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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

TU-Tagging: a method for studying cell type specific gene expression

Promotor :
dr. Helena SLAETS

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Proefschrift ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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Abstract

Cell type specific gene expression is a defining feature of multicellular organisms. With the advance tools such as microarrays and RNA-Seq, it is possible to study the dynamics of the gene expression during normal cellular processes as well as disease conditions. There are several methods available for cell type specific RNA isolation, the majority of which are physical cell isolation based methods, such as FACS and laser capture. However, some cell types have complex morphology such as glia and neurons, thus they have number of delicate processes, which might lose during mechanical steps of cell isolation. This RNA loss leads to lose of information and creates a need for a genetic based method, which allows RNA isolation without altering the dynamics of gene expression due to mechanical disruption.

TU-Tagging is a genetics technique which allows RNA labeling in specific cells types. It based on an enzyme called UPRT and owes its inability to distinguish uracil and uracil analog. When uracil analog (thio-uracil) provided, UPRT incorporates thio-uracil to UMP, which subsequently incorporated into RNA. Since thio-substituted nucleotides are not natural components of cell, it is possible to isolate them by biotin coupling and subsequent streptavidin isolation.

In this project, it was aimed to optimize and validate TU-Tagging by using *Drosophila* CNS. Basic chemistry is proven to be working while problems with cell type specificity arose and remain to be investigated.

List of Abbreviations

4-TU	4-Thiouracil
APS	Ammonium persulphate
cDNA	Complementary DNA
CNS	Central nervous system
DAPI	4',6-diamidino-2-phenylindole
Dd	Double distilled
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DOC	Deoxycholate
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
FACS	Fluorescence activated cell sorting
GFP	Green fluorescent protein
HA	Hemagglutinin
HPDP	N-[6-(Biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide
HRP	horseradish peroxidase
NTP	nucleoside triphosphate
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline-tween
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
RT-qPCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
TRAP	Translating ribosome affinity purification
UAS	Upstream binding sequence
UMP	Uridine monophosphate
UPRT	Uracil phosphoribosyltransferase
UTP	Uridine triphosphate
v/v	Volume/volume
w/v	Mass/volume

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1. Introduction

1.1. Cell type specific gene expression

Over the years, researchers developed new technologies (termed -omics) allow studying molecules that assemble a cell, tissue or organism. For instance, genomics reveal the structure of the genome, hence all the DNA sequence and genes of an organism. The 'Humane Genome Project' is one of the most important examples of application of the genomics. This project aimed to determine all the sequence of the human genome, hence complete description of all the genes in order to provide an essential reference to all genetic studies (1). The idea was to unlock the genomic information of humans, providing researchers a powerful tool with the purpose of understanding the genetic factors in human diseases and – as a result - in finding more efficient diagnostic and therapeutic approaches. However, today it is known that every cell has their own gene expression pattern, which makes them different than others (2). Therefore, products of the genome are arguably more important than genome itself and profound understanding of dynamics of a specific cell type provides insight into normal cellular processes (3) as well as explaining the causes of many diseases (4), which merely represent a breakdown of normal cellular processes.

Proteins are functional molecules found in cells; hence proteomics (the study of structure and function of the proteins) can give more information about the operation of a cell. However, despite the extensive research over the years, proteomics has several technical challenges. For instance, due to a lack of a technique, which allows amplification of proteins, proteomics is insufficient to detect low-abundant proteins. Furthermore, some proteins such as membrane proteins are difficult to study since they require carefully balanced lipophilic and hydrophilic environment whereas most proteomics methods work in water-based environment and creates a solubility problem (5,6).

On the other side, the discovery of regulatory RNAs (such as microRNAs) has changed our classical understanding of working of the cells. They are non-coding RNAs and their main function is posttranscriptional regulation of the gene expression (either allowing or repressing the translation of RNA into proteins) (7). As distinct from the proteomics, transcriptomics create a more complex picture of the cell by revealing all the expressed RNA molecules in a cell (known as the transcriptome), including regulatory RNAs. Hence, transcriptomics is the intermediate bridge between DNA and protein.

1.2. Methods for Studying Cell Type Specific Transcriptomes

To date, researchers have several powerful methods for analyzing transcriptomes. These include reverse transcription polymerase chain reaction (RT-qPCR), cDNA microarrays (chip), and RNA Sequencing (RNA-Seq).

RT-qPCR is a major development in the PCR technology, which allows quantitative detection of PCR products generated during each cycle of PCR. In RT-qPCR, RNA is transcribed into cDNA and the amount of cDNA amplification is determined with either fluorescent dyes or fluorescently tagged oligonucleotide probes (8). The fluorescent intensity during the reaction reflects the amount of amplified DNA (9). It does not require a high amount of starting material, making it highly sensitive. However, it requires custom made primers for detecting the gene of interest (means RT-qPCR allows detection of only known genes) and this is a source of bias in the experiment.

Microarrays consist of a set of oligonucleotide probes from a set of know genes attached to a solid surface. It is based on hybridization of sample (transcript labeled with a fluorescent dye) with its target probe. Microarrays are mainly developed to detect variations in gene expression levels and also used for polymorphisms screening (10). It is a powerful tool in transcriptomics, high throughput, and relatively inexpensive. Furthermore, various chips are available for different organisms such as mouse, yeast and *Drosophila*. However, it has also limitations such as cross-hybridization (base pairing between non-identical sequences), which results in the creation of false data. More importantly it only allows analyzing of known genes, meaning the obtained data relies on existing knowledge about genome. Nevertheless, with the increasing knowledge of the genomes of different organisms, microarrays seem to remain as a powerful tool in transcriptomics.

RNA-Seq is a powerful approach used for discovering, profiling and quantifying transcripts in a given sample. Even though there are different platforms for sequencing (determining the nucleotide order), generally the system called 'sequencing by synthesis' is used. This approach is based on fluorescent-labeled nucleotides and the introduction of individual nucleotides, each of which gives a unique fluorescent signal, which is recorded and reflected as sequence. It has number of advantages over microarrays. Principally, it is a hypothesis-free approach and can sequence all the transcripts in the sample, while microarrays are limited by the content on the array. Hence it has the ability to investigate both known and novel transcripts. Furthermore, it can distinguish closely related transcripts from each other, such as splice variants and mutations. Also it does not rely on pre-designed probes, thus providing unbiased analysis of transcriptomes. Owing all the advantages mentioned above, RNA-seq provides more accurate and comprehensive data, thus it has the potential to revise the many aspects of transcriptomes (3).

It is obvious that researchers have several powerful approaches for transcriptomes analysis, which creates need for robust specific cell isolation methods in order to obtain a

specific transcriptome. There are several methods to achieve this aim such as manual dissection, laser capture, fluorescence-activated cell sorting (FACS) and antigen selection by antibodies, which require dissociations of target cells from their location (11,12). FACS separates a population of cells from a mixture based on fluorescent labeling. Fluorescent labeling is achieved either with the expression of fluorescent reporters such as Green Fluorescent Protein (GFP) in specific cell types or labeling cell surface antigens with fluorescently labeled antibodies. Sorting is based on fluorescent intensity.

Unfortunately, FACS and other methods used to produce single cell type suspensions (mentioned above) have their own limitations. First of all, they can only be done by trained personnel using expensive equipment. Secondly and probably most importantly, cells such as glia and neurons (in the central nervous system) have complex morphology and large numbers of thin, delicate cellular processes (such as axons, dendrites and glial processes). In theory, they are lost during the cell preparation (13). This cellular disruption likely leads to a loss of RNA from the cell and alteration of transcription profiling, compromising the coverage of any transcriptome analysis. Therefore, an important question arises: is it possible to isolate RNA from specific cell types without the need for preceding cell isolation?

Very few genetic methods allowing cell type specific RNA isolation (without physical cell isolation) have been developed during years. Translating Ribosome Affinity Purification (TRAP) is one of methods serves for this aim, which is established at 2008 (14). It owes the fact that mRNAs bind to ribosomes in order to be translated into proteins. In TRAP, ribosomes or polysomes are tagged with enhanced Green Fluorescence Protein (EGFP) in specific cell types. EGFP is fused to the large ribosomal subunit protein L10a and cell type specific expression of EGFP-L10a transgene is driven by regulatory elements (15). Hence it becomes possible to isolate EGFP-tagged ribosomes by immunoisolation methods such as beads coated with anti-GFP antibodies (14) (Fig. 1). Besides all the advantages, it also has number of disadvantages. Even though it avoids physical cell isolation, it can isolate only ribosome bound RNA molecules, which means non-coding RNA molecules (regulatory RNAs) cannot be detected. Also, it is based on protein-antibody interaction, which is non-covalent bound, thus weaker (16). Furthermore, there is a lack of temporal control of the RNA isolation since it isolates all the mRNA molecules that are bounded to ribosomes at the isolation time point. That is because different mRNAs within the cell have different decay rate (17). In addition, there is a need for generation of multiple animal lines for each cell type of interests.

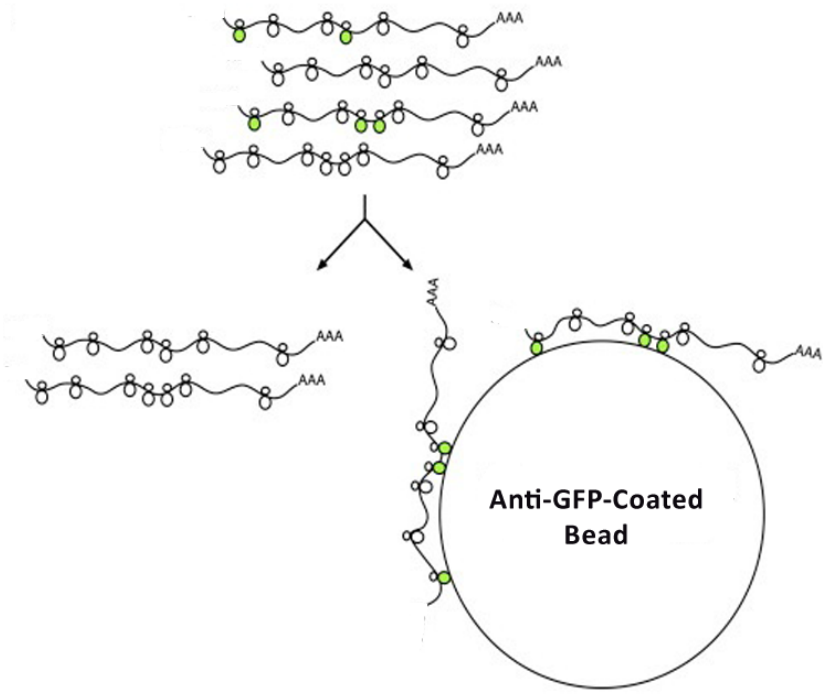

Figure 1: Affinity purification of EGFP-tagged ribosomes (green) by anti-GFP antibody-coated beads (Modified from Doyle et al. 2008).

