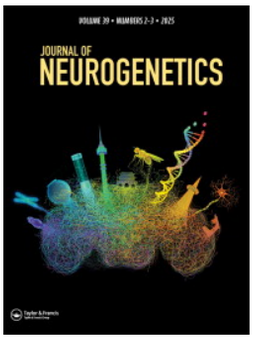

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The ataxin-2 protein is required in kenyon cells for RNP-granule assembly and appetitive long-term memory formation

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ABSTRACT

Ribonucleoprotein granules (mRNP granules) are thought to contribute to the control of neuronal mRNA translation required for consolidation of long-term memories. Consistent with this, the function of Ataxin-2 in mRNA granule assembly has been shown to be required for long-term olfactory habituation (LTH) in *Drosophila*, a form of non-associative memory. Knockdown of Ataxin-2 in either local interneurons (LNs) or projection neurons (PNs) of the insect antennal lobe disrupts LTH while leaving short-term habituation intact, leading to a model in which Ataxin-dependent translational control is required in both presynaptic and postsynaptic elements of the LN-PN synapse, whose potentiation has been causally linked to LTH. Here we use novel and established methods for cell-type specific perturbation to ask: (a) whether Ataxin-2 controls mRNA granule assembly in cell types beyond the few that have been examined; and (b) whether it functions not only in LTH, but also for long-term olfactory associative memory (LTM). We show that Ataxin-2 controls mRNP granule assembly in additional neuronal types, namely Kenyon Cells (KCs) that encode associative memory, as well as more broadly in non-neuronal cells, e.g. in nurse cells in the egg chamber. Furthermore, selective knockdown of Atx2 in α/β and α′/β′ KCs blocks appetitive long-term but not short-term associative memories. Taken together these observations support a hypothesis that Ataxin-2 dependent translational control is widely required across different mnemonic circuits for consolidation of respective forms of long-term memories.

Abbreviations: 3-OCT: 3-octanol; ALS: Amyotrophic Lateral Sclerosis; Atx2: Ataxin-2; cIDR: C-terminally located intrinsically disordered region; EB: ethyl-butyrate; FTD: frontotemporal dementia; ISO: isopentyl acetate; KCs: Kenyon cells; LNs: local interneurons; LTH: long-term habituation; LTM: long-term memory; MARCM: Mosaic Analysis with a Repressible Cell Marker; MBONs: mushroom bodies output neurons; MCH: 4-methylcyclohexanol; mRNP granules: Ribonucleoptotein granules; PBS: Phosphate Buffered Saline; PI: performance index; PNs: projection neurons; PTX: PBS containing 0.2% Triton-X100; RBPs: RNA binding proteins; SCA2: spinocerebellar ataxia-2; STM: short-term memory

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long-term memory; appetitive; RNA-binding protein; translational regulation; neuronal granules; mRNP condensate

Introduction

Long-term memories (LTMs) are distinguished from short-term memories (STMs) not only by their persistence across time, but also by the requirement for new protein synthesis in long-term synaptic plasticity that underlies LTM (Bailey *et al.*, 1996). Several lines of evidence have suggested that activity-regulated synaptic translation of stored mRNAs contributes to long-term forms of plasticity and memory (Das *et al.*, 2023; Donnelly *et al.*, 2010; Martin & Kosik, 2002; Richter & Klann, 2009). Even though the requirement of *de novo* protein synthesis has been well established, specially using protein synthesis inhibitors (Agranoff *et al.*, 1967; Hernandez & Abel, 2008; Squire & Barondes, 1972; Tully *et al.*, 1994), some key questions remain open; for example: what are the translational mechanisms involved in such process? How different are these across cell types? And what is the diversity of cell types in which activity-dependent mRNA translation, and hence a specific type of plasticity, must occur for any particular form of long-term memory.

One of the mechanisms by which *de novo* protein synthesis can be regulated is via activity-dependent modulation of ribonucleoprotein granules (mRNP granules), dynamic, membrane-less organelles, which sequester translationally repressed mRNAs together with proteins that control their translation, localization and turnover (Buchan, 2014).

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Work in the model organism *Drosophila melanogaster* has shown that several components of the neuronal-granule associated, translational-control machinery, such as: Pumilio (Vessey *et al.*, 2006), Staufen (Barbee *et al.*, 2006), EIF5C (Dubnau *et al.*, 2003), Gld2 (Kwak *et al.*, 2008), Orb2 (Hervás *et al.*, 2016; Keleman *et al.*, 2007; Majumdar *et al.*, 2012; Mastushita-Sakai *et al.*, 2010), FMRP and components of the miRNA pathway (Bolduc *et al.*, 2008; Sudhakaran *et al.*, 2014) are essential for consolidation of LTM under conditions where they are entirely dispensable for STM. Significant among these RNA-binding protein components of neuronal granules, is Ataxin-2 (Atx2), a protein altered in neurodegenerative disease, that can serve as both an activator or repressor of translation (Ciosk *et al.*, 2004; Inagaki *et al.*, 2020; Lee *et al.*, 2018; Lim & Allada, 2013; Zhang *et al.*, 2013) Atx2 is also unique in being required for the presence of a specific class of Me31B/DDX6 positive neuronal mRNP condensates, at least in inhibitory local interneurons (LNs) and excitatory projection neurons (PNs) of the *Drosophila* antennal lobes, where Atx2 must be present for olfactory LTH (Bakthavachalu *et al.*, 2018; McCann *et al.*, 2011; Sudhakaran *et al.*, 2014).

