRNA mice. No effect is found on the relationship between presynaptic stimulus strength and the magnitude of postsynaptic response. B. Decreased TBS-LTP at SC-CA1 synapses in Gld2/4 shRNA compared to control (scrambled shRNA-injected) mice. Insert: Decreased envelope of TBS-induced depolarization (average of the nine bursts of the TBS induction protocol) in Gld2/4 shRNA compared to control scrambled mice. C. Enhanced glycine-LTD at SC-CA1 synapses in Gld2/4 shRNA compared to control scrambled mice. Data are expressed as mean ± s.e.m (n = 5 mice). The fEPSP slopes acquired during distinct time intervals after LTP or LTD induction (i.e., 20–30, 60–90, 150–180 min, etc.) were averaged for each group and compared using analysis of variance (MICROCAL ORIGIN statistical tool, Microcal Software Inc.). *, $p < 0.05$; **, $p < 0.01$.

scrambled shRNA. **Table S1** shows that depletion of Gld2 in particular results in alternative splicing, exon exclusion and inclusion, of neural RNAs involved in synaptic function (e.g. postsynaptic specialization, glutamate receptor signalling pathway, synaptic vesicle recycling).

Finally, we also assessed alternative poly(A) (APA) site selection, which dictates the length of 3′UTRs. 3′UTRs encode information that drive localization of RNAs to specific compartments such as dendrites, as well as regulate translation [31]. Fig. 5A demonstrates that depletion of Gld2, but also modestly of Gld4, leads to differential use of proximal or distal poly(A) sites (i.e., AAUAAA sequences that are recognized by the nuclear poly(A) site cleavage and polyadenylation factors) [32]. A double Gld2 and Gld4 depletion has little additive effect relative to Gld2 or Gld4 depletion alone. Importantly, GO analysis shows that depletion of Gld2 in particular affects RNAs involved in neural function such as synapse organization and structure (Fig. 5B). Interestingly, depletion of Gld2 also affects factors involved in RNA splicing, suggesting that exon skipping may be a secondary effect of alternative poly(A) site selection by Gld2 depletion.

Discussion

A central tenet of neuroscience is the concept that memories are formed and stored in synaptic connections, which in turn guide animal behaviour [33–35]. Changes in these connections, synaptic plasticity, in turn modify those behaviours. Results presented in 15,and in this study (Fig. 2) show that depletion of Gld2, or Gld2 plus Gld4, results in the impairment of two forms of protein synthesis-dependent plasticity in the hippocampal dentate gyrus and area CA1: TBS-LTP and glycine-LTD. In spite of these observations, we found both here and in previous work [21] that depleting these enzymes has almost no effect on hippocampal-dependent behaviour. Expression of a dominant-negative (i.e. catalytically inactive) but not full-length Gld2 in *Drosophila*, however, impairs long-term memory [36].

Although a connection between learning and memory performance and synaptic plasticity is compelling [37], whether specific mechanisms of plasticity support particular traits of learning and memory behaviour still is an open question [2]. Behaviour is the result of coordinated activity across neural networks and systems [38]. Inhibition of certain molecular and/or cellular functions in restricted brain regions (e.g. hippocampal region CA1) may yield specific but modest behavioural alterations compared to broader (e.g. hippocampus-wide) manipulations. Moreover, manipulations that modulate synaptic function (and that could be compensated for) could have a lesser impact on behaviour than those that eliminate synaptic function altogether. Our data indicate that depleting both Gld2 and Gld4 in hippocampal area CA1 alters an important fraction of mRNAs associated with synaptic activity and learning and memory. Our functional (electrophysiological) data support the gene expression findings in that a form of NMDAR-dependent LTP and a form of NMDAR-dependent LTD are decreased and enhanced, respectively. This bidirectional LTP/LTD shift could still allow for changes in synaptic weight to occur within an operational range in response to learning [39–41], albeit with a reduced clearance for synaptic potentiation and an augmented one for synaptic depression. Notably, some studies suggest that genetically modified animals that show reduced hippocampal LTP and augmented hippocampal LTD exhibit impaired long-term spatial reference memory but enhanced spatial working

