Full title: Loss of pseudouridine synthases in the RluA family causes hypersensitive nociception in *Drosophila*

Short title: Pseudouridine synthase RluA in nociception

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Abstract

Nociceptive neurons of *Drosophila* larvae are characterized by highly branched dendritic processes whose proper morphogenesis relies on a large number of RNA-binding proteins.

Post-transcriptional regulation of RNA in these dendrites has been found to play an important role in their function. Here, we investigate the neuronal functions of two putative RNA modification genes, *RluA-1* and *RluA-2*, which are predicted to encode pseudouridine synthases. *RluA-1* is specifically expressed in larval sensory neurons while *RluA-2* expression is ubiquitous.

Nociceptor-specific RNAi knockdown of *RluA-1* caused hypersensitive nociception phenotypes, which were recapitulated with genetic null alleles. These were rescued with genomic duplication and nociceptor-specific expression of *UAS-RluA-1-cDNA*. As with *RluA-1*, *RluA-2* loss of function mutants also displayed hyperalgesia. Interestingly, nociceptor neuron dendrites showed a hyperbranched morphology in the *RluA-1* mutants. The latter may be a cause or a consequence of heightened sensitivity in mutant nociception behaviors.

Author Summary

Pseudouridine (Psi) is a C5-glycoside isomer of uridine and it is the most common posttranscriptional modification of RNAs, including noncoding tRNAs, rRNAs, snRNAs as well as mRNAs. Although first discovered in the 1950s, the biological functions of Psi in multicellular organisms are not well understood. Interestingly, a marker for sensory neurons in *Drosophila* encodes for a putative pseudouridine synthase of unknown function called *RluA-1*. Here, we report our characterization of nociception phenotypes for larvae with *RluA-1* loss of function along with that of a related gene *RluA-2*. Disrupting either or both *RluA-1* and *RluA-2* resulted in hypersensitive nociception. In addition, *RluA-1* mutants have more highly branched nociceptor neurites that innervate the epidermis. Our studies suggest an important role for the
RluA family in nociception. This may be through its action on RNAs that regulate neuronal excitability and/or dendrite morphogenesis.

Introduction

Pain serves an indispensable, protective role but when pain becomes pathological it can have a debilitating impact on human life. The total annual cost of pain to society in the United States was estimated by the Institute of Medicine to be up to $635 billion, which is greater than that of heart disease, cancer, and diabetes combined (1). It is therefore of urgent importance to uncover the basic molecular and cellular mechanisms involved in pain in order to better treat it.

Laboratory animal models of pain and nociception have played an essential role in identifying such mechanisms. Drosophila larvae respond to noxious thermal and mechanical stimuli through stereotyped rolling escape locomotion (in which the larva rotates around its long body axis), which is robust and easily distinguishable from other forms of locomotion (2). Rolling is used as a behavioral defense against attacks by parasitoid wasps which lay their eggs within the fly larvae using a sharp ovipositor (3). Successful wasp infection is lethal to the larvae and is a strong selective pressure in natural populations of Drosophila. Combined with the unparalleled genetic tools available for Drosophila melanogaster, this behavioral readout provides an excellent system to study the genetics of nociception and pain (2, 4-8). Previous studies have demonstrated a specific subset of dendritic arborization (da) sensory neurons in the peripheral nervous system, Class IV multidendritic da (cIVda) neurons, are of critical importance for thermal, mechanical and high intensity light nociception (3, 9, 10). Further evidence suggests a lesser but significant contribution of Class II (cIIIda) and Class III da (cIIIda) neurons in mechanical nociception (3, 11). In addition, great progress has been made in identifying the circuits in the larval abdominal ganglion that are involved in rolling escape locomotion (12-15).
Genes that are specifically expressed in multidendritic (md) neurons have been found to play a role in nociception. For instance, *painless* (2) is required for mechanical and thermal nociception and it is expressed in all four classes of md neurons. Mechanical nociception genes such as *pickpocket* (16), *ppk26/balboa* (17, 18) and the polymodal nociception gene *dTRPA1-C/D* (19) each show very specific expression in the clVda neurons. Forward genetic screens have also identified a set of genes with enriched expression in the clVda neurons which either inhibit or activate nociceptive pathways (20).

The historically first known genetic marker with specific expression pattern in the da neurons was the *lacZ* enhancer trap *E7-2-36* (21). Later studies reported that this enhancer trap gene was inserted upstream of the *RluA-1* gene and DNA sequences from upstream of *RluA-1* caused expression of GAL4 in multidendritic neurons (22). The *RluA1* gene encodes an enzyme that is predicted to have pseudouridine synthase activity, but how this RNA modifying protein is involved in the function of nociceptive multidendritic neurons remains unknown.

A widespread importance of RNA-binding proteins in clVda neuron dendrite morphogenesis and function was found in a recent large-scale RNAi screen that identified 88 genes encoding RNA-binding proteins whose knockdown caused aberrant dendrite morphogenesis (23). The elaborate dendrite arbors of clVda neurons project long distances from the neuronal cell body and mRNA granules are trafficked to these distant sites where they may undergo local translation. Indeed, RNA granules containing Nanos (Nos), Pumilio (Pum), Oskar (Osk), Fragile X Mental Retardation (FMRP) and other proteins have been shown to regulate the formation of higher order dendrites in these cells (24-28). Interestingly, the *nos* mRNA itself is transported into dendrites via a pathway that relies on Oskar and Rumplestiltskin, two proteins that also play a role regulating *nos* in the early embryo (28).
Mechanical nociception defects are also observed in animals with disruption in these pathways (28).

Pseudouridylation is the most common post-transcriptional RNA modification. Pseudouridine (Ψ), the C5-glycoside isomer of uridine, was initially found in many positions in rRNA, tRNA, and snRNA in all organisms that have been investigated (29). RNA-seq based global pseudouridine profiling has shown the presence of Ψ in many mRNAs and a large number of those sites were found to be dynamically regulated in yeast and human cells (30-33). Dysfunctional pseudouridylation has been linked to several human diseases (34-36). Since many sites of pseudouridylation in different organisms are evolutionarily conserved, Drosophila melanogaster may provide an excellent and genetically tractable metazoan system to elucidate some of these functions.

The isomerization of uridine to pseudouridine is catalyzed by six families of pseudouridine synthases. They function either as guide RNA directed ribonucleoprotein complexes or as stand-alone proteins (37, 38). In the Drosophila genome, 9 proteins have been identified with annotated pseudouridine synthase domains. Minfly (mfl), the RNA-dependent pseudouridine synthase homolog of human dyskerin (mouse NAP57 and yeast Cbf5), is required for somatic stem cell homeostasis and is essential for Drosophila viability and fertility (39-41). Knockout of Drosophila Pus7, the human and yeast Pus7 homolog, results in increased aggressiveness in adult flies (36). The function and specificity of other predicted pseudouridine synthases are largely unknown. Among the six families, the RluA family, which does not rely on guide RNAs, appears to be the most complex based on divergent substrate specificities in bacteria and yeast (42). RluA family members in bacteria are involved in ribosomal assembly and growth (43, 44) but their function in multicellular organisms has not been studied. Although pseudouridine synthases appear to function ubiquitously, as noted above, Drosophila RluA-1, a
member in RluA family, has been reported to be specifically expressed in md neurons (22). Thus, we have investigated the role for RluA-1 and its paralog RluA-2, in nociception pathways that are known to depend on md neurons. Our results indicate an important function for RluA-1 and RluA-2 in the regulation of nociception.

Results

RluA-1 is expressed in the multidendritic neurons of the peripheral nervous system and in the cells of brain

Previously described reporter genes for RluA-1 showed specific expression in larval multidendritic neurons (22). We replicated this finding by generating an RluA-1\(^{GAL4}\) driver at the endogenous gene locus through recombination mediated cassette exchange (RMCE) of RluA-1\(^{MI06897}\) (an intronic MiMIC) and a Trojan exon cassette (45). An mCD8GFP reporter driven by RluA-1\(^{GAL4}\) was expressed in peripheral sensory neurons in each segment of the larval body wall (Fig 1A). In the dorsal cluster, GFP-positive signals were clearly detected in all four classes of md-da sensory neurons, dorsal multiple dendrite neuron (dmd1), external sensory (ES) and dorsal bipolar dendritic (dbd) neurons (Fig 1B). In the larval ventral nerve cord, the GFP-positive signals were seen in the axonal projections of the sensory neurons (Fig 1C). GFP signal was also observed in the unidentified clusters of neurons in the larval brain (Fig 1C). In the adult brain, significant signals were detected in the optic lobes and other small cell clusters of the central brain (S1A Fig).