The formation of mRNP granules is facilitated by multivalent interactions between mRNA molecules, the binding of RNA binding proteins (RBPs) to their target mRNAs as well as RBP interactions among each other particularly through intrinsically disordered regions (Protter & Parker, 2016). A detailed structure function analysis of the *Drosophila* Atx2 protein domains in cultured cells and *in vivo* identified a C-terminally located, intrinsically disordered region (cIDR) of the protein as being required for efficient neuronal granule formation, but dispensable for animal survival (Bakthavachalu *et al.*, 2018). Significantly, similar to flies with reduced levels of Atx2, flies genetically engineered to lack only the Atx2 cIDR showed defective LTH but normal STH (Bakthavachalu *et al.*, 2018; McCann *et al.*, 2011; Sudhakaran *et al.*, 2014). These data argue for a causal role for mRNP granules in the regulation of translational processes that underly long-term habituation (Bakthavachalu *et al.*, 2018).

Neuronal mRNP condensates are not only of interest for their function in long-term memory, but also for their involvement in neurodegenerative diseases – particularly Amyotrophic Lateral Sclerosis (ALS) and frontotemporal dementia (FTD) - which typically show cell-type specificity (Ling *et al.*, 2013; Nedelsky & Taylor, 2022). Polyglutamine expansions in human Atx2 protein, which increase the stability of Atx2 condensates (Wijegunawardana *et al.*, 2025), are associated with spinocerebellar ataxia-2 (SCA2) and ALS (Elden *et al.*, 2010). Remarkably cytotoxicity seen in fly models of ALS, is greatly reduced in animals lacking the Atx2 cIDR (Bakthavachalu *et al.*, 2018) directly connecting mRNP granules function with disease progression. Further, the involvement of Atx2 in LTM has as well been observed in human patients. For instance, SCA2 patients, with a mutation in the human Atx2 locus, also show a decline in verbal memory (Rodríguez-Labrada *et al.*, 2019).

To more deeply examine Atx2's functions *in vivo*, we investigated (a) whether Atx2 contributes to the assembly of mRNP condensates in multiple neuronal and non-neuronal cells; and (b) how widely mRNP granules contribute to associative long-term memory, which extensive studies have shown to depend on plasticity of a different subset of neurons: namely, in synapses made by Kenyon cells (KCs) onto output neurons (MBONs) of the *Drosophila* mushroom body, a structure known to be the major site of associative learning and memory in insects (Campbell & Turner, 2010).

Material and methods

Fly lines and husbandry

All genotypes were raised on standard cornmeal medium under LD (12 h Light: 12 h Dark) cycles at 25 °C. For behavioural analysis the flies were grown at 18 °C until eclosion, then the animals were moved to 30 °C for a week for RNAi expression. The following Gal4 and effector lines were used: *MB247-gal4*, *c739-gal4*, *c305a-gal4*, *5-HTR1b-gal4*, *UAS-dcr* (S. Sprecher lab stock), *nos-gal4::VP16* (RRID:BDSC 4937; Rørth 1998), *tubGal80(ts)* (RRID:BDSC 7018), *hsFLP,UAS-GFP/FM7*; *TM3Ser/Sb* (G. Jefferis lab stock), *FRT82B* (RRID:BDSC 2035), $atx2^{X1}$ (Satterfield *et al.*, 2002), *Me31B::GFP* (RRID:BDSC 51530; Buszczak *et al.* 2007), *UAS-Atx2 RNAi*[1] (VDRC 34955) (McCann *et al.*, 2011; Nadimpalli *et al.*, 2022; Sudhakaran *et al.*, 2014), *UAS-Atx2 RNAi*[2] (RRID:BDSC 36114) (del Castillo *et al.*, 2022; Monteiro *et al.*, 2025; Palozzi *et al.*, 2022), *UAS-Atx2 RNAi*[3] (RRID:BDSC 67878) (Monteiro *et al.*, 2025; Palozzi *et al.*, 2022).

These RNAi lines were chosen because they target different regions of *Atx2* according to an *in silico* analysis run using the UP-TORR website (https://www.flyrnai.org/up-torr/) and because they were used in previous publications.

Immunohistochemistry

Adult brains

Drosophila adult flies were decapitated, and heads transferred to chilled S2 cell medium (Gibco). The brains were removed from the head capsule and any attached trachea and surrounding tissues were carefully removed. The dissected brains were transferred into a 0.5 ml tube containing 4% paraformaldehyde diluted in Phosphate Buffered Salin (PBS) containing 0.2% Triton-X100 (PTX) and fixed for 20 min at room temperature on a shaker.

Female germline

Mated females (~5–7 days old) were collected and fed liquid yeast paste for 24h prior to dissection. Ovarioles were isolated from the abdomen of females in PBS, transferred into a 0.5 ml tube containing 4% paraformaldehyde diluted in PBS and fixed for 20 min at room temperature on a shaker.

Fixed samples (adult brains & adult female ovarioles) were washed four times for 15 min each in PTX (PBS + 0.2% Triton), blocked with blocking solution (PTX+ 5% NGS) for 1h and then incubated in primary antibody diluted in blocking solution over night at 4 °C. Samples were washed four times for 15 min each in PTX and then incubated in secondary antibodies for 3h at room temperature diluted in

blocking solution. Adult brains were mounted in Vectashield Mounting Medium (Vecta labs) on slides using coverslips (thickness No1) as spacers. Female ovarioles in Vectashield were transferred to a microscope slide, dissociated using tungsten needles and sharp forceps and spread out to allow imaging of individual egg chambers.

Antibodies

Primary antibodies used: mouse rab-Me31B (1:100; Boster), rabbit anti-GFP (1:1000) (Invitrogen), chicken anti-GFP (1:1000) (Abcam). Secondary antibodies used: Alexa 488- and Alexa 555- conjugated anti rabbit and anti-mouse IgG (1:1000) (Invitrogen).

Image acquisition

All images were taken on a Zeiss LSM 880 confocal microscope with Airyscan for high resolution imaging. To image Me31B mRNP particles in fixed brain tissue, we used a 63x objective (Zeiss Plan-Apochromate, 1.4 Oil Ph3) and the 'digital zoom' software feature of the LSM 880 software (zoom factor between 3 and 4) that allows to scan a region of interest at a higher pixel resolution, constrained of course by the normal ~300nm resolution limit of light microscopy. Female egg chambers were imaged using a 20x (Zeiss Plan Apochromat 20x/0.8) objective.