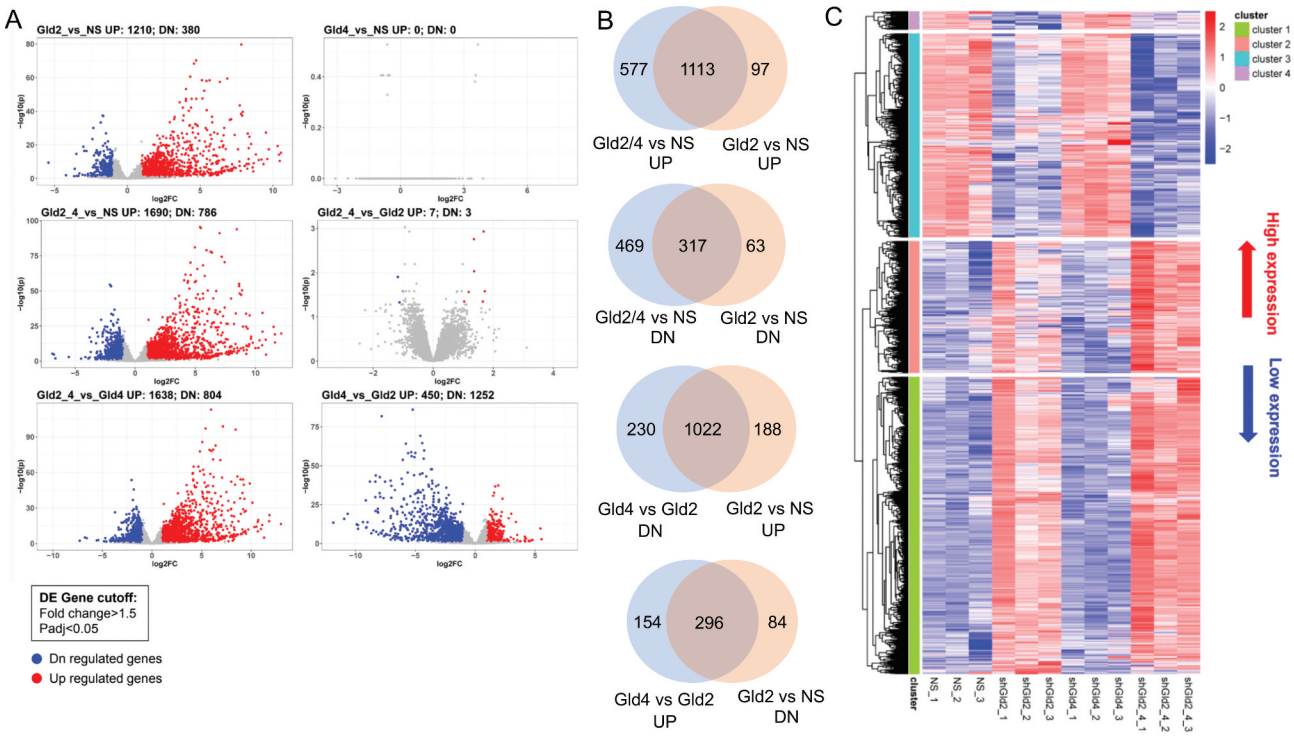

Figure 3. Noncanonical poly(A) polymerases regulate steady state RNA levels. A. Volcano plots demonstrating RNAs that are elevated (red) or reduced (blue) following injection of scrambled (NS), Gld2 shRNA, Gld4 shRNA, or Gld2 and Gld4 (Gld2_Gld4) shRNAs. Multiple pair-wise comparisons are shown; the number of RNAs up or down regulated are indicated (all changes >1.5 fold; padj <0.05). B. Venn diagrams illustrating the extent of overlap for RNAs up and down regulated in multiple pair-wise comparisons. C. Cluster analysis presented as a heat map of RNAs up and down regulated (cluster 1, 1430 RNAs; cluster 2, 634 RNAs; cluster 3, 980 RNAs; cluster 4, 88 RNAs). The colour bar at right indicates log2 fold change. Biologic triplicates for each condition are presented.