Therefore, a more convenient method for cell type specific RNA isolation is needed, which avoids prior cell isolation and allows cell type specific and temporal labeling of all RNA species in the cell. Such an approach named "TU-Tagging" has been established in *Drosophila* in 2009 (18) and applied to mice last year (13).

1.2.1. TU-Tagging

Toxoplasma gondii is a parasitic protozoan, which has the ability to infect most warm-blooded animals. When *T. gondii* infects the host cell, it employs two mechanisms to produce pyrimidine nucleotides, both of which involve synthesis of uridine monophosphate (UMP) (19). The first mechanism is the conventional *de novo* synthesis of the UMP, while the second one is the pyrimidine salvage pathway, in which the host cell provides the uracil to yield UMP (20). The pyrimidine salvage pathway is catalyzed with the uracil phosphoribosyltransferase (UPRT) enzyme. Under natural conditions, UPRT enzyme within the parasite couples ribose-5-phosphate to the N1 nitrogen of the uracil to yield UMP, which is subsequently incorporated into RNA.

Importantly, mammalians and even flies lack UPRT activity. Previously, it has been shown that UPRT enzyme can be transfected into mammalian cells and used to biosynthetically label newly synthesized RNA *in vivo* (21). The 'trick' is UPRT enzyme cannot distinguish

uracil or uracil analog 4-thiouracil (4-TU). Hence, if 4-TU is provided to the enzyme as substrate, the UMP (yielded from 4-TU) is also incorporated to RNA and creates TU-labeled RNA. Also importantly, it is shown that thio-uracil incorporation into mRNA has no effect on protein synthesis (21).

Normally thiol groups are not natural components of RNAs. Hence, with the right chemistry it is possible to isolate them from the native RNAs. In the case of this method, biotin is used, which binds to thiol group of the RNA with a disulfide bond. Furthermore, subsequent isolation of biotin is possible with streptavidin-biotin interaction. Additionally, as the disulfide bond is a covalent bond, the binding reaction will be stronger than any antibody-protein interaction. Since only newly synthesized RNA will be tagged by 4-TU, TU-Tagging has the crucial advantage to separate newly synthesized RNA from total cellular RNA. The isolated Thio-RNA can be the subject for downstream experiments such as qPCR, microarray or RNA-Seq (Fig. 2).

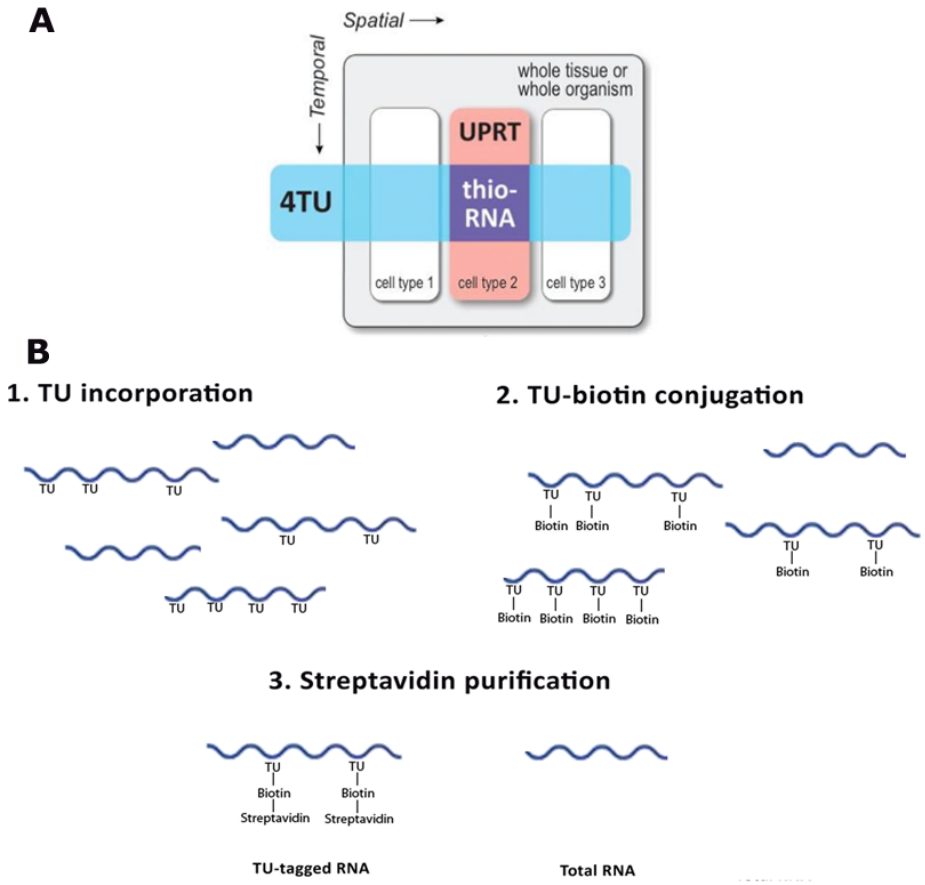

Figure 2: TU-Tagging method. **(A)** Spatial control is achieved by cell type specific expression of UPRT and temporal control is provided by pulse of 4-TU (13). **(B)** TU-tagged RNA isolation from total RNA extract. TU-Tagged RNA is labeled by biotin and further isolated by streptavidin.

Besides all the advantages, TU-Tagging also has several drawbacks. For instance, it is reported that uracil amount in the individual transcripts is a source of bias in TU-Tagging experiments (18). In order to overcome this problem, there is a need for normalization of the uracil numbers in the transcripts by bioinformatics.

1.3. *Drosophila*: A Versatile Model Organism in Biology

Drosophila melanogaster has been widely used as a model organism in research for decades. There are several reasons for that. First of all, they have relatively short generation time (about two weeks depending on the temperature) and sophisticated genetic manipulations are available (such as Gal4/UAS system, see below) (22). Additionally, many molecular mechanisms and signaling pathways are conserved between *Drosophila* and mammalians, which makes *Drosophila* an appealing subject. Furthermore, flies are increasingly used as models for human diseases since high percentage of human disease related genes are shown to have homology with fly genes (not only sequence but also functional homology) (23). All these factors make flies appropriate model organisms for transcriptomics research, in this case for TU-Tagging application.

1.3.1. Gal4/UAS System in *Drosophila*

To be able to perform TU-Tagging method in *Drosophila*, there is a need for a genetic tool which allows UPRT expression in defined cell types in flies. The Gal4/UAS system is an elegant and powerful genetic tool in *Drosophila* for targeted gene expression which was introduced in 1993 (24) and used widely since then. This system basically consists of two elements: a Gal4 gene and a UAS site.

The Gal4 gene encodes a regulatory protein of galactose-induced genes identified in *Saccharomyces cerevisiae* (25–27). It is known that Gal4 can bind specific DNA and has transcriptional activation functions (28). Gal4 regulates the transcription of the genes by recognizing and binding a 17 base pair sequence in the target genes (29), which called as Upstream Activation Sequence (UAS) element. The UAS element is an analog of enhancer element of multicellular organisms and is crucial for the transcriptional activation of Gal4-regulated genes (30). Additionally, it is shown that Gal4 expression is capable of activating transcription of a gene under the control of UAS element in *Drosophila* without any known side effects to the organism (31). By the introduction of this technique, it became possible to target gene expression in a cell type specific way *in vivo*.

In this system, expression of the gene of interest is controlled by the presence of UAS elements. To achieve the activation of gene expression a fly line containing UAS (attached to the gene of interest, in this case UPRT) is mated with a fly line containing a tissue specific promoter bound to the Gal4 gene. When Gal4 expresses, it binds to the UAS and activates the transcription of the gene of interest in the specific tissue (Fig. 3). Currently

there are large numbers of Gal4 drivers available (for different tissues) and they are obtainable easily and cheaply from the central stock centers.

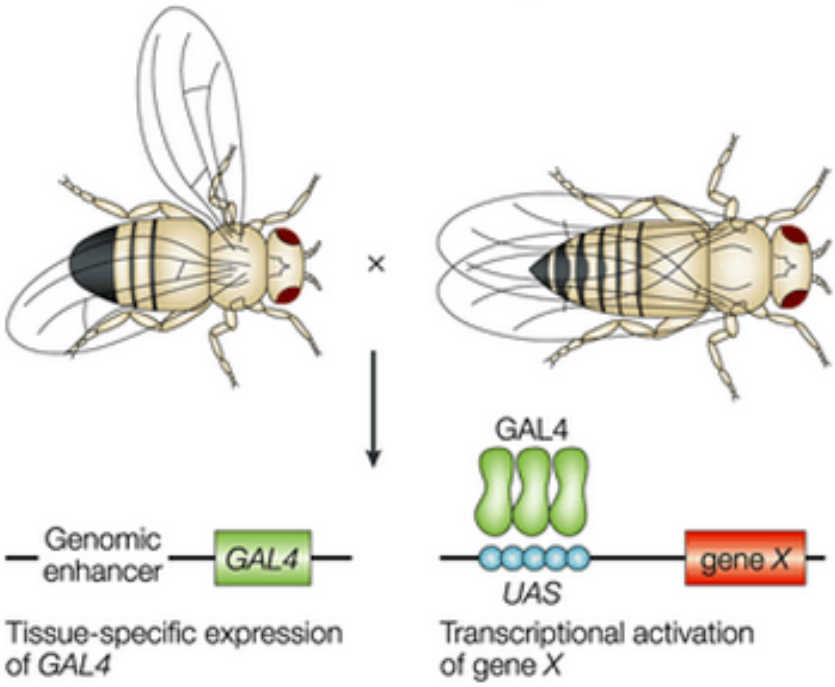

Figure 3: Gal4/UAS System. In order to achieve spatial control of gene expression, UAS-gene X transgene carrying flies crossed with tissue specific Gal4 lines. Therefore, in the progeny Gal4 will bind to UAS and activate the expression of gene X in tissue specific manner (48).

1.4. Aim of the Study

In this study I aimed to optimize and validate the TU-Tagging method in the Central Nervous System (CNS) of *Drosophila* (due to above-mentioned advantages). TU-Tagging can be done in any fly tissue, however our laboratory's long-term goal is defining glial genes in different processes (such as memory formation), which are overlooked compared to neurons over years due to primacy of neuron in most neuroscience doctrines and lack of suitable tools.

I aimed to achieve my goal by altering TU-Tagging's various components, such as streptavidin. I also aimed to determine the appropriate 4-TU feeding duration. Through this thesis, first the controlling of fly crosses (in order to see whether they have the UPRT) will be presented and it will be followed by the validation of the TU-Tagging method by various internal and external controls. Finally, the application of the TU-Tagging method to the CNS of the flies will be shown and outcomes will be discussed.