Image analysis

To quantify mRNPs in marked single cells in the adult brain (generated by MARCM) we used a combination of freely available imaging software packages, Fiji (https://fiji.sc) and iLastik (https://www.ilastik.org).

First single channel grey scale stacks for marked mRNP granules were used to train a 'pixel classification' in iLastik. After successful training confocal stacks of mRNP granules were segmented and binary images of predicted mRNP granules were exported.

In Fiji a binary mask of single cells marked by GFP was generated by a simple binarization of the GFP channel. This mask was used to subtract the 'GFP mask' from the segmented mRNP granules stack leaving only a binary mRNP granules stack that were within the marked cell boundaries.

To quantify mRNPs in single female egg chambers ROIs around the nurse cells were selected with the same size and slice thickness and the image stacks were binarized using the 'Auto local threshold (Method: Max_Entropy)' feature in Fiji.

To quantify the number and sizes of mRNP granules in both tissues the Fiji '3D Objects counter' plug in was used. All statistical analysis were performed using GraphPad Prism.

MARCM to create and visualize single KCs of defined genotype

For the induction of MARCM clones *Drosophila* crosses for both control and experimental were raised at 25 °C and the flies were transferred regularly to a new vial for timed egg collections. A heat-shock pulse at 37 °C in a water bath for 1-2h was given at optimal time points to induce single cell clones in KCs. For α'/β'KCs 2nd instar larvae (48–60h AEL), for α/β KCs late 3rd wandering instar larvae and early pupal stages (120–130h AEL) and for γ KCs early 1st instar larvae (24h AEL) were collected and shocked. Heat-shocked larvae and pupa were transferred back to 25 °C, raised to adulthood and the adult flies were dissected 1–4 days after eclosion.

Behavioural assays

Memory experiments were performed at 25 °C. Conditioning and testing were carried out in dim red light. *Drosophila* olfactory learning was tested using an olfactory classical conditioning paradigm (Krashes & Waddell, 2011; Tully & Quinn, 1985).

19–24 h before conditioning groups of 60–100 flies were put into vials with 0.75% agar on the bottom for starvation. For appetitive olfactory conditioning, flies were loaded in training tubes lined with water filter papers. After an acclimatization period of 30 s, a first odour (CS–) was presented for 2 min. Then, the odour was removed, and air was allowed to flow for 30 s. Subsequently the animals were transferred to tubes lined with 1% or 2% sucrose filter papers while they were presented with the second odour (CS+) for 2 min. The odour pairs used were ethyl-butyrate [EB] and isopentyl acetate [ISO] or 3-octanol [3-OCT] and 4-methylcyclohexanol [MCH].

For the memory tests, flies were moved to a two-arm choice point where they could choose between the CS+ and CS- for 2 min. After this period, the number of flies within each arm was counted and a preference index was calculated.

$$\mathrm{PI} = \left(\text{Number of flies in}\left(\mathrm{CS}+\right) - \text{Number of flies in}\left(\mathrm{CS}-\right)\right) / \text{Total number of flies}$$

One memory experiment consisted of two groups of flies with reciprocal conditioning, in which the odour paired with sucrose was exchanged. The preference indices from these two groups were averaged to calculate a performance index (PI).

Short-term memory was tested immediately after conditioning (3 min memory). Flies tested for long-term 24 h memory were put in food vials after conditioning for 6 h and then transferred to starvation vials for 18–20 h until the test.

When conditional expression of the RNAi was required, flies were grown at 18 °C and collected after hatching (0–3 d old flies). To induce RNAi expression, flies were moved for 6 d to 29 °C. For the starvation period before conditioning and until the test, those animals were kept at 25 °C.

All statistical analyses were performed using the GraphPad Prism (https://www.graphpad.com/) and a one-way ANOVA was conducted with a Sidak post-hoc test between the experimental group and the genetic controls.

Results

Atx2 is required for the formation of Me31B RNPs in different Kenyon cell types

Atx2 function has been shown to be essential for the formation of several types of intracellular mRNP granules, including P-bodies and stress granules (Ciosk *et al.*, 2004; J. Lee *et al.*, 2018; Petrauskas *et al.*, 2024; Singh *et al.*, 2021). Among these, are Me31B/DDX6-positive mRNP assemblies such as P-bodies and neuronal granules, the latter previously shown to be required for LTH, a form of non-associative long-term memory (Bakthavachalu *et al.*, 2018; Nonhoff *et al.*, 2007; Sudhakaran *et al.*, 2014). Given the potential significance of these assemblies for translational control in neural and non-neural tissue, we performed a MARCM (Mosaic Analysis with a Repressible Cell Marker) (Lee & Luo, 1999; Luo & Wu, 2006) analysis to examine how loss of Atx2 affects Me31B-positive mRNP granules in other cell types. Mosaic analysis using this technique relies on creating small clones of GFP-marked homozygous *atx2*-null mutant cells in *atx2*-heterozygotes via mitotic recombination (Hillebrand *et al.*, 2010; Sudhakaran *et al.*, 2014). In the MARCM system, Gal80 repressor protein represses the activity of Gal4 that would drives GFP in a targeted cell type, resulting in unmarked cells – in this case heterozygous for both Gal80 and an *atx2*-null mutation. After FLP/FRT-dependent mitotic recombination, homozygous *atx2*-null mutant cells lack Gal80 but possess an active Gal4 that drives a UAS-GFP reporter gene. Thus *atx2*-null mutant cells labelled with GFP labelled may be recognized and analysed (Lee & Luo, 1999; Luo & Wu, 2006). This system allows to analyse the effect of loss of function of Atx2 in single cells in an otherwise genetically wild-type background. Control MARCM clones, though induced identically, are wild type for *atx2*. We began by examining mushroom body Kenyon Cells (KCs) known to be involved in well-studied forms of associative memory.