To determine the localization of the RluA-1 proteins, we generated a GFSTF exon trap (46) that expresses an in frame GFP fusion with RluA-1 at the endogenous genomic locus (with RMCE of the RluA-1\(^{MI06897}\) MiMIC element) (46). Although live imaging did not detect the EGFP tagged RluA-1 protein, immunostaining with anti-GFP labelled nuclei of larval multidendritic neurons, ES and dbd neurons also expressing a membrane-localized RFP (Gal4\(109(2)80 > UAS-\)
CD8-RFP, Fig 1D). The tagged RluA-1 protein was also detected in the cell bodies of neurons in optic lobes and other yet-to-be identified cells in the adult brain (S1B Fig).

Reducing or removing RluA-1 results in a hypersensitive thermal nociception phenotype

Given our confirmation of the expression of the RluA1 gene and protein in the multidendritic neurons we tested for potential roles of RluA-1 in the regulation of nociception.

To do so, we first performed tissue-specific knockdown using GAL4/UAS based RNA interference (RNAi). A cIVda specific driver (ppk1.9-GAL4 UAS-dicer2) (47) was employed to drive an RluA-1 RNAi construct in the larval cIVda nociceptors. We assessed potential insensitive phenotypes (with a probe temperature of 46°C) and potential hypersensitive phenotypes (with a temperature of 42°C) as previously described (20). When stimulated with the higher temperature 46°C probe the RluA-1-RNAi larvae responded significantly faster than the ppk-GAL4 driver alone controls. This genotype also responded faster than the UAS-RNAi controls but this difference was not statistically significant (Fig 2A). The results suggested that reducing RluA-1 in classIV neurons may have made the larvae more sensitive to noxious heat.

Indeed, a clearly hypersensitive phenotype for the RluA1-RNAi animals was seen when testing with 42°C probe (Fig 2A). The average latency to roll in the RluA-1 knock-down animals was significantly faster than the driver alone animals or the UAS-RNAi control animals. These data combined suggest that reducing the activity of RluA-1 in the noxious heat-responsive cIVda cells caused thermal hyperalgesia.

To further test the function of RluA-1 we next generated a precise genetic deletion mutant of RluA-1 in which 11.14 kbp including the entire RluA-1 genomic region was removed by CRISPR-guided homologous recombination-directed repair (HDR) (48). Homology arms of ~1kb immediately flanking the CRIPSPR cleavage sites were used to direct the HDR (S2A Fig).
The resultant deletion mutant \((RluA-1^{del-HDR})\) was confirmed by PCR amplification and sequencing of PCR products from the targeted \(RluA-1\) locus (S2B Fig). To facilitate the behavioral comparisons, the \(RluA-1^{del-HDR}\) mutant was backcrossed six times to our most commonly used genetic background strains Canton-S (CS), \(w^{1118}\), and isogenized \(w^{1118}\) (iso\(w^{1118}\)).

As with class IV md neuron-specific RNAi knockdown, \(RluA-1^{del-HDR}\) larvae showed significantly faster responses to noxious heat stimulation of \(42^{\circ}C\) compared to the corresponding control animals, regardless of genetic background (Fig 2B). Note that for unknown reasons, these genetic backgrounds vary in their baseline responses. This genetic background effect has a significant impact on the latency of larval nociception responses to noxious heat stimuli, with the most striking differences between homozygous \(RluA-1^{+/+}\) and the relatively insensitive iso\(w^{1118}\) background, followed by that of \(w^{1118}\) background, and then the CS background (Fig 2B).

Given the sensitivity to genetic background, we performed an additional genetic test for the importance of \(RluA-1\), by generating an independent mutant allele \((RluA-1^{del-FRT})\) using FLP recombinase and FRT-bearing insertions \((49)\) \((PBac{WH}^{f02750}(+)\) and \(p\{XP\}^{s2586}(-)\), S2A Fig). Transheterozygous \(RluA-1^{del-FRT}\) \(/RluA-1^{del-HDR}\) mutant larvae displayed hypersensitivity to a \(42^{\circ}C\) stimulus (Fig 2C) indicating that \(RluA-1^{del-FRT}\) failed to complement \(RluA-1^{del-HDR}\). Failure of complementation of independently generated alleles created in distinct genetic backgrounds provides strong evidence that the hypersensitive nociceptive phenotypes observed are a consequence of the mutation of \(RluA-1\) and not due to unlinked mutations present in one background or another.

For the remainder of our behavioral studies, we relied on the isogenized \(w^{1118}\) background. This had the advantage of greater genetic uniformity relative to \(w^{1118}\) and CS, as well as showing the strongest hypersensitive mutant phenotype for \(RluA-1\).
Genetic rescue of RluA-1 mutant restores thermal nociception response

To test that the mutation in RluA-1 was the underlying cause of the hypersensitive nociception phenotype, we introduced a genomic rescue construct into the RluA-1\(^{del-HDR}\) mutant background (a Bacterial Artificial Chromosome (BAC) (P6-D7)) (50) covering the RluA-1 gene region. With the 42°C stimulus, larvae with two copies of the duplication (Dp) in the background of RluA-1\(^{del-HDR}\) showed rescue of the hypersensitivity (Fig 3A). This rescue with the genomic duplication was dosage dependent. Larvae with only one copy of the Dp in the RluA-1\(^{del-HDR}\) background (RluA-1\(^{-}\); Dp\(^{+}\)) responded more slowly than the mutant; this difference was not statistically significant (Fig 3A). Combined, the results of genomic rescue experiments support the hypothesis that mutation of RluA-1 is indeed the cause of the hypersensitive thermal nociception phenotype.

A caveat remained in that the genomic rescue construct included other genes in addition to RluA-1. Thus, genomic rescue did not rule out the possibility that a mutation tightly linked to RluA-1, but not in RluA-1 itself, was responsible for the mutant phenotype. Thus, as a final test, we generated transgenic lines to express an RluA-1 cDNA under the control of the GAL4/UAS system (UAS-RluA-1). Using the UAS-RluA-1 we specifically restored RluA-1 to clVda neurons in the RluA-1 null mutant background. When stimulated with the 42°C probe the animals with both the ppkGal4 driver and the UAS-RluA-1-cDNA transgene in the RluA-1\(^{del-HDR}\) showed a complete rescue from the hypersensitivity seen in the null mutant (Fig 3B). Neither the ppkGal4 driver alone nor the UAS-RluA-1 had an effect on the hypersensitive thermal nociception phenotype in the RluA-1 null mutant background, excluding the possibility of non-specific effects of these transgenes (Fig 3B). In addition, overexpression of UAS-RluA1 with a md neuron driver (MD-Gal4) had no effect on nociception behavior at 42°C stimulus ruling out the possibility that the increased latency seen in the rescue effect was a non-specific
consequence of over-expression (S3 Fig). Combined, these nociceptor-specific rescue experiments provide genetic confirmation that loss-of-function mutation in RluA-1 causes hypersensitive thermal nociception and localizes the site of action for RluA-1 in this process to the nociceptors.

**RluA-1 requirement for mechanosensory thresholds**

The cIVda neurons are not only required for detection of noxious heat, they also contribute to sensing harsh mechanical stimulation (3, 16, 17). Thus, we investigated the RluA-1 mutant responses to noxious mechanical stimuli. When stimulated with a 30mN/720kPa Von Frey fiber, significantly more RluA-1\textsuperscript{delHDR} null mutant larvae performed the typical nociceptive rolling behavior compared to the isow\textsuperscript{1118} controls (Fig 4A). Even when the probe strength/pressure was reduced to 15mN/360kPa, the majority of RluA-1\textsuperscript{-/-} larvae still rolled while less than half of control larvae rolled (Fig 4A), indicating the defect in RluA-1 also caused hypersensitive mechanical nociception. Loss of RluA-1 did not have any impact on behavioral responses to gentle touch (Fig 4B) suggesting a more specific involvement in nociception than for sensory processing in general.