2. Materials and Methods

2.1. Materials

2.1.1. Buffers and Solutions

Protein Extraction

Lysis Buffer	150 mM NaCl (Fisher Scientific, S/3160/65) 0.1% (w/v) Triton X-100 (Merck Millipore, 108603) 50 mM Tris-HCl pH 8 (Sigma, T5941) 1x Protease inhibitor cocktail (Cell Signaling Technology, 5871)

Protein Determination

DOC	0.15% (v/v) DOC (Sigma, D6750) (Prepared from a 1% (w/v) stock)
Solution I	670 mM Na_2CO_3 (Chem Lab, CL00.1446.1000) 1M NaOH (Merck Millipore, 1.06469.100) 70 mM Na_2-tartrate x $2H_2O$ (Merck Millipore, 1.06663.0250) 347 mM SDS (Serva, 20783)
Solution II	16 mM $CuSO_4$ x $5H_2O$ (Sigma, 209198)
Solution III	100 units solution I 1 unit solution II (Mixture is stable for 2 weeks at room temperature)
Solution IV	1 unit 2N Folin-Ciocalteu Phenol Reagent (Sigma, F9252) 1 unit ddH_2O
TCA	72% (w/v) TCA (Sigma, 91228)

Poly Acrylamide Gel Electrophoresis (PAGE)

Anode Buffer 10x (pH 8.9)	2 M Tris (Merck Millipore, 648311) Adjusted with 10 M HCl (Fisher Scientific, 7647-01-0)

Diluted with dH_2O in order to achieve 1x working concentration.

APS	10% (w/v) APS (Sigma, A9164)
Cathode Buffer 10x	1 M Tris (Merck Millipore, 648311)
(pH 8.25)	1 M Tricine (Alfa Aesar, 14695)
1x Working Buffer	1% (w/v) SDS
Gel Buffer	3 M Tris (Adjusted with 10 M HCl)
(pH 8.45)	0.3% (w/v) SDS
Glycerol	50% (v/v) Glycerol (VWR International, 24388)
Sample Buffer 5x	250 mM Tris (Adjusted with 10M HCl)
(pH 6.8)	20% (w/v) SDS
	60% (w/v) Glycerol
	0.05% (v/v) Serva Blue G (Serva, 35050) and 10% (v/v) β-Mercaptoethanol (Sigma, M7522) were added before use.

Western Blot

Blocking Solution	5% (w/v) Milk Powder (Nestlé)
	0.2% (v/v) Tween 20 (Sigma, P7949)
	1x PBS
Blot Transfer Buffer	200 mM Glycine (Sigma, G8898)
	25 mM Tris
	0.04% (w/v) SDS
	20% (v/v) Methanol (Biosolve, 13683502)
PBS 20x	137 mM NaCl
(pH 7.4)	2.7 mM KCl (Sigma, P3911)
	10 mM Na_2HPO_4 x $2H_2O$ (Sigma, 30412)
	1.76 mM KH_2PO_4 (Merck Millipore, 1.04873.0250)
	Adjusted with 10M HCl

Diluted with dH_2O in order to achieve 1x working concentration.

Ponceau S Solution	0.10% (w/v) Ponceau S (Roth, 5938.1)
	1% (v/v) acetic acid (VWR International, 20104-298)

Immunohistochemistry

Blocking Solution	1x PBS 5% (w/v) Goat Serum (Abcam, Ab7481) 0.2% (v/v) Triton X-100
Fixation Solution	4% (w/v) PFA (Sigma, P6148) in PBT
PBST	1x PBS 0.1% (v/v) Tween-20

TU-Tagging

4-TU Stock	1 M 4-TU (Sigma, 440736) in DMSO. Aliquots kept at -20°C.
Biotin	1 mg EZ-Link HPDP-Biotin (Pierce, 21341) dissolved in 1 mL DMF (Sigma, D4551). Aliquots kept at -80°C.
Tris-EDTA Buffer 10x (RNase Free)	1 M RNase-free Tris-HCl (pH 8.0) (Ambion, AM9855G) 0.5 M RNase-free EDTA (pH 8.0) (Ambion, AM9260G) Adjusted with RNase-free dH_2O (Ambion, AM9932).
Elution Buffer for Beads	100 mM β-Mercaptoethanol, freshly prepared with RNase-free dH_2O.

2.1.2. Antibodies

Table 1: Western Blot Antibodies

Antibodies	*Suppliers, Catalog Number*	*Dilution*
Mouse Anti-HA-Tag	Cell Signaling Technology, 2367	1:1000
Mouse Anti-α-Tubulin	Hybridoma Bank, 12G10	1:400
Goat Anti-Mouse, HRP	BioRad, 172-1011	1:5000

Table 2: Immunohistochemistry Antibodies

Antibodies	*Suppliers, Catalog Number*	*Dilution*
Rat Anti-HA-Tag	Roche, 1867431	1:250
Mouse Anti-Repo	Hybridoma Bank, 8D12	1:20
Goat Anti-Rat, Alexa Fluor 488	Molecular Probes, A11006	1:500
Goat Anti-Mouse, Alexa Fluor 555	Molecular Probes, A21425	1:500

2.1.3. Fly Stocks

Following fly stocks were obtained from Bloomington stock center: repo-Gal4 (III), nysb-Gal4 (III), UAS-HA:UPRT 2.1, UAS-HA:UPRT 3.2 and WIII8. UAS-HA:UPRT lines crossed with described Gal-4 lines. The numbers of the lines (2.1, 3.2 and number in the brackets) indicate the insertion chromosome of the UPRT. All crosses were established at 25°C and stocks were maintained at room temperature.

2.2. Methods

2.2.1. *In vitro* Transcription

pBS hPat1 plasmid (which served as the template for *in vitro transcription*) was obtained from Tillman Achsel (CME-KU Leuven). In order to prepare linearized template, 10 µg of plasmid used for digestion with SacII (Promega, R6221), according to the manufacturer's protocol.

For *in vitro* transcription, the reaction mix was prepared as described: 1 µg linearized pBS hPatL1 plasmid, 1x NTP mix (Roche, 11 969 064 001), 1x 4-thio-UTP (Fisher Scientific, 12859306), 1x transcription buffer (supplied with T7 RNA polymerase), 30units T7 RNA polymerase (Thermo Scientific, EP0111) and the final reaction amount was adjusted to 50 µL with RNase-free dH_2O. The mixture was then incubated for 2 hours at 37°C in the dark. Template DNA was cleaned with RQ1 RNase-free DNaseI (Promega, M6101) according to the manufacturer's protocol. Unincorporated NTP's were removed with Illustra MicroSpin G-50 column (GE Healthcare, 27-5330-01) according to the manufacturer's protocol.
RNA was extracted with the standard RNA extraction protocol starting from chloroform extraction step (see below). RNA concentration was determined using a Nanodrop 1000 (Thermo Scientific) and thio-UTP incorporation was determined by measuring the absorbance at 320 nm. RNA was used if the ratio of measured absorbance at 260nm and 280nm was ≥ 2.0 (indicating sufficient purity of the RNA). RNA was diluted to 1 µg/µL and kept at -20 °C as aliquots.

2.2.2. 4-TU treatment and RNA extraction

For 4-TU feeding, instant medium containing blue dye (Carolina Biological Supply Co., 173210) was used in order to visualize food intake in flies' intestines. Larvae were picked at their 3rd instar stage (approximately 24 hours before pupation), washed in water and transferred to blue food containing 0.5 mM 4-TU for 2-8 hours (depending on the optimization step) at 29°C. Subsequently, their brains were roughly dissected on ice and homogenized in Trizol (Ambion, 15596-026) with a 22G needle, snap frozen in liquid nitrogen and stored at -80°C until RNA purification. To treat adult flies with 4-TU, following starvation for 12-16 hours the flies were transferred to blue food containing 1 mM 4-TU for

6-8 hours at 25°C. Their heads were removed and homogenized in Trizol with a 20G and 22G needle, snap frozen in liquid nitrogen and stored at -80°C until RNA purification.

RNA extraction was performed according to the standard Trizol purification method (for both larvae and adults). 200 µL chloroform (EMSURE, 1.02445.1000): isoamyl alcohol (EMSURE, 100979) (24:1) was added to the crude homogenate, vortexed for 5 min and incubated at room temperature for 2-3 min. The mix was then centrifuged at 21100 g for 15 min at 4°C. Aqueous upper phase was transferred to a new tube and 1 vol of isopropanol (AppliChem, A3928, 1000PE) added, vortexed and incubated for 10 min at room temperature. The mix was then centrifuged at 21100 g for 20 min at 4 °C. The pellet was washed with 1 mL 70% ethanol and centrifuged at 21100 g for 10 min at 4 °C. The open tube was incubated on a tissue paper for 5 min to dry. The pellet was resuspended in 50 µL preheated (50°C) RNase free H_2O. RNA concentration was determined using a NanoDrop 1000. In order to remove residual genomic DNA, RNA was treated with TURBO DNase (Ambion, AM2238) according to the manufacturer's protocol. Concentration was determined with a NanoDrop 1000 following DNase treatment and only RNA samples with absorbance 260/280 ratios of ≥ 2.0 were used for subsequent experiments (see above).

2.2.3. Purification of TU-Tagged RNA

A standard TU-Tagging method (13) was used with minor modifications (for the modifications, see the results). Total RNA obtained from flies was mixed with 2 ng of hPatL1 mRNA (provided a positive external control for method validity). The RNA mixture was fragmented using the NEBNext RNA fragmentation kit (New England BioLabs, E6150S) according to the manufacturer's protocol with an exception of incubation at 85 °C (instead of 95 °C) (13). After fragmentation RNA was purified with RNeasy Mini Kit (QIAGEN, 74104) according to the manufacturer's protocol. 1 µL of total RNA was separated as an input and diluted in 10 µL RNase free H_2O. Concentration was determined with NanoDrop 1000.

The biotinylation reaction mix contained 1X RNase free TE Buffer and 1 µL of the HPDP-biotin solution (1 mg/mL) per 1 µg of RNA. The reaction volume was adjusted with RNase free H_2O so that the concentration of HPDP-biotin was equal to 30% of the final reaction volume (18). Reaction mixture was incubated in the absence of light at room temperature for 3 hours. Biotinylated RNA was purified with RNeasy Mini Kit according to the manufacturer's protocol.

Purification of biotinylated RNA was performed with streptavidin coated magnetic beads: either Dynabeads M-270 Streptavidin (Invitrogen, 65305) or µMACS Streptavidin Kit (Miltenyi Biotec, 130-074-101). For both magnetic beads, the manufacturer's protocols were followed. For elution of the TU-Tagged RNA from the beads 2x100 µL washes with preheated (80 °C) β -Mercaptoethanol were used. TU-Tagged RNA was then repurified

with the RNeasy MinElute Cleanup Kit (QIAGEN, 74204) according to the manufacturer's protocol and TU-Tagged RNA was finally eluted in 14 µL RNase free H_20. RNA concentration was determined with a NanoDrop 1000 in order to prepare the reverse transcription reaction.