We induced Atx2-null clones homozygous for the loss-of-function allele $Atx2^{X1}$ or $Atx2^{L3}$ in memory-relevant KCs and examined Me31B granules in these cells. Depletion of Atx2 from α'/β', α/β and γ KCs caused a clear reduction of Me31B-positive mRNP granules in those neurons [Figure 1]. We compared the total number of mRNP granules per cell

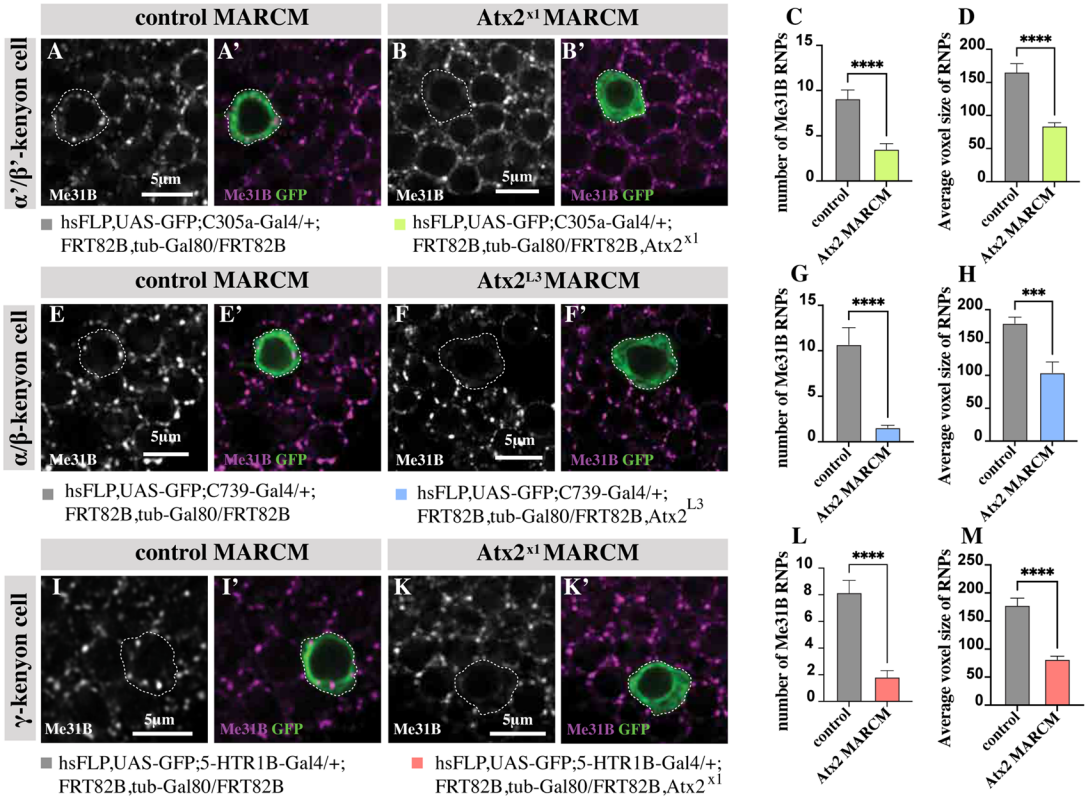

Figure 1. Atx2 is required in α'/β', α/β and γ KCs for the formation of Me31B-positive mRNP granules. (A-A') Representative image of control MARCM clones in α'/β' KCs. (B-B') Representative image of $atx2^{X1}$ MARCM clones in α'/β' KCs. (C) Significant reduction of the total number of Me31B-positive mRNP granules in α'/β' KCs between control MARCM and $atx2^{X1}$ MARCM, unpaired t-test $p < 0.0001$. (D) Significant reduction of the average voxel size of Me31B-positive mRNP granules in α'/β' KCs between control MARCM and $atx2^{X1}$ MARCM, unpaired t-test $p < 0.0001$. (C-D) Error bars are ± SEM; $^{****}P < 0.0001$ by unpaired student T-test; control MARCM $n = 18$, $atx2^{X1}$ MARCM $n = 20$. (E-E') Representative image of control MARCM clones in α/β KCs. (F-F') Representative image of $atx2^{L3}$ MARCM clones in α/β KCs. (G) Significant reduction of the total number of Me31B-positive mRNP granules in α/β KCs between control MARCM and $atx2^{X1}$ MARCM, unpaired t-test $p < 0.0001$. (H) Significant reduction of the average voxel size of Me31B-positive mRNP granules in α/β KCs between control MARCM and $atx2^{L3}$ MARCM, unpaired t-test $p = 0.0002$. (G-H) Error bars are ± SEM; $^{***}P < 0.001$, $^{****}P < 0.0001$ by unpaired student T-test; control MARCM $n = 28$, $atx2^{L3}$ MARCM $n = 26$. (I-I') Representative image of control MARCM clones in γ KCs. (K-K') Representative image of $atx2^{X1}$ MARCM clones in γ KCs. (L) Significant reduction of the total number of Me31B-positive mRNP granules in γ KCs between control MARCM and $atx2^{X1}$ MARCM, unpaired t-test $p < 0.0001$. (M) Significant reduction of the average vox el size of Me31B-positive mRNP granules in γ KCs between control MARCM and $atx2^{X1}$ MARCM, unpaired t-test $p < 0.0001$. (L-M) Error bars are ± SEM; $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ by unpaired student T-test; control MARCM $n = 14$, $Atx2^{X1}$ MARCM $n = 10$.