**Expression pattern and nociception functions for RluA-2**

The RluA-2 locus is adjacent to RluA-1 on the second chromosome of Drosophila melanogaster. RluA-2 has significant sequence similarity with RluA-1 within the evolutionarily conserved pseudouridine synthase domain. This amino acid similarity of RluA1 and RluA2 suggested possible functional overlap for the encoded proteins. To investigate the expression of RluA-2 we generated a GFSTF line with RMCE of the RluA-2\textsuperscript{M122981} mimic element (S4A Fig) (46). Immunostaining with anti-GFP labelled nuclei in all of the cell types that we observed in third instar larvae (Fig 5A), including md neurons (Fig 5B).
To test the function of RluA-2 we generated a deletion (RluA-2\textsuperscript{del-HDR}) to remove its pseudouridine synthase domain via CRISPR/Cas9 HDR (S\textsubscript{4}A Fig). Also, since RluA-1 and RluA-2 are both expressed in the md neurons (Fig 1D and Fig 5B), we generated a double mutant (RluA-1\textsuperscript{del-HDR} RluA-2\textsuperscript{del-HDR}) by injecting the RluA-2\textsuperscript{del-HDR} constructs in the RluA-1\textsuperscript{del-HDR} null mutant background (S\textsubscript{4} Fig). The double mutant allowed us to completely remove RluA pseudouridine synthase gene activity from the flies. Prior to functional assessment, the single mutant and the double mutant were backcrossed six times to the genetic background of isow\textsuperscript{1118}.

We tested each single mutant (RluA-1\textsuperscript{del-HDR} and RluA-2\textsuperscript{del-HDR}) together with the double mutant RluA-1\textsuperscript{del-HDR} RluA-2\textsuperscript{del-HDR} side by side in thermal nociception assays with the 42°C thermal stimulus. RluA-1\textsuperscript{del-HDR} larvae (RluA-1\textsuperscript{-/-}) again responded significantly faster than the genetic background control (Fig 6). The RluA-2\textsuperscript{del-HDR} single mutant larvae also displayed a faster response to the stimulus (Fig 6) and similar hypersensitivity was also seen in an independent allele for RluA-2 that we generated by FLP/FRT mediated recombination (RluA-2\textsuperscript{del-FRT}) allele (S\textsubscript{5} Fig). Finally, the double mutant RluA-1\textsuperscript{del-HDR} RluA-2\textsuperscript{del-HDR} larvae showed a faster response than the control larvae (Fig 6) to the same extent as the single mutant of RluA-2\textsuperscript{del-HDR}. These results indicated that RluA-2, like RluA-1, negatively regulates nociception. The finding that the double mutant did not show a more severe phenotype than either single mutant suggests that RluA-2 and RluA-1 have non-redundant functional roles, and that they may function in the same molecular pathway. When this pathway is disrupted, hypersensitive nociception results.

**RluA-1 regulates neuronal dendrite morphology of nociceptors**

A nociceptor-specific RNAi screen with thermal nociception assay discovered dozens of genes whose reduction caused either insensitive or hypersensitive thermal nociception (20). Interestingly, some of those genes targeted with RNAi showed a reduced or increased
branching of Class IV neuron dendrites. Reduced dendrite branching was often seen with nociceptive insensitivity while increased branching was found in some hypersensitive genotypes. Thus, regulation of Class IV neuron dendrite morphology is a commonly affected developmental pathway that is related to nociception phenotypes. Given this, we investigated the dendrite morphology of the cIVda neuron dendrites in the RluA-1<sup>del-HDR</sup> mutant. In mutant ddaC neurons visualized with ppk-CD4-tdTomato, we observed a modest but significant increase in the number of dendrite branches (normalized by neuron size) and shorter average branch length in comparison to control animals (Figs 7A and 7B). We also found that dendritic branches in the RluA-1<sup>del-HDR</sup> ddaC neurons had higher frequency of isoneuronal cross-over events compared to the control (Figs 7C and 7D). This latter phenotype is suggestive of an isoneuronal tiling defect. Increased isoneuronal crossovers are also seen in mutants that affect dendrite attachment to the basal lamina (51-54). Whether or not these dendrite abnormalities play a causal role in the hypersensitive nociception phenotypes of the RluA-1 mutant will be an interesting subject for future investigation.

Discussion

Given the well-established nociceptive role of md-neurons, we have investigated the historically first known molecular marker for md-neurons in nociception pathways. This gene encodes the RluA-1 protein in the RluA family of pseudouridine synthases. Our studies clearly demonstrate that loss of function for either RluA-1 or RluA-2 produce hyperalgesia in third instar Drosophila larvae. Tissue-specific RNAi, genetic null mutant, and cDNA rescue experiments all indicate that loss of the RluA-1 gene from whole animals, or specifically from nociceptors, results in hyperalgesia. A newly generated RluA-1<sup>GAL4</sup> driver showed specific expression in larval multidendritic and ES neurons. As well, a GFP exon trap for RluA-1 protein localized to the nuclei of these neurons. A small number of unidentified neurons in the larval
brain were also revealed by $RluA-1^{GAL4}$ and we observed expression of $RluA-1^{GAL4}$ driven mCD8GFP and GFP tagged RluA-1 in cells of the adult brain, which included the optic lobe.

Although loss of $RluA-2$ also caused hyperalgesia, its expression pattern was ubiquitous and included multidendritic neurons. Like the RluA-1 GFP exon trap, the RluA-2 GFP exon trap labelled nuclei. The nuclear localization for both RluA-1 and RluA-2 may indicate that these proteins act on RNA targets prior to export of the nucleus, or that they predominantly act upon nuclear localized RNAs. A caveat to this interpretation is that we have yet to demonstrate that these molecules function as genuine pseudouridine synthases, and this function remains a hypothesis that is based on the known function of evolutionary homologues. This hypothetical function requires future biochemical verification.

We also observed that $RluA-1$ mutants showed an increase in the number of dendrite branches relative to control genotypes as well as an increase in isoneuronal crossovers. Transcription factors, cytoskeletal regulators, motor proteins, secretory pathways and cell adhesion molecules all function in concert to develop and maintain optimum dendrite morphology (55). RluA-1 may modify RNAs for those genes that regulate dendritic morphology, or changes in the dendrite morphology could be an indirect consequence of neuronal sensitivity that is regulated by RluA-1. It is noteworthy that prior studies have noted a potential link between the degree of dendrite branching and the sensitivity of nociception behaviors (20). In other cases, axonal factors such as the ion channel SK have been found to be important in regulating the cIVda neuron excitability (56, 57). Whether the dendrite branching phenotype that we observe in $RluA-1$ mutants is a cause or a consequence of hypersensitivity will be an interesting question for future studies.

A large body of literature indicates that RNA trafficking and local translation is important in dendrites and axons of neurons (58, 59). Relative to uridine the pseudourine base
is believed to have enhanced rotational freedom which may alter conformation of RNA secondary structures. As well, an additional hydrogen bond donor present in pseudouridine may favor alternative base-pairing interactions in RNA. These properties may consequently alter RNA localization, stability and/or efficiency of translation (60-62). Pseudouridines can also influence decoding during translation as pseudouridylation of nonsense codons has been shown to suppress translation termination both in vitro and in vivo (63). Thus, another possible function for pseudouridylation in nociceptive neurons could be to favor read-through of pseudouridylated stop codons to generate novel sequences at protein carboxy termini.

The precise mechanism explaining the involvement of RluA proteins in nociception can only be elucidated by identifying the RNA targets of these enzymes. Several groups have developed methods using next generation sequencing methods to identify the pseudouridine sites in transcriptomes (30-33, 64). Future investigations applying these methods to wild type and RluA mutants in Drosophila will help us to identify the RluA targets and to further define the underlying mechanisms.