memory [42,43]. The near absence of overt behavioural alterations upon Gld2/Gld4 depletion could be the result of the delicate role these translational regulators have on synaptic function, specifically, on maintaining an operational plasticity range. Interestingly, animals that lack the translational regulator FMRP show changes in plasticity yet roughly normal spatial learning and memory until challenged with high demand memory tasks that test for cognitive flexibility [44]. Therefore, by analogy, it is possible Gld2 and/or Gld4 may be most in demand when supporting synaptic function that underlies complex memory tasks.

We ran a battery of widely used standard behaviours to characterize Gld2 and Gld4 depletion in area CA1 of the hippocampus and found no overt phenotype. Among the various possibilities discussed here, we subscribe to the idea that translational control may directly contribute to the capacity of synapses to manage information [45]. Behavioural measures might look normal until that capacity is overwhelmed; a notion that invites a new set behavioural studies to bridge the gap between translational control and behaviour.

We report several important molecular findings in this study. First, Gld2 depletion dramatically affects RNA levels in the hippocampus, which we surmise is probably at the level of stability rather than transcription because this enzyme modulates poly(A) tail length that in turn controls RNA degradation [46]. Gld2 could be directly affecting RNA levels through tethering to transcripts by RNA-binding proteins such as CPEB1 [15,22], however, it should be borne in mind that there are four members of the CPEB family of proteins [47], any or all of which could tether Gld2. Moreover, other RNA binding proteins with different sequence specificities could also tether Gld2 to RNA [48]. Second, Gld2 controls alternative splicing, but whether the enzyme acts directly on pre-mRNA substrates or through other effector molecules is unknown. Gld2 is predominantly cytoplasmic [22,49], but it may also shuttle between nucleus and cytoplasm [50,51] and thus could act on pre-mRNA. Third, Gld2 controls alternative poly[A] site selection, but the underlying mechanism remains is unclear. Importantly, 51,have shown that the related nuclear protein TUT-1 (Star-PAP], a related nuclear protein, also modulates alternative poly-(A) site selection, possibly by associating with the nuclear cleavage and polyadenylation specificity factor (CPSF) and or cleavage stimulatory factor (CSF), which are multi-component complexes that recognize and catalyse RNA cleavage 20–30 nucleotides downstream of the hexanucleotide recognition sequence AAUAAA [32]. Fourth, Gld4 has substantially less activity in the hippocampus compared to Gld2, but appears to potentiate Gld2 activity in that simultaneously depleting Gld2 and Gld4 has a slight additive effect compared to Gld2 depletion alone (c.f., marble-burying behaviour (Fig. 1G), and the reduced RNA levels (Fig. 3C)). It is also important to note that Gld2 and/or Gld4 can 3′ monoadenylate and thereby stabilize miRNAs [21,52] and add uridine and other residues to mRNAs to control rate of degradation [46,53,54]. Indeed, these and other members of the nucleotidyltransferase superfamily have a number of activities [55].

The observation that depleting both Gld2 and Gld4 impairs two forms of impaired synaptic plasticity may be related to