2.2.4. RT-qPCR

Reverse transcription was performed with SuperScript II Reverse Transcriptase (Invitrogen, 18064-014) according to the manufacturer's protocol. Random hexamers (Integrated DNA Technologies) and dNTP mix (Bioline, BIO-39044) were included to the mixture, as they are not provided with the kit. Identical amounts of TU-Tagged RNA (varied from experiment to experiment as the recovered amount of the RNA was different) and input were used in order to allow for comparing the results within the experiment. A 1:25 dilution of cDNA (produced from the RT reaction) was used for all the RT-qPCR experiments.

Custom-made primers (Integrated DNA Technologies) were designed using Primer-BLAST (National Center for Biotechnology Information). Primers were designed specifically to span for an exon-exon junction (such primers do not bind to genomic RNA). Amplicon size adjusted to 70-90 base pairs. Primers' efficiency calculated using standard linear regression analysis (32).

Table 3: Primers were used in this study

Marker	Pair 1	Pair 2
Repo	3'CAGCCAAAAAGGACGGAAGC-5'	5'-AGGCAGTAAAGGTGGTTCTCG-3'
Moody	3'-CGCAGCAGAGAAATGGAAAGA-5'	5'-CGACATCTGGGAGACGATTG-3'
Anachronism	3'-TGGAGCGTTTACCGAACAGA-5'	5'-TAGCCTTGGTTTTCTTGGCA-3'
Elav	3'-ACACCGAGCGAAATACGGC-5'	5'-CTCCTCCTTGCTCTCTGCTT-3'
Syt	3'-GAACTGACCAAAAGCAAGTCG-5'	5'-TTCGTGATGGGCTATGCGT-3'
Nsyb	3'-GCAAGTTTTCGCAGTTGGC-5'	5'-TGATGTGTATGTGTGCGGGT-3'
UPRT	3'-CGTCTTCTACGCCGACCG-5'	5'-CCAGAGGGGTTGTCACTTCC-3'
Gal4	3'-ACCTTCGCATCGCTCAGTC-5'	5'-CACCAAACAAAGCAGACGGG-3'
hPatL1	3'-AGCATTACCAAGGCGGTCAA-5'	5'-ATCTGTTTAGGTGGGGTGCC-3'

qPCR experiments were performed with a LightCycler 480 SYBR Green I Master Kit (Roche Applied Science, 04707516001) and a LightCycler 480 instrument (Roche Applied Science, 05015278001) according to standard protocol as follows: pre-incubation (95 **°**C, 1 cycle), amplification (60 **°**C, 45 cycles), melting curve (95 **°**C, 1 cycle) and cooling (40 **°**C, 1 cycle). Abs Quant-2nd Derivative Max analysis was performed on a LightCycler 480 Software in order to calculate Ct numbers (the value which indicates the cycle number on which the fluorescence level exceed the threshold and was used for the analysis). Fold

enrichment was calculated with following equation: $\log_2$concentration=$\log_2$(Δ-Ct x $\log_2$primer efficiency).

2.2.5. Protein Extraction

For Western Blot analysis, protein was extracted from adult fly heads. For each protein preparation ~20 flies were decapitated and homogenized in 50 µL lysis buffer. Homogenate was mixed on a wheel rotor for 40 min at 4 °C and centrifuged at 21100 g for 10 min at 4 °C. Supernatant was taken and kept at -20 °C until protein determination.

2.2.6. Lowry Protein Determination

The standard Lowry-Peterson protein determination method was used for protein detection (See supplementary section A-1).

2.2.7. Poly Acrylamide Gel Electrophoresis

PAGE was carried out using a mini-Protean® III Tetra cell system (BioRad). A Schägger Gel System was used as it gives good separation in the weight range of 10-100 kDa (33). (For gel preparation: see supplementary section A-2).

For the electrophoresis, the electrophoresis chamber was set and the cathode buffer was poured between two casting frames and the anode buffer was poured to the outside of the frames. For each sample, 10 µg of protein was mixed at a 5:1 ratio with 5x sample buffer to dilute it to 1x. Samples were heated at 95 °C for 10 minutes to ensure complete protein denaturation and 10 µg of protein was loaded into wells. To indicate approximate molecular weights, 5 µl PageRuler (Thermo Scientific, 26616) was loaded per gel. Protein stacking was achieved by running the gel at 60 V for 15 minutes. After proteins had entered the resolving gel, the voltage was increased to 120 V and the gel was allowed to run for approximately 45 minutes in order to separate proteins.

2.2.8. Western Blot

In order to transfer proteins from gel to a nitrocellulose membrane, a semi-dry transfer system (Phase PEGASUS with a BioRad Power-Pac basic power supply) was used. Stacking gels were removed and resolving gels put into transfer buffer along with the nitrocellulose membranes and 4 Whatman papers for each blot. Blotting sandwich was built using 2 Whatman papers, the gel, a nitrocellulose membrane and 2 Whatman papers (from bottom to top). Each layer had soaked with transfer buffer in order to ensure electrical conductivity. After built up the sandwich, it was rolled over by a plastic pipet to avoid air bubbles. The transfer was carried out at a constant current of 0.8 mA/cm2 nitrocellulose

membrane. At the end of the transfer step the membrane was put into Ponceau S solution for 10-15 minutes on a shaker in order to visualize the quality of the transfer. Subsequently, Ponceau S was removed and the membrane was washed with PBS 3 times and put into blocking solution for 1 hour at room temperature to block off all the free protein binding sites on the membrane in order to prevent non-specific signal.

Antibody solutions were prepared in blocking solution (Table 1). First, the membrane was incubated overnight with primary antibodies at 4 °C. Membrane was then washed 3 times with blocking solution and 3 times with PBS at room temperature in order to remove unbound antibodies. Appropriate secondary antibodies were then applied in blocking solutions to the membrane for 1 hour at room temperature. Subsequent washing was performed as described above. For detection of the antibodies Western Lightning ECL Reagent (Perkin Elmer, NEL104001) was used and bands were visualized with LAS-3000 mini (Fujifilm). Images were taken with Fujifilm Image Reader and processed with PowerPoint (Microsoft).

2.2.9. Immunohistochemistry and Imaging

Adult fly brains were dissected in PBS at room temperature and fixed for 15 minutes at room temperature. They were washed with 3xPBT for 15 minutes each. Brains were put into 500 µL blocking solution for 1 hour at 4 °C (rotating). Antibody solutions were prepared in blocking solution (Table 2). First, brains were incubated with primary antibodies overnight at 4 °C. Brains were then washed with 3xPBT as described above. Subsequently, they were then incubated with secondary antibodies for 2 hours at room temperature. The brains were then washed with 3xPBT and mounted in Vectashield Mounting Medium with DAPI (Vector Laboratories, H-1200).

Leica TCS SP5 II Confocal Microscope and Leica LAS AF Software (Leica Microsystems) were used for imaging of the brains. Excitation wavelengths used were 488 and 555 nm (with standard excitation and emission filters) for visualization of secondary antibodies. Images were taken with the standard Leica 40x and 60x objectives and images were imported into ImageJ for subsequent processing (National Institutes of Health).

3. Results

In this thesis, two different Gal4 lines were used in order to achieve the expression of the UPRT in the cell type of interest. Reverse polarity (repo) is a glia specific protein in *Drosophila* required for the glial development (34). Hence, the repo-Gal4 line was used to drive the expression of the UPRT in the glial cells. On the other hand, neuronal synaptobrevin (nsyb) is a key protein for synaptic vesicle exocytosis (35). Therefore, the nsyb-Gal4 line was used for the neurons. Apart from the drivers, two different UAS-HA:UPRT lines were used: UAS-HA:UPRT 2.1 and UAS-HA:UPRT 3.2. UAS-HA:UPRT 3.2 line reported as more efficient in larvae and adult flies (with lower unintended expression in other cells) (Official communication: Bloomington stock report).

Additionally, UPRT is fused to hemagglutinin (HA) tag to facilitate the detection and isolation the enzyme. HA is the receptor binding and membrane fusion surface glycoprotein, which is required for the infectivity of the influenza virus (36). HA-tag is derived from HA protein (amino acids 98-106) and has been widely used to tag recombinant proteins.

First, the experiments with UAS-HA:UPRT 2.1 flies will be presented and they will be followed by UAS-HA:UPRT 3.2 experiments.

3.1. UAS-HA:UPRT 2.1 Experiments

3.1.1. Biochemical and Immunohistochemical Analysis of the crosses with UAS-HA:UPRT 2.1 line

After establishing the crosses, it was crucial to prove whether the progeny were carrying UPRT since the expression of the UPRT in the right tissue was critical for the TU-Tagging experiments. For the 2.1 line, the majority of the experiments were done with repo-Gal4 driver as it was focused to establish the TU-Tagging in the glia cells.

3.1.1.1. Western Blot

Western blot analysis was done in order to prove the presence of the UPRT enzyme in the progeny (repo-Gal4 X UAS-HA:UPRT 2.1) as well as the absence of the enzyme in the individual lines (repo-Gal4, UAS-HA:UPRT 2.1) (Fig. 4). For this experiment, proteins were isolated from the head of the adult flies and 10 µg protein was used for each fly line. Adult flies were used instead of larvae, as more protein was available from the adult head.

As there is no antibody against the UPRT, the HA-tag antibody was used for visualization of the UPRT enzyme in the flies. Additionally, alpha-tubulin antibody was used as a loading

control. The expected molecular weight of the UPRT is 27 kDa (37) and 55 kDa for alpha-tubulin (38).