Materials and methods

Fly strains and husbandry

The following fly strains were obtained from Bloomington Stock Center: (w^{1118}; Pbac{vas-Cas9}VK00027), Mi{MIC}RluA-1^{[Mi12981]}, Mi{MIC}RluA-2^{[Mi12981]}, Df(2L)Exel7048/Cy O, (yw; Sp/CyO; pC-(lox-attB2-SA-T2A-Gal4-Hsp70)3), (yw hs-cre, vas C31; Sp/CyO; Sb/TM3Ser), (P[ry-hsFLP], yw Mi{vas-int.B}ZH-2A; Sp/CyO; P[FRT-attB-[GfSTF]-attB(w+)-FRT]), ppk-CD4-tdTom, md-Gal4, UAS-mCD8::RFP, 40XUAS-mCD8::GFP. The following fly strains were obtained from Exelixis collection at Harvard Medical School: Pbac{WHj}^{112750}, P[XP]d2586, Pbac{WHj}Grip75^{[f05483]}, Pbac{WHj}RluA-2^{[f177022]}. The RNAi lines targeting RluA-1 (31719-R1, 31719-R2) were obtained from Kyoto Stock Center. The genetic duplication line (BAC ID: CH321-
49P21) covering RluA-1 (starting at 24,819,420 and ending at 24,910,132 on 2R) was obtained from Genetivision. The nos-Cas9 line used for generating RluA-2\(^{del-HDR}\) and double mutant RluA-1\(^{del-HDR}\) RluA-2\(^{del-HDR}\) [y sc v; {nos-Cas9} attP2 (TH00787.N)] was kindly provided by the Norbert Perrimon lab. All larvae used in experiments were reared on the Bloomington Drosophila medium in an incubator with controlled temperature (25°C) and humidity (70%) on a 12h light/12h dark cycle. Strains were otherwise maintained at room temperature.

**CRISPR targeting of RluA-1 and RluA-2**

HDR for mutagenesis used the CRISPR/Cas9 (Gratz et al. 2013) to generate RluA-1\(^{del-HDR}\), RluA-2\(^{del-HDR}\) and the double mutant RluA-1\(^{del-HDR}\) RluA-2\(^{del-HDR}\). Target sites were selected using the flyCRISPR Optimal Target Finder. For the precise deletion of RluA-1, the gRNA target sites are 5' end 5'-CCACTG⏐TGCAAGCGGAAAATTCACT-3' (where “⏐” represents the Cas9 cut site, which is 71bp upstream of the RluA-1 transcriptional start) and 3' end: 5' -ACATATATTCAAAGCT⏐CTTTGG-3' (cut site at 60bp downstream of RluA-1 3'UTR). We used the following primer pairs to amplify the 885bp RluA-1 homology ARM1: RluA-1-ARM1F: 5' -AATACACCTGCATTATCGCTGGTCCCTGTGGCTTTGCAC-3', RluA-1-ARM1R: 5' -AATACACCTGCATTACTAACGAGCTGCTGCTTGATGGGCAAACCGCATTT-3'. We used RluA-1-ARM2F: 5'-GACTGCTCTTCGTATCTTTGGATGGTGCTTAAAC-3' and RluA-1-ARM2R: 5' -ATTAGCTCTTCTGACCTTACACTCCTTGCTGATGCTTACACAC-3' to amplify the 979bp RluA-1 homology ARM2. For RluA-2, we used a target site at the 5' end: 5'-GCAATCTATAGGTCTGGGGAAGC-3' (cut site at bp 5 of exon 3) and at the 3' end: 5'AAAGATAAACACTACAGAGCCCGGG-3' (cutting site in the middle of exon 8) so that the conserved pseudouridine synthase domain (located in exon 5) would be deleted. Primer pairs RluA-2-ARM1F: 5'CTAACACCTGCAATTCATCGCTGCTCGAAACCCATTTGATGGGAGCG-3' and RluA-2 ARM1R: 5' CATTCAACCTGATTACTACGCAGACCTTGTTATGGAATG-3' were used to amplify the 979bp
RluA-2 homology ARM1, RluA-2-ARM2F: 5’ CAGTGCTCTTCTGATCCGCCGCAAAAGGATCTCA-3’, and RluA-2-ARM2R: 5’ GACTGCTCTTCCGACATTTCAATGGCTTTGGCCAA-3’ were used to amplify the RluA-2 ARM2 (998bp). Rapid dsDNA donor cloning was carried out with the pHD-DsRed-attP vector (65) and the guide RNA (gRNAs) were cloned into pU6-Bsbl-chiRNA vector (66). Embryos of vas-Cas9 on chromosome III (w^{118B}; PBac[y+mDint2]=vas-Cas9]VK00027, “injection line 1”) were injected with RluA-1 dsDNA donor and gRNAs to generate G₀ founders for RluA-1\textsuperscript{del-HDR}. Embryos with nos-Cas9 on chromosome III [y sc v; {nos-Cas9}attP2 (TH00787. N), “injection line 2”) were injected with RluA-2 dsDNA donor and gRNAs to create the G₀ founders for RluA-2\textsuperscript{del-HDR}. For generating the double mutant RluA-1\textsuperscript{del-HDR}, RluA-2\textsuperscript{del-HDR}, nos-Cas9 was first introduced to the RluA-1\textsuperscript{del-HDR} mutant background and the DsRed marker in RluA-1\textsuperscript{del-HDR} removed with CRE recombinase and the resultant homozygous strain [yw; RluA-1\textsuperscript{del-HDR}, ΔDsRed; {nos-Cas9}attP2 (TH00787. N), “injection line 3”] was injected with the RluA-2 dsDNA donor and gRNAs. All embryo injections of the dsDNA donor (at a concentration of 500ng/ul) and gRNAs (100ng/ul for each) were performed by the Model system Injections (modelsysteminjections@flymsi.com).

To identify the desired HDR mutants, G₀ flies were crossed to w^{118B} and single F₁ founders were identified with DsRed fluorescence in the eyes (from 3XP3-DsRed reporter) and mated with a second chromosome balancer strain to establish independent lines. Molecular testing for the desired events was performed by PCR on gDNA extracted from candidates placed over a deficiency (Df(2L)Exelixis7048) covering the RluA-1 and RluA-2 region (primer pairs marked in S2A and S4A Figs). PCR products sequenced across the attP-loxP-3XP3-DsRed-SV40-loxP fragment (S2B and S4B Figs, Fig 4C) confirmed accurate targeting of the loci. For behavioral analysis, the original deletion mutants were backcrossed to CS, w^{118B} or isow^{118B} for six times. For each generation, five heterozygous females were selected for six successive
backcrosses in vials and about 10 heterozygous females were used to cross to a second chromosome balancer to produce balanced mutant males in bottles and finally heterozygous mutant virgins and balanced males were crossed “en masse” in bottles to establish the balanced and homozygous mutant lines. In all crosses the dsRed fluorescence marker was used to follow the presence of the mutant.

**Generation of deletion line in **RluA-1** and **RluA-2** using FRT-mediated deficiency**

FRT bearing insertions {WH4}^{fo2750} and {XP-}^{d2586} and in RluA-1 (locations marked in S2A Fig) were used for generating a deficiency allele RluA-1^{del-FRT}. Insertions of {WH-}RluA-2^{F07702} and {WH-}Grip75^{fo5483} (locations marked in S4A Fig) were used for generating the deficiency allele RluA-2^{del-FRT}. Crossing and heat-shock schemes followed (49). Hybrid PCR with corresponding primers (WH5’ plus / XP5’ minus left and right primers) was used to screen for candidate lines with w- deletion in RluA-1^{del-FRT} and two-sided PCR with left and right primers for WH5’ minus/WH5’ minus was used for screening for candidate lines with w+ deletion in RluA-2^{del-FRT} (49).

Molecular testing for the deletion was performed by PCR on gDNA extracted from positive candidates placed over a deficiency (Df(2L)Exelixis7048) covering the RluA-1 and RluA-2 region. The primers used for PCR and sequencing verification in RluA-1^{del-FRT} are RluA-1-d2586-up-F: 5’-AAAAATGCCTGTTTGCCC-3’, located upstream of the {XP-}d2586 insertion site and RluA-1-fo2750-down-R: 5’-AAGGGGTAAACAAAAGGTCCAG-3’, downstream of {WH+}fo2750 insertion site.

**Generation of RluA-1^{Gal4} using “Trojan-exon”**

A triplet Trojan exon donor line on the 3rd chromosome (yw; Sp/CyO; pC-(lox-attB2-SA-T2A-Gal4-Hsp70)3) and an RluA-1 insertion line containing an intronic MiMIC element (Mi[MiC]RluA-1^{MboBB97}) were used to generate the RluA-1^{Gal4} driver using a crossing scheme as described by (45). Candidate males who have lost the y’ selection marker associated with MiMIC were crossed to 40UAS-mCD8::GFP line and animals expressing GFP were selected to
establish a stable line. The line with the correct linker (phase 0) was confirmed with sequencing of PCR products amplifying the left side with primers \textit{RluA-1-5494F}: 5'-TGATGTTGCCCCATAACG-3' and \textit{T2A-Gal4-Seq-1R}: 5' CGCTATCGATGCTACGGTC-3' and the right side with the primers \textit{T2A-Gal4-4F}: 5'-ACACGTGCTGCTAGGC-3' and \textit{RluA-1-5907R}: 5'-GAAAACATCGACATCTGG-3' of the \textit{RluA-1} genome-\textit{T2A-Gal4} insertion bordering region.