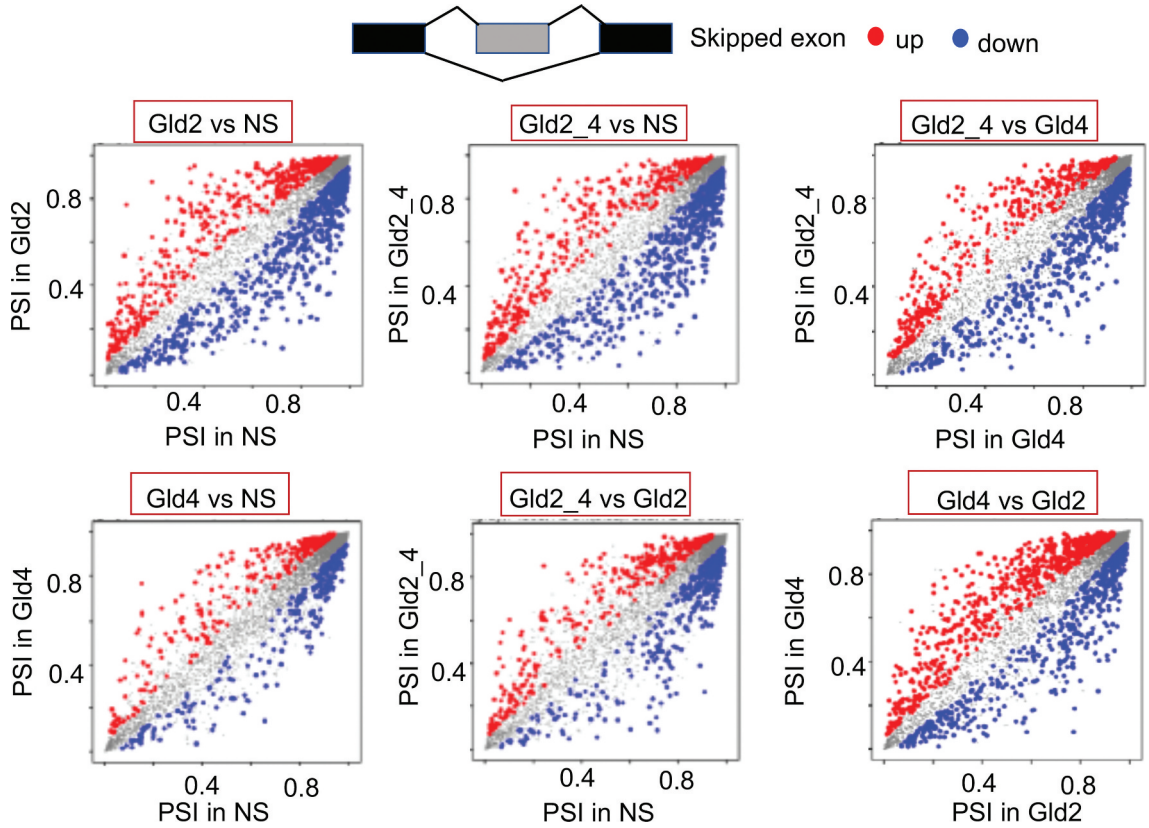

Figure 4. Noncanonical poly(A) polymerases regulate exon alternative RNA processing. Multiple pair-wise comparisons of skipped exons following noncanonical poly(A) polymerase depletion. Gld2 vs scrambled control (NS) (No change, NC, grey 3986 RNAs, skipped exons (blue), 669; included exons (red) 440); Gld4 vs NS (NC (grey), 5530; skipped exons (blue) 270; included exons (red) 270; Gld2/4 vs NS (NC, grey 3982; skipped exons (blue) 638; included exons (red) 391); Gld2/4 vs Gld2 (NC, grey 4396; skipped exons (blue) 385; included exons (red) 352); Gld2/4 vs Gld4 (NC, grey 4319; skipped exons (blue) 635; included exons (red) 421); Gld4 vs Gld2 (NC, grey 43,239; skipped exons (blue) 518; included exons (red) 704). PSI, percent spliced in.

the aberrant expression of numerous RNAs involved in synaptic function. For example, GO analysis shows that Gld2 depletion results in reduced mRNAs regulating membrane potential, calcium homoeostasis, and neurotransmitter transport (Figure S1). Gld2 depletion also causes alternate splicing changes of mRNAs regulating postsynaptic specialization organization, regulation of postsynaptic membrane neurotransmitter receptors levels, and presynaptic endocytosis (Table S1). Further, depleting Gld2 alters 3′ poly(A) site selection of RNAs encoding synapse organization, positive regulation of neuron projection organization, and axogenesis (Fig. 5B). Gld4 depletion causes similar, albeit less dramatic, changes in RNAs with related functions. These observations suggest that depleting noncanonical poly(A) polymerases – particularly Gld2 – in the hippocampus leads to the misregulated expression of numerous mRNAs that thereby alters synaptic plasticity.