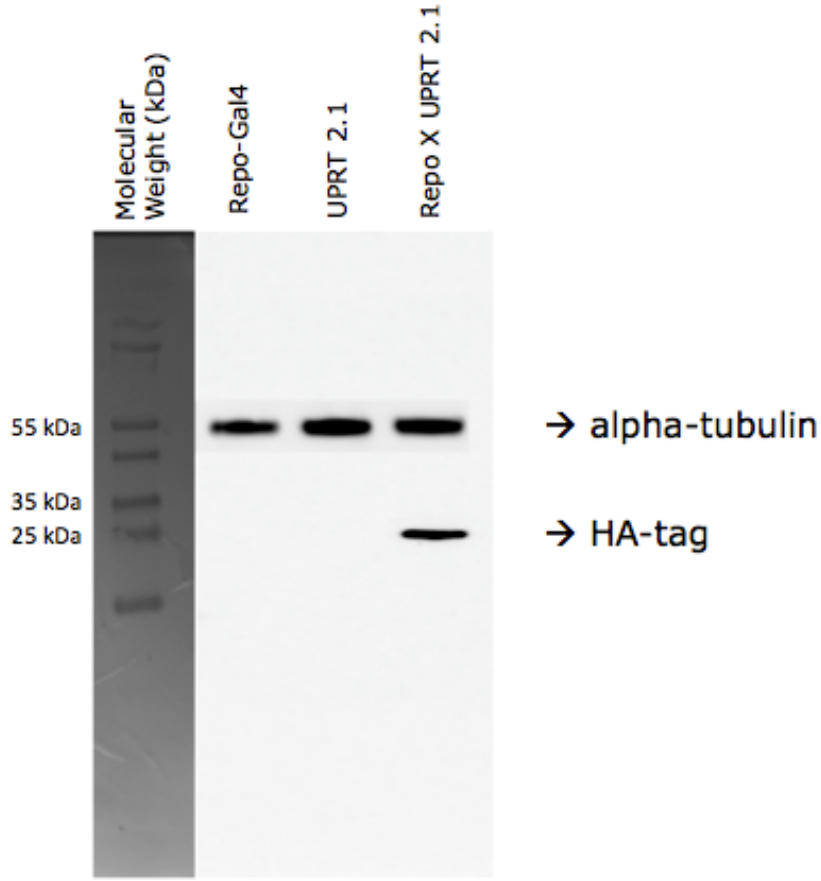

Figure 4: Western Blot analysis of UPRT expression in different fly lines. Bands show the presence of the proteins.

It is clear that UPRT is not expressed in the individual fly lines (repo-Gal4 and UPRT 2.1), meaning the Gal4 expression is necessary for the expression of the enzyme as discussed. On the other hand, UPRT is present in the progeny, which indicates the success of the crosses. It is worth noting, however, that the blot shows the expression of the enzyme, not the activity and it does not demonstrate in which cell types the UPRT is expressed.

3.1.1.2. Immunohistochemistry

Immunohistochemistry analysis was done in order to visualize the absence and the presence of the UPRT in the adult fly brain cells.

Repo antibody (Alexa Fluor 555; red channel) was used to label the glia cells while the HA-tag antibody (Alexa Flour 488; green channel) was used to label the enzyme and therefore indicate cell types labeled in the brain. Additionally, repo is localized to cell nucleus (39) while UPRT (HA tag in this case) is localized to both cytoplasm and nucleus (40).

Theoretically, in the case of crosses with the repo Gal-4 driver, the UPRT should be expressed in the glia cells. Thus, the repo and the HA-tag should be co-localized in the glia cells (Fig. 5 and 6 A-C). Additionally, the UAS-HA:UPRT 2.1 line was stained in order to test the degree of the expression in the absence of the driver (Fig. 7 A-C) and repo-Gal4 line was stained in order to prove there is no expression of the UPRT without the UAS line. (Fig. 8 A-C).

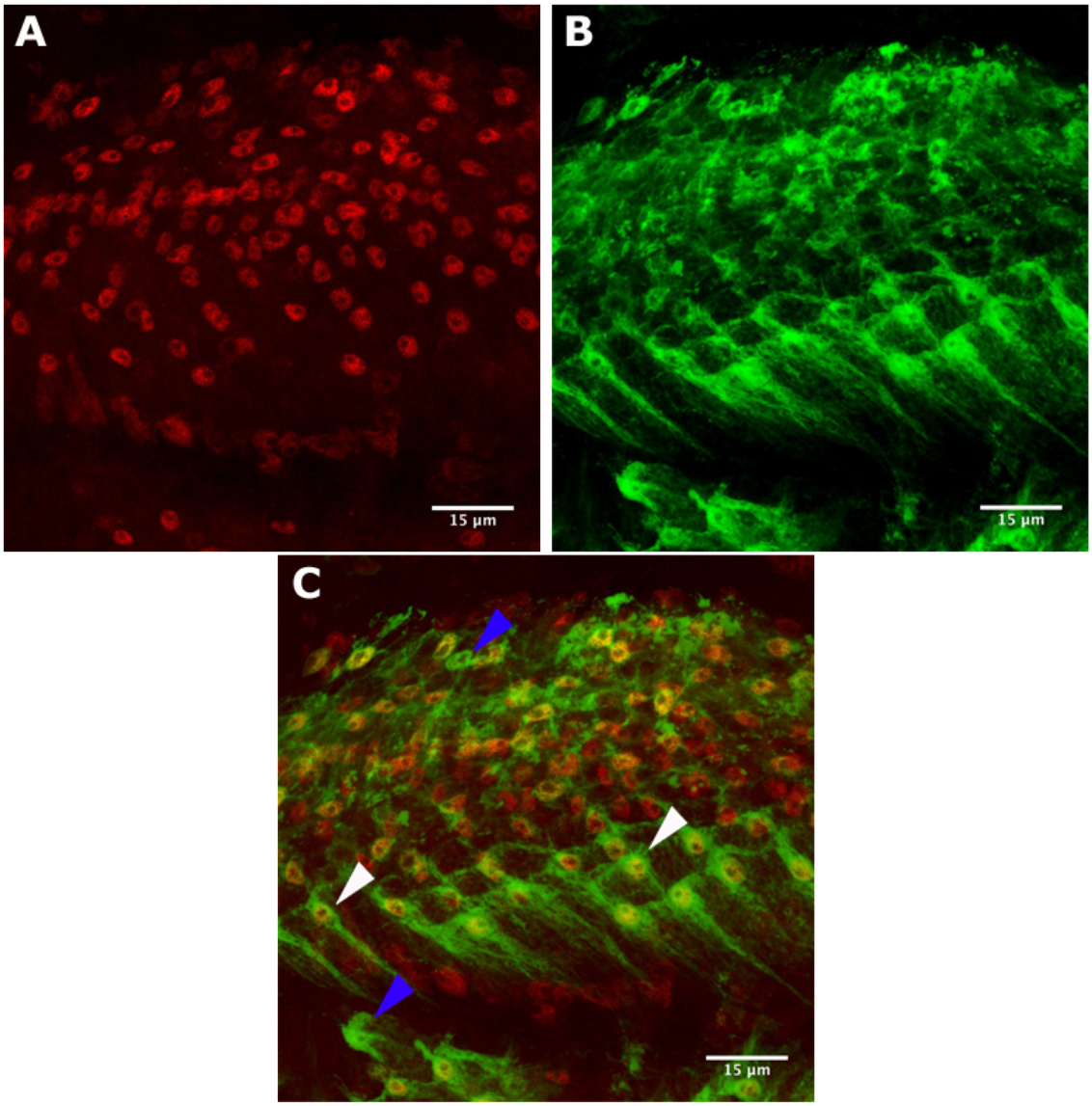

Figure 5: Repo-Gal4 x UAS-HA:UPRT 2.1 progeny staining, optic lobe of the brain. **(A)** Repo staining; Alexa Flour 555. **(B)** HA staining; Alexa Flour 488. **(C)** Merged version of A and B. White arrows show the co-localization while the blue arrows show HA staining alone.

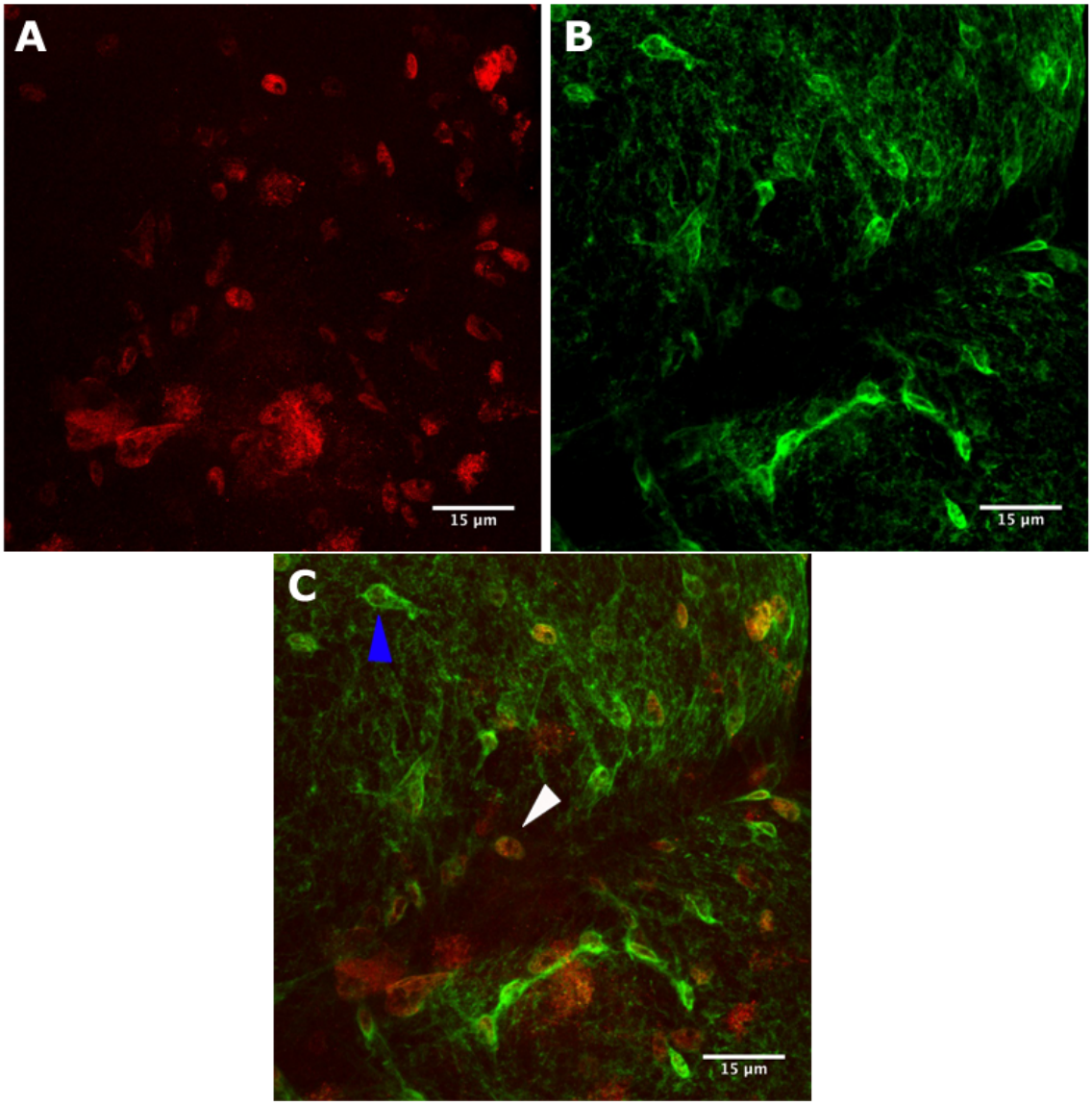

Figure 6: Repo-Gal4 x UAS-HA:UPRT 2.1 progeny staining, mid brain. **(A)** Repo staining; Alexa Flour 555. **(B)** HA staining; Alexa Flour 488. **(C)** Merged version of A and B. White arrows show the co-localization while the blue arrows show HA staining alone.