**Generation of GFSTF insertions in RluA-1 and RluA-2 by recombination mediated cassette exchange (RMCE)**

Crossing, heat shocking and screening for EGFP tagged MiMIC lines in \textit{RluA-1} and \textit{RluA-2} were essentially carried out as described (46). Males carrying a MiMIC insertion in a coding intron of \textit{RluA-1} (\textit{RluA-1-MI06897}) or \textit{RluA-2} (\textit{RluA-2-MI12981}) were crossed to females carrying the \textit{hs-FLP} and \textit{vasa-\phi C31} integrase on the X chromosome and a frame-specific ("phase 0") \textit{FRT} flanked multiple tag (GFSTF) cassette on chromosome III. Candidate males with mosaic \textit{w-} eyes and \textit{y-} bodies were individually crossed to \textit{w; Sco/CyO; Sb/TM3 Ser} balancers to establish stocks. The presence and direction of the insertion were tested by PCR assays described (67). Since the original MiMIC insertion in \textit{RluA-1} or \textit{RluA-2} and the respective gene are in the same orientation, positive PCR reaction 1 (with primers MiLF and TagR) and 4 (with primers MiLR and TagF) as described (67) indicated a successful RMCE event and resulted in expression.

**Generation of UAS-RluA-1**

\textit{Drosophila RluA-1} full length cDNA clone for \textit{RluA-1} transcript A (Flo4540) was obtained from the \textit{Drosophila} Genome Resource Center (DGRC). The \textit{UAS-RluA-1} expression constructs were generated with the ENTR/gateway system following the instructions of the manufacturer (Invitrogen). The \textit{RluA-1-cDNA-F} (5'-CACCAGTGAAGATTCCGGCT-3'), \textit{RluA-1-cDNA+STOP-R} (5'-TCATGGCGAGTCTAAGTG-3') primers were used to amplify the open reading frame and cloned into \textit{pENTR-D-TOPO} vector. The sequence was verified and then cloned to
the P-element based destination vector \( pTW \). Model System Injections performed injections into \( w^{118} \) embryos. \( F_0 \) flies were crossed to \( w^{118} \) and single \( F_1 \) founders were identified based on the \( w^1 \) marker. Individual lines were mapped and balanced to establish stable stocks. A \( UAS-RluA-1 \) line with relatively weak expression evaluated by Q-PCR in \( RluA-1 \)-cDNA lines driven by \( md\text{-GAL4} \) was used for the cIV-neuron specific \( RluA-1 \)-cDNA rescue experiment (Fig 3B).

**Larval behavioral analyses**

Wandering 3\(^{rd} \) instar larvae were washed out from vials and acclimated for 5 min in petri dishes before testing. Larval thermal and mechanical nociception response assays were conducted as previously described (4, 17, 56). Behavioral recording and scoring were performed with the observer blinded to the genotype.

For gentle touch assays, early L3 larvae were scooped out from the top layer of the fly food in the vials and 5-10 larvae were briefly rinsed with PBS and allowed to acclimate on 1% agarose in a plate for 5 min before testing with an eyelash fixed to the end of a paintbrush. Each larva was brushed with the eyelash on segments T1-A3 for 4 times and the responses were recorded and summarized using a gentle touch scale (68).

**Immunohistochemistry and microscopy**

The following primary antibodies were used for immunofluorescence: rabbit anti-GFP (ab6556, Abcam, 1:500), mouse anti-GFP (ab38689, Abcam, 1:500), Rabbit anti-HRP (1:100), mouse anti-nc82 (DSHB, supernatant, 1:30). Alexa Fluor 488, 633 were used at 1:1000 as secondary antibodies. Detailed immunostaining protocol is available on request. Images for immunostained tissues were taken on a Zeiss LSM 5 LIVE confocal microscope using a 40X objective except for Fig 1D and S1B Fig, which were taken on a Zeiss LSM880 using the 63X objective.

**CIV dendrite imaging, tracing and analysis**
To image class IV dendrites, the DsRed marker in RluA-1^{del-HDR} was first removed with CRE recombinase (w^{118}; RluA-1^{del-HDR},ΔDsRed) and ppk1.9-CD4::tdTom was introduced to the mutant background and a stable homozygous stock was established. For CIV dendrite analysis, six virgins and three males were crossed in each vial for the mutant (w^{118}; RluA-1^{del-HDR},ΔDsRed; ppk1.9-CD4::tdTom) and control (ppk1.9-CD4::td-Tom). Wandering larvae were anesthetized with diethyl ether in a sealed glass chamber for 15min before being arranged on a slide and covered with 50 mm glass coverslip. Neurons expressing the fluorescently tagged markers were visualized on a Zeiss LSM 5 Live confocal microscope with a 40X oil objective (Plan Apo M27, NA 0.8). Images were collected as 5x3 tiles scans of z-stacks with 512x512 resolution. A MatLab build was used for initial automatic tracing of the ddaC neuron dendrites from the confocal z-stack series TIFF images (69). The generated SWC files were overlaid onto the maximum intensity projected image of the neuron in neuTube (70) and manually curated to eliminate tracing errors made by MatLab. The corrected images were then analyzed with MatLab to extract neuron features of interest including number of branches, average branch length, and neuron size (the estimated size of the neuron, defined as the area of the minimum bounding circle) (69). Iso-neuronal crossover events were quantified manually from the traced dendrites for each genotype (n=6 neurons).

**Statistical analysis**

Statistics were performed using GraphPad Prism 4. Thermal and gentle touch behavioral data were compared with an unpaired non-parametric Mann-Whitney test when comparing two groups and Kruskal-Wallis test when comparing three or more groups. Mechanical nociception behavioral data were compared with Fisher's exact test. Dendrite morphology data were compared with the Student's t test. Error bars represent S.D. in all the figures unless otherwise specified.
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References

1. Gaskin DJ, Richard P. The economic costs of pain in the United States. J Pain. 2012;13(8):715-24.
2. Tracey WD, Wilson RI, Laurent G, Benzer S. painless, a Drosophila gene essential for nociception. Cell. 2003;113(2):261-73.
3. Hwang RY, Zhong L, Xu Y, Johnson T, Zhang F, Deisseroth K, et al. Nociceptive neurons protect Drosophila larvae from parasitoid wasps. Curr Biol. 2007;17(24):2105-16.
4. Caldwell JC, Tracey WD. Alternatives to Mammalian Pain Models 2: Using Drosophila to Identify Novel Genes Involved in Nociception. Methods Mol Biol. 2010;617:19-29.
5. Milinkeviciute G, Gentile C, Neely GG. Drosophila as a tool for studying the conserved genetics of pain. Clin Genet. 2012;82(4):359-66.
6. Im SH, Galko MJ. Pokes, sunburn, and hot sauce: Drosophila as an emerging model for the biology of nociception. Dev Dynam. 2012;241(1):16-26.
7. Tracey WD, Jr. Nociception. Curr Biol. 2017;27(4):R129-R33.
8. Khuong TM, Wang QP, Manion J, Oyston LJ, Lau MT, Towler H, et al. Nerve injury drives a heightened state of vigilance and neuropathic sensitization in Drosophila. Sci Adv. 2019;5(7):eaaw4099.
9. Xiang Y, Yuan Q, Vogt N, Looger LL, Jan LY, Jan YN. Light-avoidance-mediating photoreceptors tile the Drosophila larval body wall. Nature. 2010;468(7326):921-6.

10. Grueber WB, Jan LY, Jan YN. Tiling of the Drosophila epidermis by multidendritic sensory neurons. Development. 2002;129(12):2867-78.

11. Hu C, Petersen M, Hoyer N, Spitzweck B, Tenedini F, Wang D, et al. Sensory integration and neuromodulatory feedback facilitate Drosophila mechanonociceptive behavior. Nat Neurosci. 2017;20(8):1085-95.

12. Ohyama T, Schneider-Mizell CM, Fetter RD, Aleman JV, Franconville R, Rivera-Alba M, et al. A multilevel multimodal circuit enhances action selection in Drosophila. Nature. 2015;520(7549):633-9.