[Figure 1 C-G-L; (C) unpaired t-test $p<0.0001$, (G) unpaired *t*-test $p<0.0001$, (L) unpaired t-test $p<0.0001$], average size of RNP granules per cell [Figure 1 D-H-M; (D) unpaired t-test $p<0.0001$, (H) unpaired *t*-test $p=0.0002$, (M) unpaired t-test $p<0.0001$]. Each of these analyses revealed significant reduction in both number and size of Me31B mRNP granules in Atx2 null mutant MARCM clones in comparison to control MARCM clones that contain WT Atx2.

To further examine whether Atx2 has a broader role in the assembly of Me31B positive mRNP granules across cell types, we tested whether Atx2 in nurse cells of the female germline is required for assembly of well-studied Me31B-positive granules, essential for the localization and timely translation of mRNAs encoding determinants of embryonic pattern (Nakamura *et al.*, 2001; Richter & Lasko, 2011; Voronina *et al.*, 2011). Atx2 knockdown, achieved by expression of either of two different Atx2 RNAi lines during egg development, significantly reduced the efficiency of Me31B-positive mRNP granule assembly in the female germline. This was calculated by analysing the total number of RNP granules [Figure S1 D-F; (D) unpaired t-test $p<0.0001$, (F) unpaired t-test $p=0.0046$, (F) unpaired t-test $p<0.0001$] and the average voxel size of RNP granules [Figure S1 E-G; (E) unpaired t-test $p<0.0001$, (G) unpaired t-test $p=0.0021$]. These results, together with previous observations in olfactory projection neurons, local interneurons and cultured S2 cells (Bakthavachalu *et al.*, 2018; Hillebrand *et al.*, 2010; Sudhakaran, Hillebrand *et al.*, 2014), establish Atx2 as a general regulator of Me31B-positive mRNP granules not only across neuronal types, but also across *Drosophila* tissue types.

Atx2 function is required for the formation of associative LTM

Several translational regulators have been shown to influence a long-term but not a related short-term form of memory (Bolduc *et al.*, 2008; Keleman *et al.*, 2007; McCann *et al.*, 2011; Roselli *et al.*, 2021; Sudhakaran *et al.*, 2014). However, very few such regulators have been tested for roles in more than one type of LTM, let alone in neuronal subtypes required for each form of memory. To examine whether Atx2, previously shown to be required for LTH, is also required for long-term appetitive associative olfactory memory, we knocked-down the protein in different Kenyon cell (KC) populations. RNAi expression was restricted to the adult stage by co-expressing a temperature-sensitive inhibitor of Gal4 ($Gal80^{ts}$) under the control of a tubulin promoter (McGuire *et al.*, 2003), along with Gal4-driven RNAi transgenes. In flies carrying *tubGal80^{ts}* transgenes, Gal4 is inhibited at low temperatures (18 °C) at which $Gal80^{ts}$ is functional, but active when flies are shifted to higher temperatures that are non-permissive for $Gal80^{ts}$ (29 °C). After eclosion flies are shifted to 29 °C for 6 days, following the animals were moved to a starvation vial at 25 °C for 12 h and then trained for appetitive memory. STM was tested 3 min after training while LTM was tested 24 h after training [Figure 2A].

Adult-specific expression of Atx2 RNAi using the KCs driver, *mb247-gal4*, which strongly expresses in α/β and γ KCs and faintly in α'/β' KCs (Aso *et al.*, 2009; Boto *et al.*, 2014; Zars *et al.*, 2000), led to a significant decrease in LTM performance in comparison to genetic control flies [Figure 2C; one-way ANOVA $p=0.0048$], without affecting STM [Figure 2B; one-way ANOVA $p=0.9485$]. Behavioural control experiments, using the same experimental procedure as schematized in Figure 2A, were performed to determine whether the animals could sense and avoid the odorants used for conditioning, as well as for their ability to sense and be attracted to sugar. In all cases, the experimental groups had similar levels of sugar sensitivity, MCH odour and OCT odour avoidance [FigureS 2; (A) one-way ANOVA $p=0.7436$, (B) one-way ANOVA $p=0.8015$, (C) one-way ANOVA $p=0.9889$] compared to the controls, indicating a selective role for Atx2 in mediating in KC plasticity processes required for a long-lasting association between behaviourally paired odorants and sugar reward.

To define the specific KCs populations in which Atx2 is required for appetitive LTM we used the experimental procedure schematized in Figure 2A, while using Gal4 drivers that target distinct KCs populations: *c739-gal4* for α/β KCs