It is obvious from the figures 5-6C that there is some UPRT expression in the repo negative cells of the repo-Gal4 X UAS-HA:UPRT crosses. The leakage of the Gal4 to other cell types might be the reason for this undesirable UPRT expression.

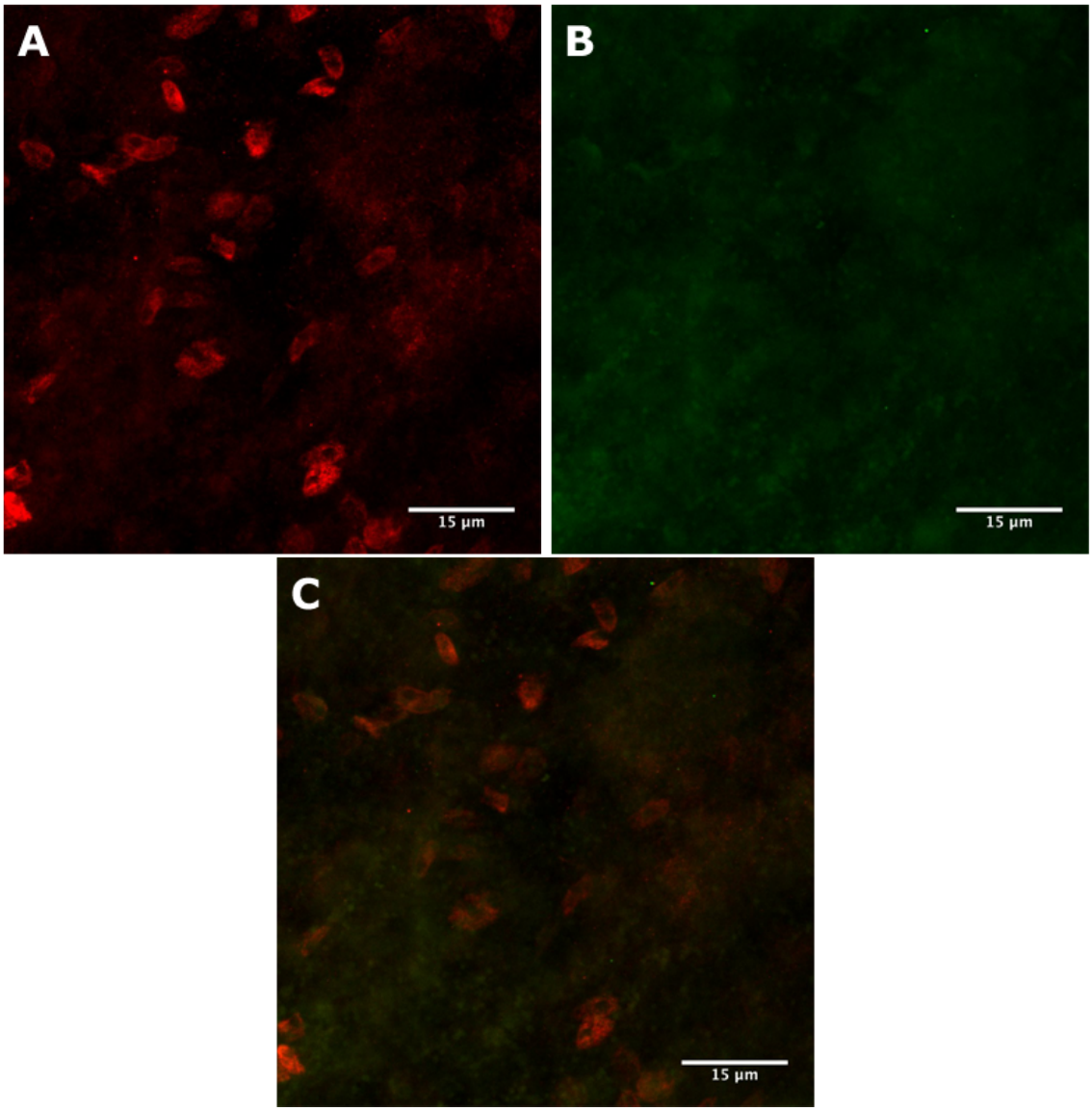

Figure 7: Repo-Gal4 individual line staining. **(A)** Repo staining; Alexa Flour 555. **(B)** HA staining; Alexa Flour 488. **(C)** Merged version of A and B.

As seen in figure 7, repo-Gal4 flies do not show the expression of the UPRT when not crossed with the UAS-HA:UPRT line. The green view on the panel B represents autoflourescence of the staining, not a specific signal.

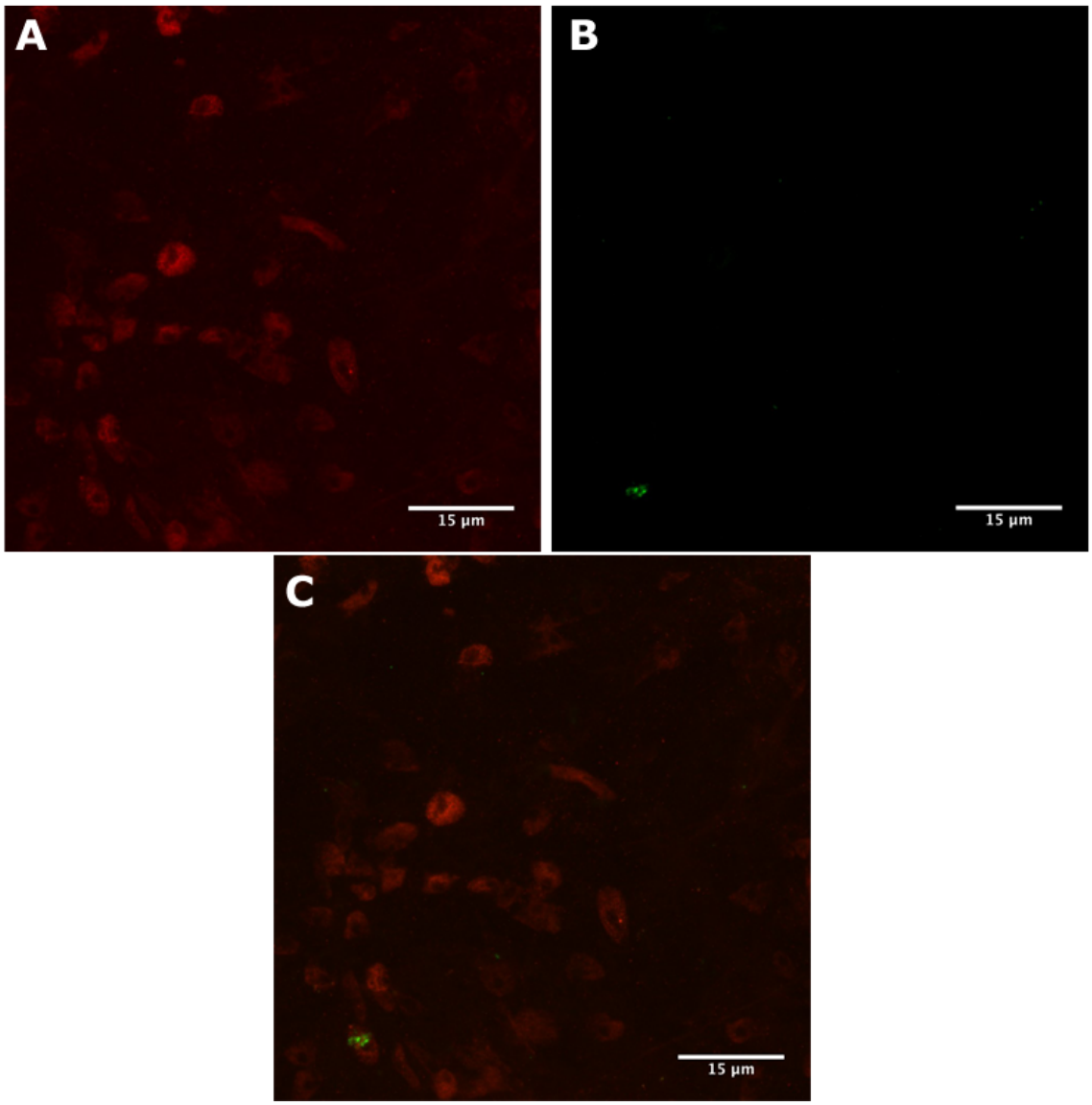

Figure 8: UAS-HA:UPRT 2.1 individual line staining. **(A)** Repo staining; Alexa Flour 555. **(B)** HA staining; Alexa Flour 488. **(C)** Merged version of A and B.

As desired, the UAS-HA:UPRT 2.1 line did not show the expression of the UPRT without a driver. The western blot and immunohistochemistry results together show that crosses were successful (UPRT expression is activated) and Gal4 drives the UPRT expression.

3.1.2. TU-Tagging Experiments

For the validation of the TU-tagging chemistry, there was a need for an external positive control. Human protein associated with topoisomerase II homolog 1 (yeast) (hPatL1) was the chosen gene as the control since it is not expressed in *Drosophila*. The hPatL1 mRNA was expressed *in vitro* by using 4-TU to ensure it is fully labeled with 4-TU. After isolating

the total RNA from flies, the same amount of hPatL1 mRNA was added to the total RNA and all TU-Tagging experiments performed in the presence of hPatL1 mRNA.

Classical neuronal and glial markers were used in the TU-Tagging experiments in order to show the cell type specificity of the RNA isolation. They were repo, anachronism and moody for glia and embryonic lethal abnormal vision (elav), nsyb and synaptotagmin (syt) for neurons. Absence or the presence of these cell type specific markers was determined with RT-qPCR after TU-Tagging RNA isolation. As TU-Tagging is supposed to isolate cell-type specific RNA (TU-Tagged RNA) from a pool of total CNS RNA, in theory cell-type specific markers should be enriched in TU-Tagged RNA compared to total isolate. Results were presented as fold enrichment change in the expression level. For repo-Gal4 based crosses, results were normalized to 1 in elav while for nsyb-Gal4 based crosses they normalized to 1 in repo as they are well know cell type specific markers (34,41).