13. Chin MR, Tracey WD, Jr. Nociceptive Circuits: Can't Escape Detection. Curr Biol. 2017;27(16):R796-R8.

14. Burgos A, Honjo K, Ohyama T, Qian CS, Shin GJ, Gohl DM, et al. Nociceptive interneurons control modular motor pathways to promote escape behavior in Drosophila. Elife. 2018;7.

15. Ohyama T, Jovanic T, Denisov G, Dang TC, Hoffmann D, Kerr RA, et al. High-throughput analysis of stimulus-evoked behaviors in Drosophila larva reveals multiple modality-specific escape strategies. Plos One. 2013;8(8):e71706.

16. Zhong L, Hwang RY, Tracey WD. Pickpocket is a DEG/ENaC protein required for mechanical nociception in Drosophila larvae. Curr Biol. 2010;20(5):429-34.

17. Mauthner SE, Hwang RY, Lewis AH, Xiao Q, Tsubouchi A, Wang Y, et al. Balboa Binds to Pickpocket In Vivo and Is Required for Mechanical Nociception in Drosophila Larvae. Curr Biol. 2014;24(24).
18. Gorczyca DA, Younger S, Meltzer S, Kim SE, Cheng L, Song W, et al. Identification of Ppk26, a DEG/ENaC Channel Functioning with Ppk1 in a Mutually Dependent Manner to Guide Locomotion Behavior in Drosophila. Cell Rep. 2014;9(4):1446-58.

19. Zhong L, Bellemer A, Yan H, Ken H, Jessica R, Hwang RY, et al. Thermosensory and nonthermosensory isoforms of Drosophila melanogaster TRPA1 reveal heat-sensor domains of a thermoTRP Channel. Cell Rep. 2012;1(1):43-55.

20. Honjo K, Mauthner SE, Wang Y, Skene JH, Tracey WD, Jr. Nociceptor-Enriched Genes Required for Normal Thermal Nociception. Cell Rep. 2016;16(2):295-303.

21. Brewster R, Bodmer R. Origin and specification of type II sensory neurons in Drosophila. Development. 1995;121(9):2923-36.

22. Wang CC, Lo JC, Chien CT, Huang ML. Spatially controlled expression of the Drosophila pseudouridine synthase RluA-1. Int J Dev Biol. 2011;55(2):223-7.

23. Olesnicky EC, Killian DJ, Garcia E, Morton MC, Rathjen AR, Sola IE, et al. Extensive use of RNA-binding proteins in Drosophila sensory neuron dendrite morphogenesis. G3 (Bethesda). 2014;4(2):297-306.

24. Ye B, Petritsch C, Clark IE, Gavis ER, Jan LY, Jan YN. Nanos and Pumilio are essential for dendrite morphogenesis in Drosophila peripheral neurons. Curr Biol. 2004;14(4):314-21.

25. Pan L, Zhang YQ, Woodruff E, Broadie K. The Drosophila fragile X gene negatively regulates neuronal elaboration and synaptic differentiation. Curr Biol. 2004;14(20):1863-70.

26. Brechbiel JL, Gavis ER. Spatial regulation of nanos is required for its function in dendrite morphogenesis. Curr Biol. 2008;18(10):745-50.

27. Bianco A, Dienstbier M, Salter HK, Gatto G, Bullock SL. Bicaudal-D regulates fragile X mental retardation protein levels, motility, and function during neuronal morphogenesis. Curr Biol. 2010;20(16):1487-92.
28. Xu X, Brechbiel JL, Gavis ER. Dynein-dependent transport of nanos RNA in Drosophila sensory neurons requires Rumpelstiltskin and the germ plasm organizer Oskar. J Neurosci. 2013;33(37):14791-800.

29. Ge J, Yu YT. RNA pseudouridylation: new insights into an old modification. Trends Biochem Sci. 2013;38(4):210-8.

30. Carlile TM, Rojas-Duran MF, Zinshteyn B, Shin H, Bartoli KM, Gilbert WV. Pseudouridine profiling reveals regulated mRNA pseudouridylation in yeast and human cells. Nature. 2014;515(7525):143-+.

31. Lovejoy AF, Riordan DP, Brown PO. Transcriptome-Wide Mapping of Pseudouridines: Pseudouridine Synthases Modify Specific mRNAs in S. cerevisiae. Plos One. 2014;9(10).

32. Schwartz SJ, Bernstein DA, Mumbach MR, Jovanovic M, Herbst RH, Leon-Ricardo BX, et al. Transcriptome-wide mapping reveals widespread dynamic-regulated pseudouridylation of ncRNA and mRNA. Cell. 2014;159(1):148-62.

33. Khoddami V, Yerra A, Mosbruger TL, Fleming AM, Burrows CJ, Cairns BR. Transcriptome-wide profiling of multiple RNA modifications simultaneously at single-base resolution. Proc Natl Acad Sci U S A. 2019;116(14):6784-9.

34. Knight SW, Heiss NS, Vulliamy TJ, Greschner S, Stavrides G, Pai GS, et al. X-linked dyskeratosis congenita is predominantly caused by missense mutations in the DKC1 gene. Am J Hum Genet. 1999;65(1):50-8.

35. Fujiwara T, Harigae H. Pathophysiology and genetic mutations in congenital sideroblastic anemia. Pediatr Int. 2013;55(6):675-9.

36. de Brouwer APM, Abou Jamra R, Kortel N, Soyris C, Polla DL, Safra M, et al. Variants in PUS7 Cause Intellectual Disability with Speech Delay, Microcephaly, Short Stature, and Aggressive Behavior. Am J Hum Genet. 2018;103(6):1045-52.
37. Hammal T, Ferre-D’Amare AR. Pseudouridine synthases. Chem Biol. 2006;13(11):1125-
38. Koonin EV. Pseudouridine synthases: four families of enzymes containing a putative
39. Phillips B, Billin AN, Cadwell C, Buchholz R, Erickson C, Merriam JR, et al. The Nop60B
gene of Drosophila encodes an essential nucleolar protein that functions in yeast. Mol Gen
40. Giordano E, Peluso I, Senger S, Furia M. minifly, a Drosophila gene required for
41. Vicidomini R, Petrizzo A, di Giovanni A, Cassese L, Lombardi AA, Pragliola C, et al.
Drosophila dyskerin is required for somatic stem cell homeostasis. Sci Rep. 2017;7(1):347.
42. Hoang C, Chen JJ, Vizthum CA, Kandel JM, Hamilton CS, Mueller EG, et al. Crystal
structure of pseudouridine synthase RluA: Indirect sequence readout through protein-induced
RNA structure. Mol Cell. 2006;24(4):535-45.
43. Gutgsell NS, Deutscher MP, Ofengand J. The pseudouridine synthase RluD is required
for normal ribosome assembly and function in Escherichia coli. Rna. 2005;11(7):1141-52.
44. Raychaudhuri S, Niu L, Conrad J, Lane BG, Ofengand J. Functional effect of deletion
and mutation of the Escherichia coli ribosomal RNA and tRNA pseudouridine synthase RluA. J
Biol Chem. 1999;274(27):18880-6.
45. Diao F, Ironfield H, Luan H, Diao F, Shropshire WC, Ewer J, et al. Plug-and-play genetic
access to drosophila cell types using exchangeable exon cassettes. Cell Rep. 2015;10(8):1410-21.
46. Nagarkar-Jaiswal S, DeLuca SZ, Lee P-T, Lin W-W, Pan H, Zuo Z, et al. A genetic toolkit
for tagging intronic MiMIC containing genes. Elife. 2015;4.
47. Ainsley JA, Pettus JM, Bosenko D, Gerstein CE, Zinkevich N, Anderson MG, et al. Enhanced locomotion caused by loss of the Drosophila DEG/ENaC protein Pickpocket1. Curr Biol. 2003;13(17):1557-63.

48. Ran FA, Hsu PD, Wright J, Agarwala V, Scott DA, Zhang F. Genome engineering using the CRISPR-Cas9 system. Nat Protoc. 2013;8(11):2281-308.

49. Parks AL, Cook KR, Belvin M, Dompe NA, Fawcett R, Huppert K, et al. Systematic generation of high-resolution deletion coverage of the Drosophila melanogaster genome. Nat Genet. 2004;36(3):288-92.

50. Venken KJ, Carlson JW, Schulze KL, Pan H, He Y, Spokony R, et al. Versatile Pacman BAC libraries for transgenesis studies in Drosophila melanogaster. Nat Methods. 2009;6(6):431-4.