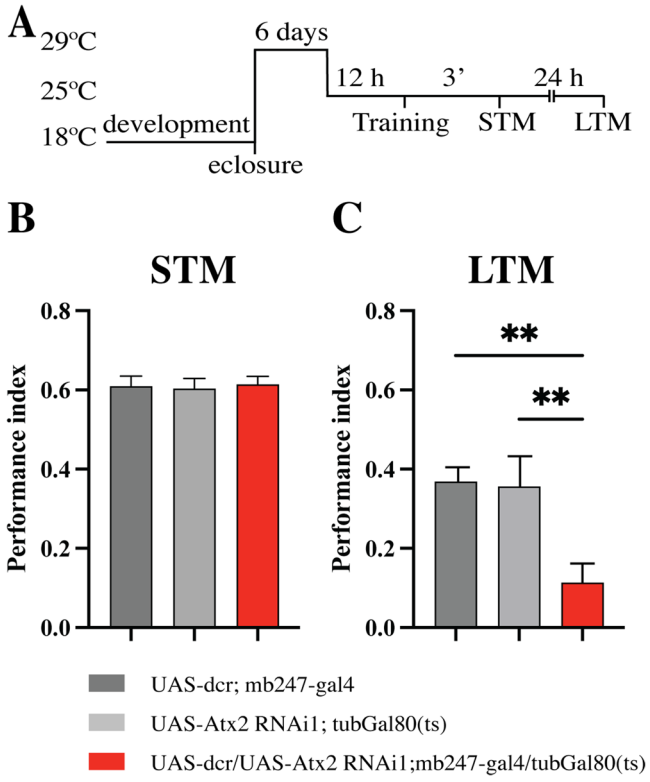

Figure 2. Ataxin-2 (Atx2) protein is required in Kenyon cells (KCs) for expression of olfactory appetitive long-term memories. In adult flies, Atx2 was k/d in a large population of KCs, using the driver *mb247-gal4*. (A) Time course of the experiment. As described in material and methods, the developing animals were kept at 18 °C, once eclosed they were collected in group of 60–100 and kept at 29 °C to allow RNAi expression for 6 days. Following the animals were moved at 25 °C for 12 h and then trained for appetitive memory. STM was tested 3 min after training while LTM was tested 24 h after training. This experiment time course was used also for the experiments shown in Figures 3 and S2. (B) No difference in olfactory memory performance was found between experimental and genetic control flies at 3 min post training (short-term memory), one-way ANOVA $p=0.9485$; Sidak $p=0.9847$, $p=0.9360$. (C) Significant difference in olfactory memory performance was found between experimental and genetic control flies at 24 h post training (long-term memory), one-way ANOVA $p=0.0048$; Sidak $^{**}p=0.0069$, $^{*}p=0.0104$. Data are mean performance indices ± SEM; $^{*}p<0.05$, $^{**}p<0.01$ by one-way ANOVA and Turkey's multiple comparison test; $n=12$.