3.1.2.1. Optimization of TU-Tagging

The first TU-Tagging experiments performed with repo-Gal4 x UAS-HA:UPRT 2.1 larvae did not work (neither hPatL1 nor cell type specific markers were enriched) (Data not shown). The first concern was whether larvae were eating the food containing 4-TU. To prove the food intake, blue dye-containing food was used since larvae are transparent and food can be visualized in the intestines. After feeding larvae's bellies were blue meaning the food was eaten (See supplementary section B). The second concern was whether the feeding duration was long enough. In order to test the correlation between the feeding duration and the fold enrichment, parallel experiments were designed. 3 groups of larvae were fed with 4-TU food for 2, 4 and 8 hours, respectively. As a result, increasing fold enrichment over the feeding duration was observed (Data not shown). Therefore, it was decided to perform 8 hours feeding (it was changed into 5 hours afterwards as more feeding duration would give more background incorporation of 4-TU).

At first, the protocol from Gay et. al 2013 was followed. In that protocol, 25 µg of biotin was used for the biotinylation independently from the starting RNA amount. However, as they only use mRNA (instead of total RNA), the biotin amount might be enough to bind all the mRNA in the reaction (mRNA is around 5% of the total RNA). Therefore, the biotinylation conditions were changed in order to adapt it to total RNA by increasing the biotin amount. Instead of a fixed 25 µg of biotin for every experiment (independent from the starting RNA amount), more flexible experimental conditions were applied which includes biotin amount based on the total RNA concentration. For instance, 100 µg of biotin were used for 100 µg of total RNA (Detailed description is available in the methods section). Following this optimization, TU-Tagging started working (Fig. 9). For the TU-Tagging experiments, larvae RNA were used (hPatL1 was always added into total RNA before performing TU-Tagging). In the experiments two groups of RNA were compared: TU-Tagged RNA and input (total RNA). Fold enrichment represents the gene expression

level difference between TU-Tagged RNA and input. Fold enrichment was normalized to the elav value (as this experiment was performed in repo-Gal4 based flies).

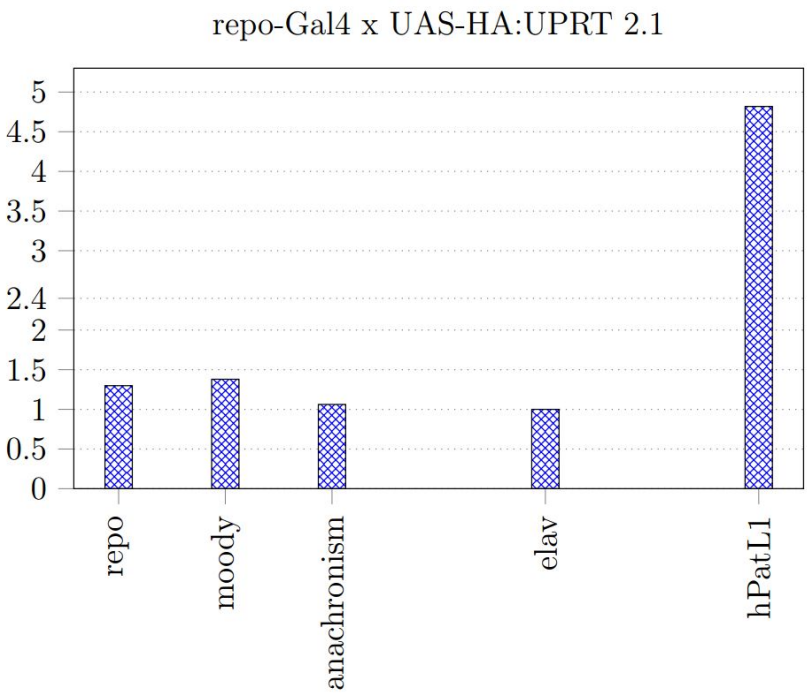

Figure 9: Bars represent the fold enrichment of TU-Tagged RNA from larvae. First 3 markers are glial markers while the next two markers are for neurons and external positive control, respectively.

The first thing to note in the TU-Tagging experiments was the hPatL1 mRNA as it is the external control for method chemistry. In figure 9, hPatL1 showed a high enrichment meaning TU-Tagging chemistry worked (Note, only the experiments in which hPatL1 enriched have been included in this study). The second thing to note is the enrichment of the cell type specific markers. As this cross was based on repo-Gal4, glial markers should have enriched more compared to neuronal marker. However, glial markers and neuronal markers were showed similar enrichment profiles. On the other hand, as transient elav expression was reported in glia cells during embryonic development of *Drosophila* (42), It was decided to perform TU-Tagging with adult flies (Fig. 10).

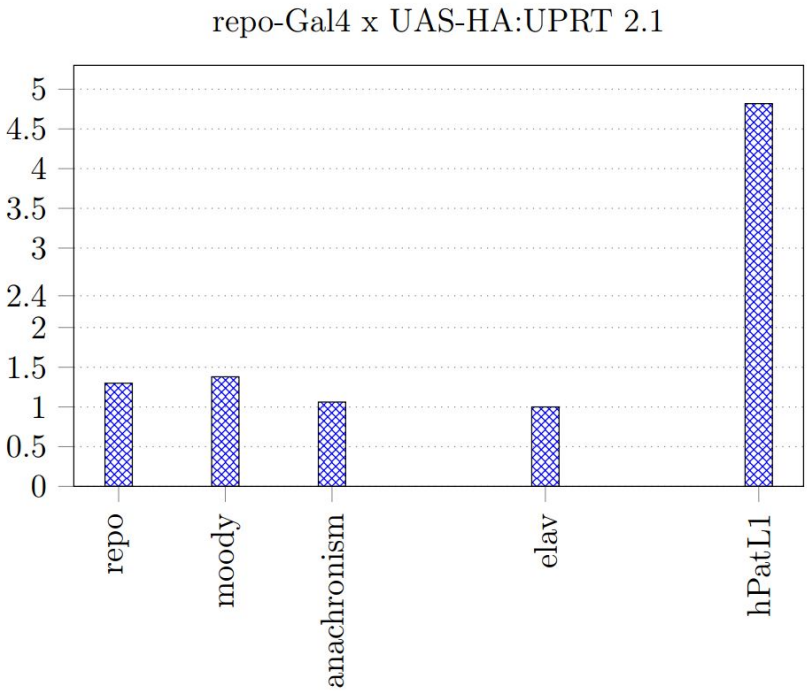

Figure 10: Bars represent the fold enrichment of TU-Tagged RNA from adults. First 3 markers are glial markers while the next two markers are for neurons and external positive control, respectively.

Figure 10 represents that both glial and neuronal markers showed similar enrichment profiles to the ones found in the previous experiment (which was performed with larvae). Changing larvae into adult did not alter the fold enrichment profile. Note that, in both experiments anachronism was enriched less than repo and moody and similar to elav. Additionally, it is important to remember that staining (Figure 5-6 C) showed UPRT expression in repo negative cells, which might be the cause of the RNA labeling in both cell types. Therefore, it was decided to change to a different cell-type specific driver (nsyb-Gal4) to perform TU-Tagging experiment. Since using larvae or adult did not change the enrichment levels in repo-Gal4 based progeny, larvae was used due to practical reasons (more RNA obtained). Two more neuronal markers were added to the experiments in order to broad the range of the markers (Fig. 11).

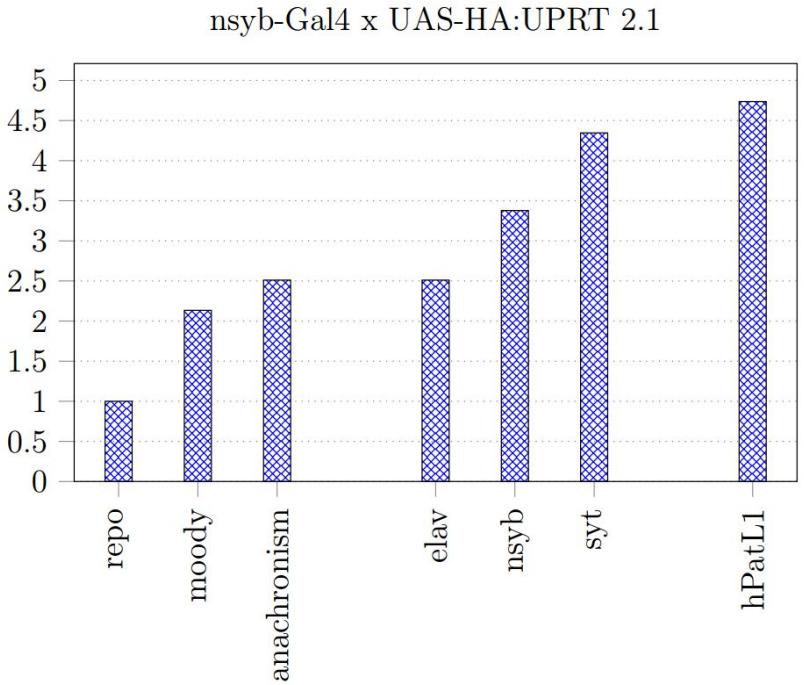

Figure 11: Bars represent the fold enrichment of TU-Tagged RNA from adults. First 3 markers are glial markers while the next 3 markers are for neurons and the last one is for the external positive control.

Normalization was performed according to the glial marker repo. Graph shows that the fold enrichment of the neuronal markers was more than glial markers, however, the glial markers were all enriched.

Additionally, 2 control TU-Tagging experiments were designed. In the first experiment, WIII8 (wild type) larvae were fed with 4-TU in order to determine whether 4-TU is incorporated without UPRT (Fig. 12).

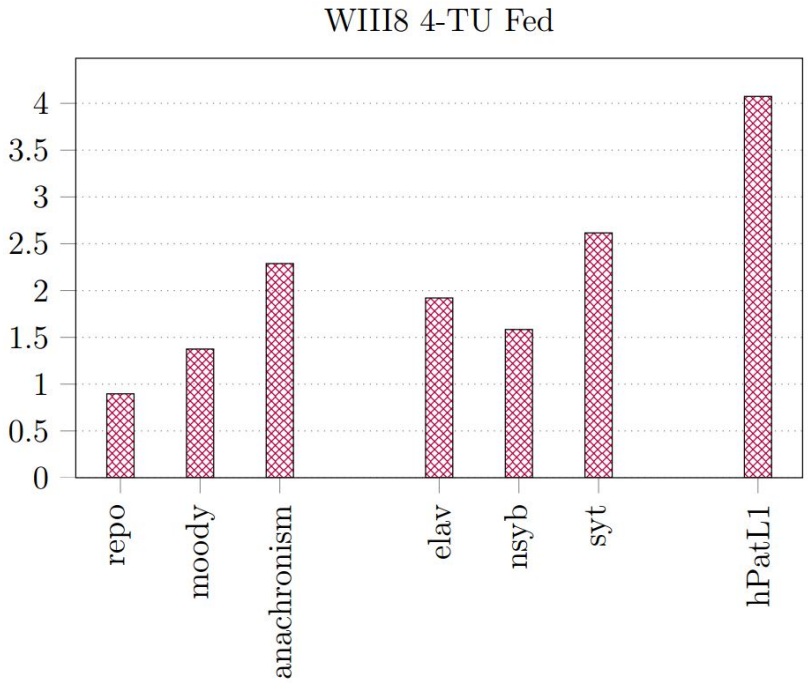

Figure 12: Bars represent the fold enrichment of RNA from larvae. First 3 markers are glial markers while the next 3 markers are for neurons and the last one is for the external positive control.