51. Han C, Wang D, Soba P, Zhu S, Lin X, Jan LY, et al. Integrins regulate repulsion-mediated dendritic patterning of drosophila sensory neurons by restricting dendrites in a 2D space. Neuron. 2012;73(1):64-78.

52. Kim SE, Coste B, Chadha A, Cook B, Patapoutian A. The role of Drosophila Piezo in mechanical nociception. Nature. 2012;483(7388):209-12.

53. Tenenbaum CM, Misra M, Alizzi RA, Gavis ER. Enclosure of Dendrites by Epidermal Cells Restricts Branching and Permits Coordinated Development of Spatially Overlapping Sensory Neurons. Cell Rep. 2017;20(13):3043-56.

54. Meltzer S, Yadav S, Lee J, Soba P, Younger SH, Jin P, et al. Epidermis-Derived Semaphorin Promotes Dendrite Self-Avoidance by Regulating Dendrite-Substrate Adhesion in Drosophila Sensory Neurons. Neuron. 2016;89(4):741-55.

55. Corty MM, Matthews BJ, Grueber WB. Molecules and mechanisms of dendrite development in Drosophila. Development. 2009;136(7):1049-61.
56. Walcott KCE, Mauthner SE, Tsubouchi A, Robertson J, Tracey WD. The Drosophila Small Conductance Calcium-Activated Potassium Channel Negatively Regulates Nociception. Cell Rep. 2018;24(12):3125-32 e3.

57. Onodera K, Baba S, Murakami A, Uemura T, Usui T. Small conductance Ca(2+)-activated K(+) channels induce the firing pause periods during the activation of Drosophila nociceptive neurons. Elife. 2017;6.

58. Rangaraju V, Tom Dieck S, Schuman EM. Local translation in neuronal compartments: how local is local? EMBO Rep. 2017;18(5):693-711.

59. Glock C, Heumuller M, Schuman EM. mRNA transport & local translation in neurons. Curr Opin Neurobiol. 2017;45:169-77.

60. Arnez JG, Steitz TA. Crystal structure of unmodified tRNA(Gln) complexed with glutaminyl-tRNA synthetase and ATP suggests a possible role for pseudo-uridines in stabilization of RNA structure. Biochemistry-U. 1994;33(24):7560-7.

61. Newby MI, Greenbaum NL. Sculpting of the spliceosomal branch site recognition motif by a conserved pseudouridine. Nat Struct Biol. 2002;9(12):958-65.

62. Kariko K, Muramatsu H, Welsh FA, Ludwig J, Kato H, Akira S, et al. Incorporation of pseudouridine into mRNA yields superior nonimmunogenic vector with increased translational capacity and biological stability. Mol Ther. 2008;16(11):1833-40.

63. Karjolich J, Yu YT. Converting nonsense codons into sense codons by targeted pseudouridylation. Nature. 2011;474(7351):395-+.

64. Li XY, Zhu P, Ma SQ, Song JH, Bai JY, Sun FF, et al. Chemical pulldown reveals dynamic pseudouridylation of the mammalian transcriptome. Nat Chem Biol. 2015;11(8):592-U93.

65. Beumer KJ, Carroll D. Targeted genome engineering techniques in Drosophila. Methods. 2014;68(1):29-37.
66. Gratz SJ, Cummings AM, Nguyen JN, Hamm DC, Donohue LK, Harrison MM, et al. Genome engineering of Drosophila with the CRISPR RNA-guided Cas9 nuclease. Genetics. 2013;194(4):1029-35.

67. Venken KJT, Schulze KL, Haelterman NA, Pan H, He Y, Evans-Holm M, et al. MiMIC: a highly versatile transposon insertion resource for engineering Drosophila melanogaster genes. Nat Methods. 2011;8(9):737-43.

68. Tsubouchi A, Caldwell JC, Tracey WD. Dendritic filopodia, Ripped Pocket, NOMPC, and NMDARs contribute to the sense of touch in Drosophila larvae. Curr Biol. 2012;22(22):2124-34.

69. Gulyanon S, Sharifai, N., Kim, M. D., Chiba, A., Tsechpenakis, G. CRF formulation of active contour population for efficient three-dimensional neurite tracing. 2016 IEEE 13th International Symposium on Biomedical Imaging 2016. p. 593-7.

70. Feng L, Zhao T, Kim J. neuTube 1.0: A New Design for Efficient Neuron Reconstruction Software Based on the SWC Format. eNeuro. 2015;2(1).

Figure Legends

Fig 1. RluA-1 gene and protein expression in Drosophila melanogaster. (A) A low magnification confocal micrograph of RluA-1 gene expression pattern in the larval PNS (third instar w[11]; RluA-1‡ UAS-mCD8::GFP). Note that GFP positive signals are detected in peripheral sensory neurons in each segment along the larval body wall. Scale bar=500μm. (B) Higher magnification of an abdominal dorsal PNS cluster. RluA-1‡ is expressed in the cell body and dendrites of all classes of multidendritic neurons, external sensory (ES), dorsal multiple dendrite neuron (dmd1) and dorsal bipolar dendritic (dbd) neurons (ddaD and ddaE (Class I), ddaB (Class II), ddaA and ddaF (Class III), and ddaC (Class IV), ES, dmd1 and dbd neurons are labeled). Scale bar = 50μm. (C) RluA-1‡ driving expression of UAS-mCD8::GFP/+ in the
larval CNS. Labelling is observed in axonal projections of sensory neurons in the larval ventral nerve cord and unidentified clusters of neurons in the larval brain. Scale bar = 50 μm. (D<sup>3</sup>) anti-GFP immunohistochemistry of a third instar larval fillet preparation of *RluA-1-GFSTF/Gal4<sup>109(2)80</sup>*>UAS-mCD8-RFP<sup>, </sup>immunoreactive signals are detected in the nuclei of multi-dendritic neurons (D<sup>3</sup> green, GFP) surrounded by the membrane-localized RFP signal (D<sup>3</sup> magenta, RFP) and merged image in D<sup>3</sup>. Scale bar = 50 μm.

**Fig 2.** Thermal nociception in animals with *RluA-1* loss of function. (A). Class IV specific knock-down in *RluA-1* results in significant hypersensitive thermal nociception in larvae compared to the *ppk-GAL4* driver alone animals at 46°C (average latency of 1.33 ± 1.10 sec for *ppk-Gal4* > *UAS-RluA-1-RNAi (31719-R1)*, n=38 vs. 2.04 ± 1.89 sec for *ppk-GAL4* driver alone, n=37. P<0.05), albeit not statistically significant compared to the UAS-RNAi alone (1.96 ± 1.32 sec for UAS-RNAi alone, n=27). These differences are more pronounced at 42°C (average latency of 1.92 ± 1.16 sec for *ppk-Gal4* > *UAS-RluA-1-RNAi*, n=35 vs. 6.25 ± 2.51 sec for *ppk-GAL4* driver alone controls, n=42 or 5.20 ± 3.28 sec for UAS-RNAi alone controls, n=49, P<0.0001). Kruskal-Wallis test with Dunn’s multiple comparison tests, only significant comparisons were labeled.

(B). Homozygous null mutant *RluA-1<del-HDR* larvae showed significantly faster response to noxious heat stimulation of 42°C compared to the corresponding control animals of *Canton-S* (average latency of 1.29 ± 0.78 sec in *RluA-1<sup>-/-</sup>* , n=54 vs. 2.22 ± 1.32 sec in *CS*, n=39), *w<sup>1118</sup>* (2.23 ± 1.57 sec in *RluA-1<sup>-/-</sup>* , n=51 vs. 4.16 ± 2.31 sec in *w<sup>1118</sup>* , n=36) and *isow<sup>1118</sup>* (4.82 ± 2.53 sec in *RluA-1<sup>-/-</sup>* , n=30 vs. 11.99 ± 3.98 sec in *isow<sup>1118</sup>* , n=17). Significance of comparisons are marked as **** (p<0.0001). Data were analyzed using Mann-Whitney non-parametric test. (C).