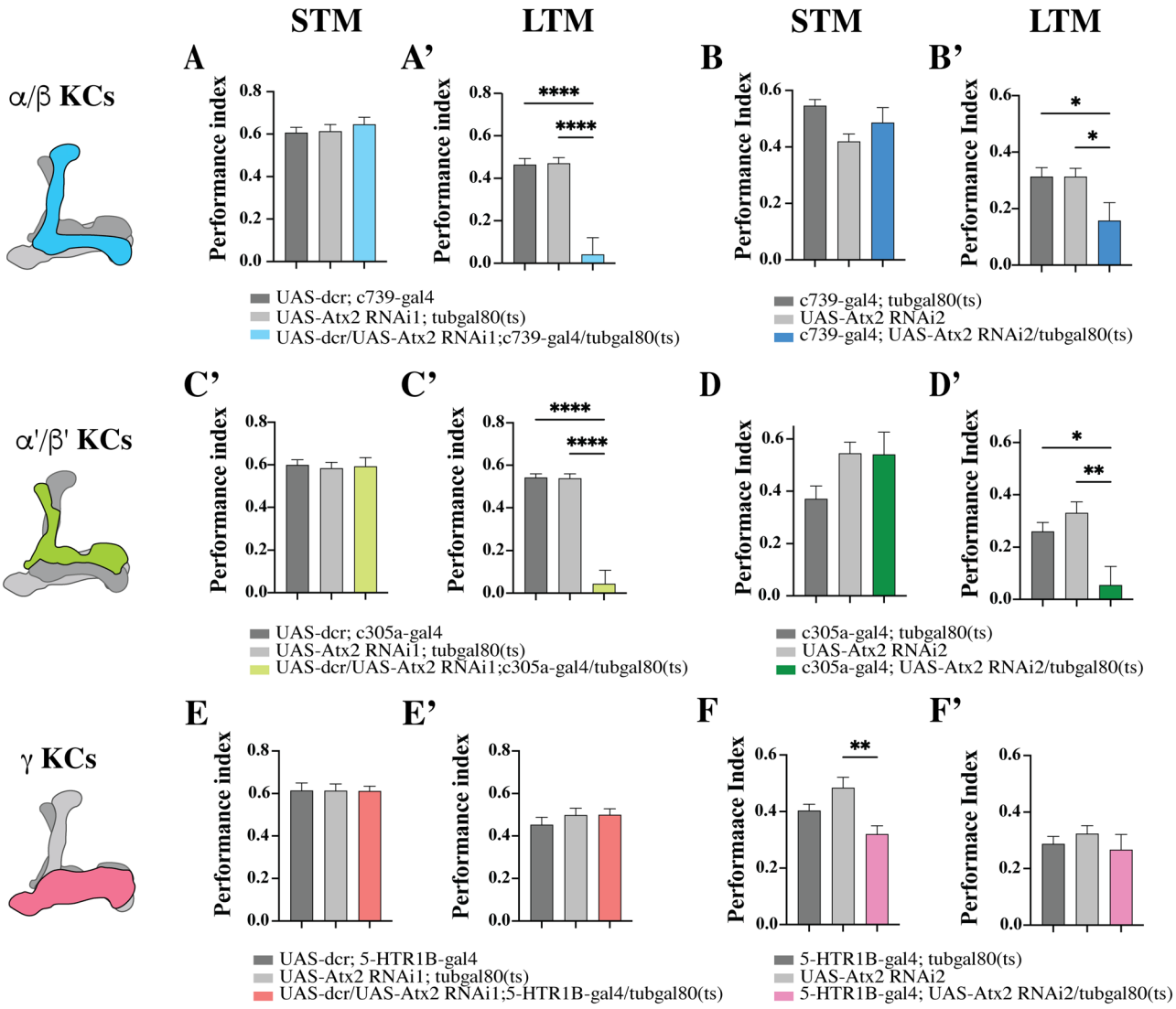

Figure 3. Atx2 is required in α/β and α′/β′ Kenyon cells (KCs) for expression of olfactory appetitive long-term memories. In adult flies, Atx2 was k/d using two RNAis in different KCs populations. (A-A′) Atx2 was k/d using RNAi1 in α/β KCs (*c739-gal4*). (A) No significant difference in STM, one-way ANOVA $p=0.6237$, Sidak $p=0.5983$, $p=0.7029$. (A′) Significant difference in LTM, one-way ANOVA $p<0.0001$, Sidak ****$p<0.0001$, ****$p<0.0001$. (B-B′) Atx2 was k/d using RNAi2 in α/β KCs (*c739-gal4*). (B) No significant difference in STM, one-way ANOVA $p=0.0623$, Sidak $p=0.4382$, $p=3676$. (B′) Significant difference in LTM, one-way ANOVA $p=0.0227$; Sidak *$p=0.0311$, *$p=0.0311$. (C-C′) Atx2 was k/d using RNAi1 in α′/β′ KCs (*c305a-gal4*). (C) No significant difference in STM, one-way ANOVA $p=0.9386$, Sidak $p=9857$, $p=9744$. (C′) Significant difference in LTM, one-way ANOVA $p<0.0001$, Sidak ****$p<0.0001$, ****$p<0.0001$. (D-D′) Atx2 was k/d using RNAi2 in α′/β′ KCs (*c305a-gal4*). (D) No significant difference in STM, one-way ANOVA $p=0.0759$, Sidak $p=0.1077$, $p=0.9983$. (D′) Significant difference in LTM, one-way ANOVA $p=0.0018$; Sidak *$p=0.0149$, **$p=0.0012$. (E-E′) Atx2 was k/d using RNAi1 in γ KCs (*5-HTR1B-gal4*). (E) No significant difference in STM, one-way ANOVA $p=0.9991$, Sidak $p=0.9991$, $p=0.9992$. (E′) No significant difference in LTM, one-way ANOVA $p=0.4982$, Sidak $p=0.5112$, $p=0.9992$. (F-F′) Atx2 was k/d using RNAi2 in γ KCs (*5-HTR1B-gal4*). (F) Significant difference in STM between one control and the experimental line, one-way ANOVA $p=0.031$, Sidak $p=0.1675$, $p=0.0015$. (F′) No significant difference in LTM, one-way ANOVA $p=0.5623$, Sidak $p=9171$, $p=5023$. Data are mean performance indices ± SEM; *$p<0.05$, **$p<0.01$, ****$p<0.0001$ by one-way ANOVA and Turkey's multiple comparison test; $n=8$–12.

(Akalal *et al.*, 2006), *c305a-gal4* for α′/β′ KCs (Krashes *et al.*, 2007), and *5-HTR1B-gal4* for γ KCs (Aso *et al.*, 2012), albeit with some additional expression in neuronal populations outside of KCs. Expression of either of two independent Atx2 RNAi lines in α/β [Figure 3A'-B'; (A') one-way ANOVA $p<0.0001$, (B') one-way ANOVA $p=0.0227$] or α′/β′ [Figure 3 C'-D'; (C') one-way ANOVA $p<0.0001$, (D') one-way ANOVA $p=0.0018$] KCs, reduced olfactory appetitive LTM measured 24 h after training without affecting STM [Figure 3 A-B-C-D; (A) one-way ANOVA $p=0.6237$, (B) one-way ANOVA $p=0.0623$, (C) one-way ANOVA $p=0.9386$, (D) one-way ANOVA $p=0.0759$]. Consistent with prior work showing that γ KCs are mostly involved in short-term appetitive memory rather than in long-term appetitive memory (Cervantes-Sandoval *et al.*, 2013), Atx2 RNAi-expression in γ KCs had no measurable effect on olfactory appetitive LTM [Figure 3 E'-F'; (E') one-way ANOVA $p=0.4982$, (F') one-way ANOVA $p=0.5623$].

Thus, Atx2 function is required not only in olfactory PNs and LNs for long-term habituation (Bakthavachalu *et al.*, 2018; McCann *et al.*, 2011; Sudhakaran *et al.*, 2014), but also in KCs subtypes for appetitive long-term olfactory associative memory.

Discussion

This study has investigated the role of the translational regulator Atx2 in mRNP granules assembly and in long-term memory formation. We can draw two main conclusions

from these experiments: (a) Atx2 contributes to the assembly of different types of Me31B-positive granules in multiple neuronal and non-neuronal cells; and (b) Atx2 is a fundamental component of plasticity processes that is required for long-term forms of memory.

Atx2 regulates the formation of Me31B-positive mRNP granules in different tissues

Ribonucleoprotein (mRNP) granules are a diverse group of RNA–protein assemblies. Distinct types of mRNP granules can have different localizations, RNA and protein compositions, as well as different roles in RNA metabolism, biological function and disease (Protter & Parker, 2016; Tauber *et al.*, 2020). For instance, nucleolar granules are sites of rRNA biogenesis, stress granules mediate aspects of translational arrest and metabolic change in response to oxidative, radiational, nutritional or viral stress (Buchan & Parker, 2009; Tauber *et al.*, 2020) and cytoplasmic P-bodies contain RNA decay machinery that mediate mRNA turnover (Parker & Sheth, 2007; Stoecklin & Kedersha, 2013). In addition, there are tissue-specific and highly specialized granules including germline and neuronal granules thought to operate as hubs for posttranscriptional regulation of gene expression required for germ cell and early embryonic development, neuronal asymmetry, and localized mRNA translation (Kato & Nakamura, 2012; Trcek *et al.*, 2015; Voronina *et al.*, 2011). While some types of neuronal granules are used for transport of translational arrested mRNAs, other related types may be required for local storage and translational control of synaptic mRNAs – a process that is required for long-term forms of synaptic plasticity (Bakthavachalu *et al.*, 2018; Grzejda *et al.*, 2022; Krichevsky & Kosik, 2001). What remains unclear is the range of neuronal granule types that mediate local translational control, as well the diversity of mechanisms and their dynamics *in vivo*.