Finally, it is particularly interesting that Gld2/Gld4 regulate poly(A) site selection. Alternative 3′UTRs control RNA localization and stability in neuronal dendrites as well as mediate synaptic plasticity [31,56,57]. However, the factors that control this process are unknown. Our studies indicate that noncanonical poly(A) polymerases play critical roles in this regulation and future experiments will explore the mechanism(s) by which they recognize specific RNAs.

Materials and methods

Stereotaxic injection of shRNA-expressing AAV in the mouse hippocampus

All animal experimentation was carried out in accordance with institutional animal care and use protocols and conforms to all applicable federal and NIH guidelines. Male C57BL/6 mice (10–12 weeks old) were anaesthetized intraperitoneally with ketamine/xylazine and positioned in a stereotaxic apparatus (Stoelting Co.). 2×10^{11} AAV9 particles (expressing shScrambled (i.e., non-specific, NS), shGld2, shGld4, shGld2 + shGld4 as well as GFP) were injected bilaterally at a constant flow rate of 0.2 µL/min using a 10 µL neuron syringe (Hamilton). In order to reach the CA1 region of hippocampus, the following stereotaxic coordinates were used: −1.75 mm AP, −1.65 mm DV, ± 1.3 mm. The needle was maintained in place for 5 min after injection. The injected animals were used for behavioural and electrophysiological experiments 3 weeks after injections. Some animals were also used for RNA-seq.

Animals were injected in three batches. Batch 1 was used for behaviour analysis only, batch 2 was used for behaviour analysis, after which the animals were sacrificed and used for RNA extraction and sequencing, and batch 3 was used for electrophysiological analysis.