Transheterozygote *RluA-1<del-HDR*/*RluA-1<del-FRT* showed hypersensitive thermal nociception responses compared to the controls (average latency of 3.07 ± 1.72 sec in *RluA-1<del-HDR*/*RluA-1<del-FRT*, n=195 vs. 6.17 ± 3.95 sec in +/-, n=125). The genetic background is *w<sup>1118</sup>* for *RluA-1<del-HDR* , and
isow<sup>1118</sup> for RluA<sup>-1</sup><sub>det-HDR</sub>. For the RluA<sup>-1</sup><sub>det-HDR</sub>/RluA<sup>-1</sup><sub>det-FRT</sub> transheterozygotes, data were pooled from the progeny from reciprocal crosses of RluA<sup>-1</sup><sub>det-HDR</sub> to RluA<sup>-1</sup><sub>det-FRT</sub>. To generate the control larvae, reciprocal crosses were made between the genetic background of w<sup>1118</sup> and isow<sup>1118</sup> and the data from the progeny of these crosses were pooled. Significance of the comparison is marked as **** (exact p<0.0001). Data were analyzed using Mann-Whitney U test.

Fig 3: RluA-1 nociception phenotypes with (A) chromosome duplication or (B) Class IV-specific expression of UAS-RluA-1 cDNA. (A). The hypersensitive nociception phenotype with 42°C thermal stimulus in RluA-1<sup>-1</sup><sub>det-HDR</sub> null mutant was rescued by introducing a duplication (Dp) on the third chromosome which covers the RluA-1 gene region (average latency of 8.02 ± 3.61 sec in RluA-1<sup>-1</sup>; Dp/Dp, n=38, which is similar to the isow<sup>1118</sup> genetic background (+/+ , average latency of 10.16±3.87 sec, n=22), but significantly slower than the latency of 5.31 ± 2.16 sec in RluA-1<sup>-1</sup>, n=34 (p=0.02)). Larvae with one copy of duplication showed slower response (average latency of 6.90 ± 4.81 sec in RluA-1<sup>-1</sup>; Dp/+, n=45) without statistical significance compared to RluA-1<sup>-1</sup><sub>det-HDR</sub>. Data were analyzed using Kruskal-Wallis test with Dunn’s multiple comparison tests. (B). The hypersensitive thermal nociception phenotype in RluA-1<sup>-1</sup><sub>det-HDR</sub> larvae was completely reversed to that of the heterozygous RluA-1 by Class IV specific expression of full length RluA-1-cDNA (RluA-1<sup>-1</sup>; ppk-Gal4<sup>+/+</sup>; UAS-RluA-1<sup>+/+</sup>, average latency of 12.37 ±6.48 sec, n=30, is similar to RluA-1<sup>-1</sup>; ppk<sub>1.9-Gal4</sub><sup>+/+</sup>, average latency of 11.04 ± 5.44 sec, n=20 but significantly slower than either the driver alone (RluA-1<sup>-1</sup>; Ppk<sub>1.9-Gal4</sub><sup>+/+</sup>, average latency of 3.78±2.11 sec, n=25 or transgene alone controls (RluA-1<sup>-1</sup>; UAS-RluA-1<sup>-1</sup>, average latency of 2.86 ± 1.39 sec, n=33) at 42°C thermal stimulus. Significance of the comparisons are marked as **** (p<0.0001). Data were analyzed using Kruskal-Wallis test with Dunn’s multiple comparison tests.

Fig 4: RluA-1 mechanical nociception and gentle touch responses. (A). In response to the noxious mechanical stimulus of 30mN/720kPa, all (100%) of the RluA-1<sup>-1</sup><sub>det-HDR</sub> null mutant larvae
(RluA-1^−/−, n=28) rolled compared to the 78.57% of control larvae (iso^w1118 n=28) rolling. At the reduced stimulus of 15mN/360kPa, 95.56% of RluA-1^−/− (n=45) rolled while only 48.53% of control (n=68) rolled. Significance of the comparisons are marked as *(p<0.05) and **** (p<0.0001). Data were analyzed using Fisher’s exact test and presented as percentages ± 95% confidence intervals. (B). RluA-1^del-HDR larvae (RluA-1^−/−) had a gentle touch response score (6.70 ± 2.48, n=36) similar to the control larvae (iso^w1118, 7.83 ± 2.69, n=30). ns, not significant (p>0.05). Data were analyzed with Student’s t test.

Fig 5: RluA-2 expression in Drosophila larvae. (A). In third instar larvae of RluA-2-GFSTF line, anti-GFP (green) immunoreactive signals are detected in the nuclei of all types of cells (neurons are labelled with anti-HRP (red)). Scale bar = 100μm. (B). In the larval abdominal dorsal PNS cluster of RluA-2-GFSTF line, GFP signal (green) is also detected in the nuclei of the md neurons, whose membranes are marked with anti-HRP (magenta). Arrow points to Class IV ddaC neuron. Scale bar = 50μm.

Fig 6. Thermal nociception responses of RluA-1 and RluA-2 single mutant, and RluA-1 RluA-2 double mutant. Larvae of single mutant RluA-1^del-HDR (RluA-1^−/−), RluA-2^del-HDR (RluA-2^−/−), double mutant RluA-1^del-HDR RluA-2^del-HDR (RluA-1^−/−RluA-2^−/−) and the genetic background iso^w1118 (+/+), were stimulated with noxious heat probe of 42ki//. RluA-1^−/−, RluA-2^−/−, and RluA-1^−/−RluA-2^−/− all displayed faster responses to the stimulus compared to controls (RluA-1^−/− average latency of 4.32 ± 2.80 sec, n= 79; RluA-2^−/−, 6.63 ± 3.31 sec, n=68; (RluA-1^−/−RluA-2^−/−), 6.26 ± 3.46 sec, n=57; +/+, 13.72 ± 6.03 sec, n=46). Significance of comparisons are marked as ** (p<0.01), *** (p<0.001) or **** (p<0.0001). Data were analyzed using Kruskal-Wallis test with Dunn’s multiple comparison tests.

Fig 7. RluA-1 ddaC neuron dendrite morphology. (A-C). Quantification of the branch number (A), average branch length (B), and isoneuronal cross-over points (C). (A). Homozygous RluA-
del-HDR larvae showed a higher normalized branch number (total number of branches / (neuron size x 10^4 μm^2)), 32.56 ± 7.83 in RluA-1^-; ppk-tdTom, n=6 vs 23.10 ± 4.96 in ppk-tdTom control, n=6, p<0.05). (B). The mean ddaC dendritic branch length in homozygous RluA-1^del-HDR was shorter than that of the control (average branch length is 14.01 ± 1.29 μm in RluA-1^-; ppk-tdTom, n=6 vs 16.30 ± 1.07 μm in ppk-tdTom control, n=6, p<0.01). (C). The number of ddaC dendritic isoneuronal crossovers in homozygous RluA-1^del-HDR was higher compared to that of the control (normalized crossover points = crossover points / (neuron size x 10^7 μm^2)), 25.03 ± 8.24 in RluA-1^-; ppk-tdTom, n=6 vs 13.48 ± 4.08 in ppk-tdTom control, n=6, p<0.05). Significant differences are marked as * (p<0.05) and ** (p<0.01). Data were analyzed with Student’s t test. (D). Representative traced dendritic structure of a class IV ddaC neuron of an L3 larva expressing ppk-GAL4 > UAS-td-Tom in homozygous RluA-1^del-HDR (RluA-1^del-HDR, left) in comparison with that of the ppk-tdTom control (+/+; right). The position of the cell body is marked with blue circle and axon with red circles.
Figure 1
**Figure 2**

### A

- **ppk-GAL4, UAS-dicer2**
  - +
  - -
  - +
  - +
- **UAS-RluA-1-RNAi**
  - -
  - +
  - +

### B

- **/+ RluA-1<sup>1</sup>**
  - **CS**
  - **w<sup>1118</sup>**
  - **isow<sup>1118</sup>**

### C

- **/+**
  - **RluA-1<sup>1</sup>del-HDR**
  - **RluA-1<sup>1</sup>del-FRT**
Figure 3

A

Average latency (sec)

+/- RluA-1+/+; Dp/+ RluA-1+/+; Dp/Dp RluA-1+/+

B

Average latency (sec)

RluA-1+/+ ppk-GAL4 UAS-RluA-1

- + - + +
Figure 4

A

Percentage rolling (%)

+/+  RluA-1^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^-^...
Figure 5
Figure 6
Figure 7

A) Normalized branch number

B) Average branch length (µm)

C) Normalized crossover points

D) Comparative diagrams showing different branch patterns and normalized branch numbers between +/+ and RluA-1/+.