We focus here on a class of neuronal RNP granule that contains Me31B, a DEAD-Box RNA helicase homologous to human DDX6, and requires Atx2 for their normal assembly. While such granules exist in multiple neuronal subtypes, it is important to note that because different granule types can share common components and regulators (Gruidl *et al.*, 1996; Tindell *et al.*, 2020), there could be more than one subtype of Me31B granule targeted in our experiments. For instance, while germline and neuronal Me31B-positive granules regulate the translation of respective target mRNAs and depend on Atx2 for their assembly, they are distinctive in composition and biological function [Figure 1, Figure S1]. Further, the observation that the few Me31B RNPs that do remain in KCs after loss of Atx2 are on average smaller in size compared to the control (Figure 1 D-H-M; S1 E-G) hints that those could represent a class of RNPs that are independent of Atx2 function. Interestingly for a different RNA binding protein, Staufen, it has been shown that the architectural non-coding small RNA *mimi* is required for the formation of large Staufen RNPs, but is dispensable for the formation of smaller RNPs (Grzejda *et al.*, 2022). It is tempting to speculate that Atx2 could similarly function in the formation of Me31B-positive large RNPs that depend on specific RNA-protein interactions. However, we acknowledge that substantial and extensive analyses, involving defining mRNP subtypes in terms of their protein and RNA contents *in vivo*, will be required to definitively address this hypothesis as well as how and whether they differ in their respective mechanisms of assembly.

Nonetheless our experiments unequivocally demonstrate that Atx2 is necessary for the assembly of Me31B foci in multiple neuronal types, not only in PNs and LNs of the antennal lobe implicated in long-term habituation, but also crucially in KCs known to encode long-term associative memory.

Atx2 dependent translational control in long-term memory

Our data support models for two aspects of molecular mechanisms that underlie long-term memory formation in the mushroom body circuit. First, they provide new support for a model in which LTM of odor-sugar association requires long-term plasticity in synapses of two different types of KCs. Second, by demonstrating that Atx2 is required for long-term plasticity in different brain regions, that Atx2 dependent translational control is broadly required for different forms of long-term memory, namely olfactory habituation (Bakthavachalu *et al.*, 2018; McCann *et al.*, 2011; Sudhakaran *et al.*, 2014) and associative appetitive memory.

The circuit underlying LTM allows experience-induced synaptic plasticity to be studied in the context of behavioural memory. Previous and extensive work on *Drosophila melanogaster* has pointed to the KCs as the main coding centre for associative olfactory memories in the fruit fly (Dubnau & Tully, 2001; Heisenberg *et al.*, 1985; McGuire *et al.*, 2001; Trannoy *et al.*, 2011). New gene expression in the MB (Jones *et al.*, 2018; Kramer *et al.*, 2011; Petruccelli *et al.*, 2020; Widmer, Bilican, *et al.*, 2018) and *de novo* protein synthesis in KCs has been shown to be required for LTM of associative conditioning (Chen *et al.*, 2012; Hirano *et al.*, 2013; Yu *et al.*, 2006). Additionally, the function of multiple translation control molecules such as dFMR/FMRP are required in KCs for the formation of aversive LTM (Bolduc *et al.*, 2008).

Prior observations that synaptic outputs from both α/β and α'/β' KCs populations are required for LTM are suggestive of plasticity being required in multiple circuit locations for LTM (Cervantes-Sandoval *et al.*, 2013; Owald *et al.*, 2015; Trannoy *et al.*, 2011). More detailed studies of the spatial requirement for gene expression showed that activity of the transcriptional regulator CREB2 is needed in two specific subpopulations of the KCs, α/β and α'/β' for consolidating LTM of appetitive conditioning (Krashes & Waddell, 2008; Widmer, Fritsch, *et al.*, 2018; Hirano *et al.*, 2016; Yin *et al.*, 1994). This indicates a need for protein-synthesis dependent plasticity in these two classes of KCs for appetitive LTM formation. This hypothesis is further supported by our observation that the translational and mRNP granule regulator Atx2 is required in α/β and in α'/β' adult stage KCs for olfactory appetitive LTM (Figure 3 A'–D') without affecting STM or olfactory responses [Figure 3 A-D].

While our data do not formally demonstrate that Atx2 functions in local translation of synaptic mRNAs in KCs, it demonstrates a specific requirement for Atx2, an RNA-associated

translational control protein, in LTM but not STM, providing a strong argument for local translation of mRNAs being the underlying mechanism by which these proteins regulate long-term plasticity of α/β and in α'/β' KCs *in vivo*.

Future studies will be needed to show the translation *in vivo* of Atx2 target mRNAs and will aim to investigate whether Atx2 could be required in other neuronal populations, which have been implicated in translational regulation of LTM such as mushroom body output neurons or DAL (Chen *et al.*, 2012; Crocker *et al.*, 2016; Hirano *et al.*, 2013; Wu *et al.*, 2017) for LTM formation. Both appetitive and aversive LTM share overlapping, yet distinct neural circuits within the mushroom body and it is relevant to address whether Atx2-dependent translational regulation mechanism is relevant in other types of long-term memory paradigm, such as aversive conditioning. While our observation that Atx2 is required for Me31B-RNPs formation in γ KCs, could hint to the fact that, even if Me31B-dependent translational regulation in these neurons is not required for appetitive LTM it could be required for other forms of memories. For example, the γ KCs are specifically involved in long-term courtship conditioning, where other translational regulators, such as Orb2 and Imp, have been shown to have a role (de Queiroz *et al.*, 2025; Keleman *et al.*, 2007; Krüttner *et al.*, 2012, 2015). We hypothesize that Atx2, being a translational regulator which is generally involved in LTM formation, could also have a role in long-term courtship conditioning in an Me31B-dependent manner.

Taken together with prior work showing that long-term olfactory habituation requires Atx2 in specific subgroups of projection neurons and local interneurons (Bakthavachalu *et al.*, 2018; McCann *et al.*, 2011; Sudhakaran *et al.*, 2014), these findings support a central and broad role for Atx2 in the control of protein synthesis dependent long-term plasticity across brain systems in *Drosophila* and potentially across species.

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