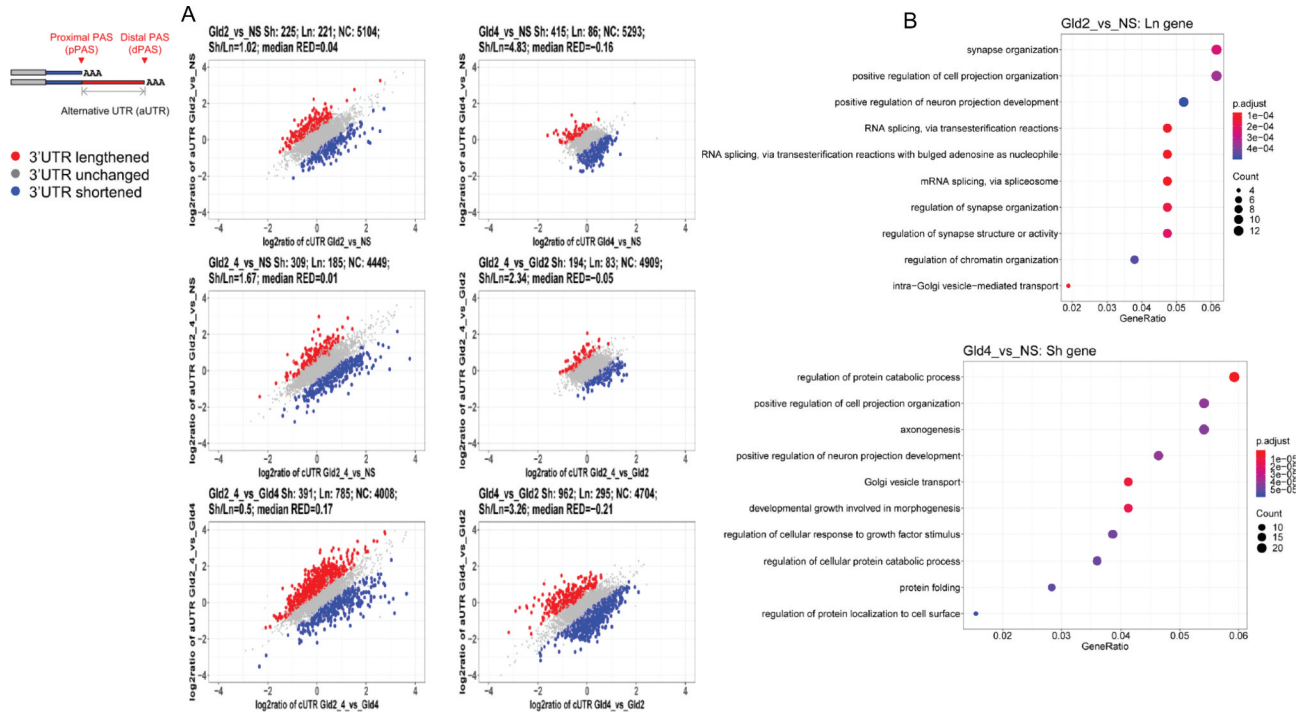

Figure 5. Noncanonical poly(A) polymerases regulate alternative poly(A) site selection (APA). A. Pair-wise comparisons of RNA with lengthened (i.e., using a distal poly(A) site, red) or shortened (i.e., using a proximal poly(A) site, blue) 3′UTRs. The number of RNAs in each category are indicated (NC, no change; Sh, shortened; Ln, lengthened). Plots are log2 ratios of constitutive 3′ UTR (cUTR) vs. alternative 3′ UTR (aUTR). B. GO analysis of RNAs with long 3′UTRs following Gld2 depletion compared to scrambled (NS). Also shown is GO analysis of RNAs with short 3′UTRs following Gld4 depletion compared to scrambled control (NS). All are padj<0.05. Gene ratio refers to the total of differentially processed RNAs in the give GO term.

Behavioural experiments

The first batch of behaviour assays was performed in the following order: elevated plus maze, open field test, novel object recognition test, T-maze spontaneous alternation, and passive avoidance. Batch two included elevated plus maze, open field test, marble burying, and three-chamber sociability test. The different behaviour tests were performed with 2–3 days interval between each assay. Mice were previously habituated to the room where experiments were performed before each behaviour assay started, with the exception of passive avoidance where one mouse was transported to the room at a time. All behaviour assays were conducted during the light phase of the light/dark cycle. All the different apparatus and objects were thoroughly cleaned with 70% ethanol between animals. All animal behaviour statistical tests employed a one-way non-parametric ANOVA (Kruskal–Wallis test).

Elevated plus maze

The elevated plus maze is a widely used test aimed to evaluate anxiety-related behaviour of laboratory animals. Mice were placed at the intersection of a plus-shaped maze elevated above the ground. Two opposite arms had high walls (closed arms) while the other two arms had no walls (open arms). Animals were free to explore the maze for 5 min. Time spent in each arm and junction was measured. A preference for the closed arms over the open arms indicates an increase in anxiety.

Open field test

The open-field test allows the assessment of general locomotor activity as well as anxiety-related behaviour. Mice were placed in the centre of an open field arena which could be explored for 10 minutes. The exploration was recorded and analysed using the Ethovision^{TM} video tracking software (Noldus) that allows the division of the open field in centre and periphery. The time and distance travelled in each zone were measured. An increase in the time spent in the centre of the open field correlates with lower levels of anxiety.

Novel object recognition test

The novel object recognition test is a useful behavioural assay for the assessment of memory. On day 1, mice were placed in an open field arena with two identical objects that could be freely explored for 10 minutes. Twenty-four hours later, mice were returned to the open field where one of the objects was replaced by a novel one so long-term recognition memory could be assessed. The position of the novel object in the left or right side was alternated between trials. The percentage of time the animals spent with the novel object relative to total time spent with both objects was measured. A percentage higher than 50% indicates the ability of animals to recognize and remember the familiar object and to spend more time with the novel object.

T-maze spontaneous alternation

The T-maze Spontaneous Alternation allows the evaluation of working memory. The apparatus consists of a T-shaped maze containing a start arm and two T arms with retractable doors. The mouse was placed in the start arm and allowed to explore and choose either the right or left arm. Once the animal chose one of the sides, the door of the opposite arm was manually closed forcing the animal to return to the start position, when

the arm door was opened again. The mouse had to complete a total of 15 choices. The experiment was performed in two consecutive days. The percentage of alternation between right and left arm was calculated. An alternation percentage higher than 50% indicates that the mouse prefers to visit a new arm rather than the previously explored arm.

Marble burying

The marble-burying test allows the assessment of anxiety, obsessive-compulsive, and/or repetitive-like behaviours. In the first day, a habituation step was performed where mice were individually placed in cages filled with 6 cm bedding for 30 minutes. In the day after, 20 glass marbles were placed on top of the bedding evenly spaced in a pattern of 4 × 5. The mice were allowed to explore the cage and bury marbles for 30 minutes. The number of buried marbles (at least 2/3 of the area) was counted after 30 minutes.

Three-chamber sociability test

The sociability test was performed in a box divided into three interconnected chambers. The first day consisted of habituation where mice were placed in the middle compartment and allowed to freely explore all the 3 empty chambers for 10 minutes. On the second day, animals were given the choice to explore one of the chambers containing an unfamiliar mouse enclosed in a wire cup or the opposite chamber with an empty wire cup for 10 minutes. The mice used as strangers were previously habituated to enclosure in the wire cup. Also, the position of the stranger mouse in the left or right side of the chamber was alternated between trials. The time spent in each chamber was measured and analysed using the Ethovision^{TM} video tracking software (Noldus). A higher time spent in the chamber with the stranger mouse indicates a preference for social interaction.

Passive avoidance

Passive avoidance is a robust behavioural assay for testing emotional learning and memory. Mice learn to suppress their innate preference for dark areas where they are exposed to an aversive stimulus. This assay was performed in an apparatus divided in two compartments connected by a door, one side was lit and the other one was dark (Gemini^{TM} Avoidance System, SD Instruments). The mouse was placed in the light compartment with the door closed. After 1 min, the door was opened. As soon as the animal crossed to the dark compartment, the door was closed and a 0.3 mA foot shock was given for 1s. The mouse was kept in the dark compartment for 30s before returning it to its home cage. Twenty-four hours later, the mouse was placed back in the light compartment and the latency to enter the dark compartment was measured. Mice that remember the aversive experience will take longer to enter the dark compartment when compared to the latency from the first day.

shRNA sequences

NS:
GATCCGCGCTTGGGCGAGAGTAAGTTCAAGAGACTT-ACTCTCGCCCAAGCGTTTTTTG

Gld2:
5GATCCATGCACAATTCAACTTTCATTCAAGAGATGA-AAGTTGAATTGTGCATTTTTTTG

Gld4:
GATCCGCCAAAGATGAAGTACAGATTCAAGAGAT-CTGTACTTCATCTTTGGCTTTTTTG

Electrophysiology

Transverse hippocampal slices (400 μm) from AVV-injected mice were incubated at room temperature with oxygenated artificial CSF (ACSF) (in mM: 119 NaCl, 4 KCI, 1.5 $MgSO_4$, 2.5 $CaCI_2$, 26.2 $NaHCO_3$, 1 NaH_2PO_4, and 11 glucose) and allowed to equilibrate for 60 min. The slices were then placed in an interface chamber with ACSF at 28 ± 1°C for at least 20 min before recording. Field EPSPs (fEPSPs) were recorded at CA3–CA1 synapses via stimulation of the Schaffer collateral axons with a bipolar electrode CBAPD75 (FHC) and recording with a 4–5 MΩ glass pipette (A-M Systems). The stimulating and recording electrodes were positioned at the beginning (CA2/CA1 border) and the ending (CA1/subiculum border) of the CA1 area, respectively. For each slice recording, a stimulus-response curve was generated in order to adjust the stimulation intensity (square pulse, 50 μs duration) to give fEPSP slopes of □40% of maximum. Throughout the duration of each recording, fEPSPs responses were sampled once per minute at this intensity. The LTP induction protocol for theta-burst stimulation was a single theta-burst episode, which consisted of nine bursts of four pulses at 100 Hz with 200 ms interburst intervals. The LTD induction protocol consisted of bath application of 1 mM glycine for 20 minutes. For all experiments, two slices from approximately the same hippocampal area were recorded in parallel, one control and one stimulated. For statistical analysis, The fEPSP slopes acquired during distinct time intervals after LTP or LTD induction (i.e. 20–30, 60–90, 150–180 min, etc.) were averaged for each group (Gld2/4 depletion and non-specific control) and compared using analysis of variance (MICROCAL ORIGIN statistical tool, Microcal Software Inc.). In all electrophysiological experiments, *n* indicates the number of animals. In the text, the electrophysiological data were presented as mean ± S.E. The difference between two experimental data sets was considered significant at $P < .05$.

RNA extraction and RNA-seq library preparation

For RNA extraction, the brain was sectioned with a tissue chopper and the GFP-expressing CA1 region was microdissected under a microscope. The microdissected sample was immediately frozen and the RNA was extracted with Trizol, and precipitated with isopropanol and glycogen overnight at −80 °C. A Fragment Analyser was used to determine an RNA Integrity Number (RIN), which was between 8 and 10. Two hundred forty ng of RNA was poly(A)-selected using Bioo Scientific NEXTflex poly(A) beads and then used for a Bioo Scientific NEXTflex rapid directional qRNA-seq library preparation following the manufacturer's instructions. Twelve cycles were used for the final PCR amplification. The libraries were quantified with a KAPA Library Quantification Kit and

the quality was examined with a Fragment Analyser. Libraries were pooled with equal molar ratios, denatured, diluted, and sequenced with NextSeq 500/550 High Output Kit v2 (Illumina, 150bp pair-end runs for RNA-seq, #FC-404-2004) on a NextSeq500 sequencer (Illumina).

RNA-Seq data processing and expression analysis

Raw sequencing fastq files were uploaded to the Dolphinnext platform [58] at the UMMS Bioinformatics Core for mapping and quantification. The raw reads were first quality-filtered with Trimmomatic (0.32). The initial cleaned reads mapped to mouse rRNA by Bowtie2 (2.1.0) were further filtered out. Cleaned reads were next mapped to the Refseq mm10 genome and quantified by RSEM (1.2.11). Estimated counts on each gene were used for the differential gene expression analysis by DESeq2 (1.16.1). After the normalization by median of ratios method, only the genes with minimal 5 counts average across all samples were kept for the DEG analysis.

Alternative splicing and poly(A) site selection analysis

In addition to gene expression analysis, alternative splicing (AS) and alternative polyadenylation (APA) will also be analysed using rMATS (v4.0.2) [59] and APAlyzer (v1.0.0), respectively. Significant AS events will be identified as those with a delta percent spliced-in (PSI) > 5% and p-value < 0.05. Significant APA events will be those with a relative abundance change > 5% and p-value < 0.05. We included genes that had at least one read of the differentially AS or APA in both genotypes for the downstream functional enrichment analysis.

GO enrichment

EnrichGo was used from the clusterProfiler package [60] to obtain GO term enriched for the differentially DE genes, AS events and APA events. To remove redundancy in reporting, each reported GO term was required to have at least 25% of genes that were not associated with another term with a more significant FDR adjusted p value.

Quantification and statistical analysis

For Quantification and statistical analysis, all grouped data are presented as mean ± s.e.m. Student's t test was used to determine statistical significance between groups. The Kolmogorov–Smirnov (K-S) test was used to compare the distributions in the volcano plots. When exact p values are not indicated, they are represented as follows: *p < 0.05; **p < 0.01; *** p < 0.001; n.s., p > 0.05.

Data and code availability

Codes and scripts used for quantification analysis were written in Python or R and will be provided upon request to the Lead Contact. The Gene Expression Omnibus (GEO) accession number for the data reported in this paper is GSEXXXXX.

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Disclosure

The authors have no conflict of interest

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Data availability

All sequence data are deposited on GEO. Other information may be obtained from the contact author.

Geolocation:

Disclosure statement

No potential conflict of interest was reported by the authors.

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