Figure 12 shows a high level of fold enrichment of both the neuronal and glial markers. However, since in WIII8 flies there is the absence of UPRT, the question is whether 4-TU was incorporated into RNA without the UPRT (maybe with the internal machinery) or beads were binding to the RNA unspecifically. To further investigate this, repo-Gal4 x UAS-HA:UPRT 2.1 larvae were used without feeding them with 4-TU. The aim of this experiment was to show whether there is unspecific binding of the RNA to the streptavidin beads (Fig. 13).

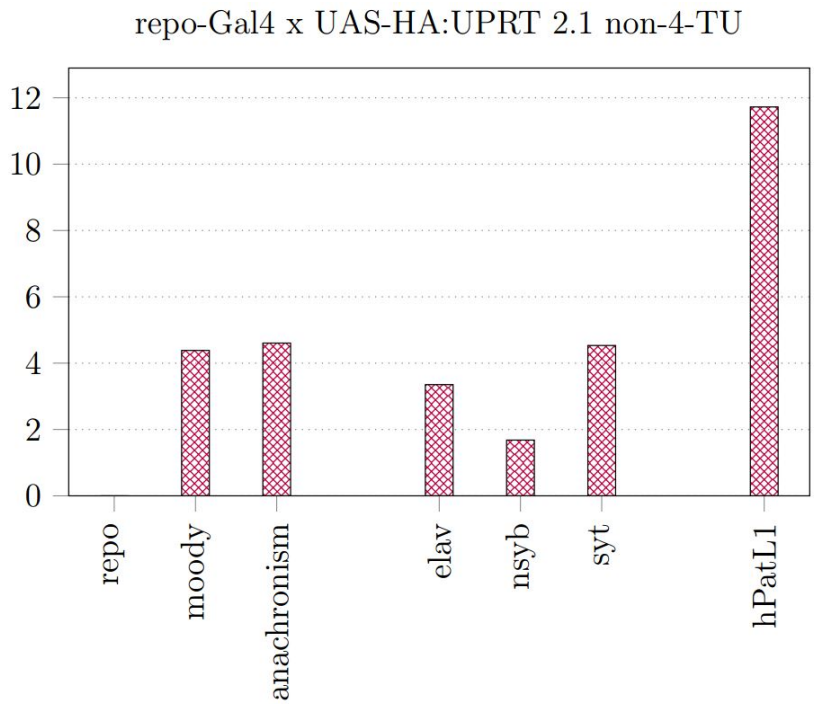

Figure 13: Bars represent the fold enrichment of RNA from larvae. First 3 markers are glial markers while the next 3 markers are for neurons and the last one is for the external positive control.

Figure 13 shows that even without 4-TU, there is fold enrichment for both neuronal and glial markers. It means either RNA binds to the beads even unspecifically or biotin binds something other than the thiol group.

In the all above-mentioned experiments, Dynabeads M-270 Streptavidin (2.8 µm in diameter) were used for the isolation of the biotinylated TU-Tagged RNA. The other option was µMACS Streptavidin beads (50 nm in diameter). This difference in diameters makes Dynabeads 3136 times bigger by the surface area than µMACS beads. Higher surface area might be a cause for more unspecific RNA binding to the beads. Therefore, it was decided to compare two beads in parallel experiments. For this experiment, all the conditions were same, just the beads used were different (Fig. 14).

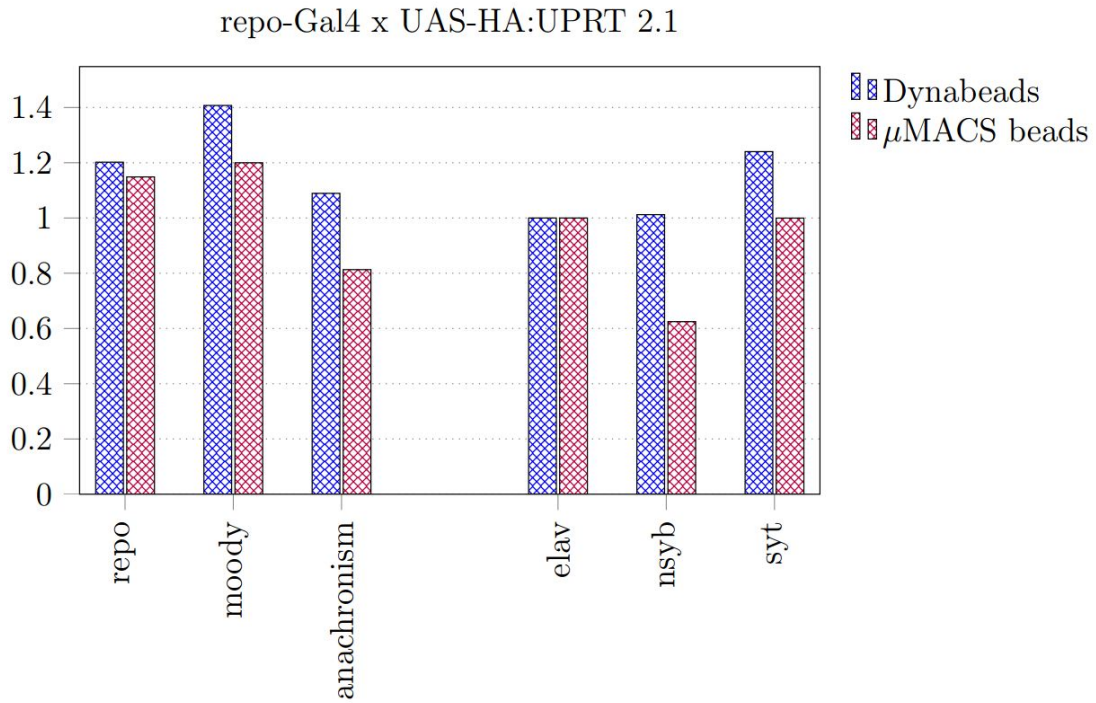

Figure 14: Bars represent the fold enrichment of RNA from larvae with two different streptavidin beads. First 3 markers are glial markers while the next 3 markers are for neurons.

Figure 14 shows the comparison of the two different streptavidin beads. According to the bars, Dynabeads provide higher enrichment compared to Dynabeads. The reason of the more binding might be the unspecific binding of Dynabeads to the RNA due to Dynabeads' huge surface area. To avoid any unspecific binding risk, it was decided to use μMACS Streptavidin beads for further experiments.

3.2. UAS-HA:UPRT 3.2 Experiments

In the second part of the study, the UAS-HA:UPRT 2.1 line was changed into the 3.2 line due to the above mentioned advantage of the UAS-HA:UPRT 3.2 line and the unintended UPRT expression in repo negative cells with UAS-HA:UPRT 2.1 cross (Figure 5-6 C).

3.2.1. Biochemical and Immunohistochemical Analysis of the crosses with UAS-HA:UPRT 3.2 line

After establishing the new crosses with UAS-HA:UPRT 3.2 line (with same drivers: repo-Gal4 and nsyb-Gal4), it was crucial to show the success of the crosses as it was done with the UAS-HA:UPRT 2.1 line.

3.2.1.1. Western Blot

Western blot analysis was done in order to show the existence of the UPRT in the crosses. As it was done in the previous western blot, adult fly heads were used for protein extraction and 10 µg protein was used for each line. Additionally, in order to allow comparison between two UPRT lines within the same experiment, UAS-HA:UPRT 2.1 line was also included (Fig. 15).

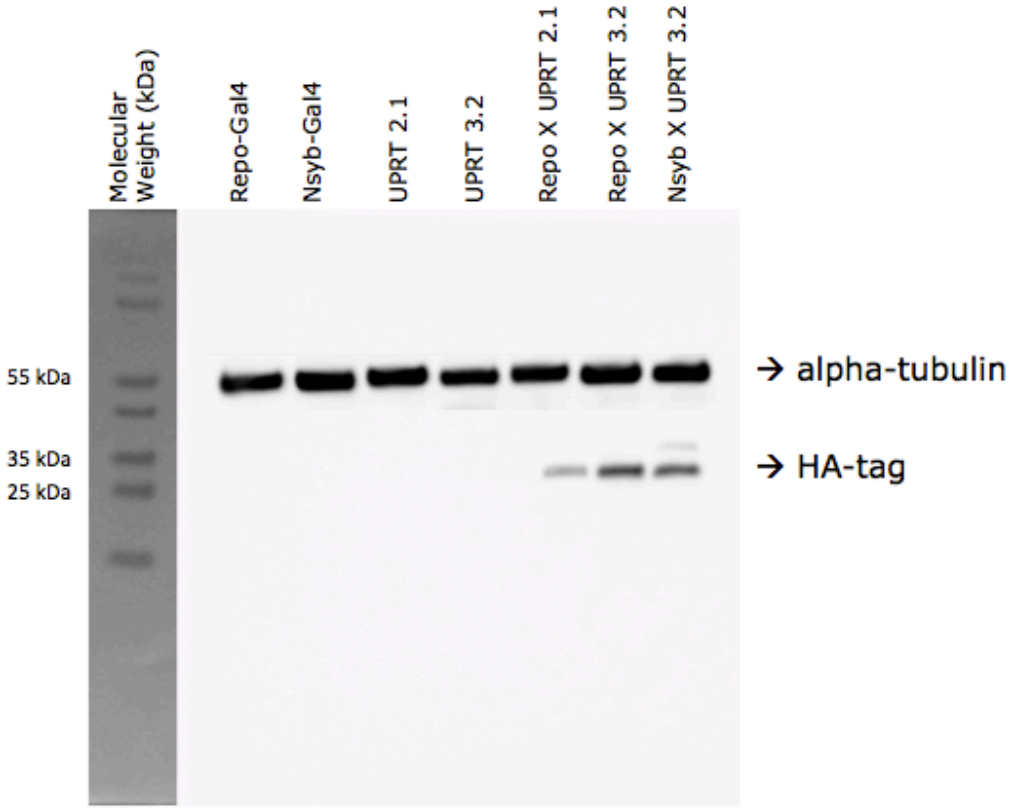

Figure 15: Western Blot analysis of UPRT expression in different fly lines. Bands show the presence of the proteins.

Figure 15 shows that UPRT is present in the crosses, which indicates the success of the crosses. Furthermore, it is clear that in the repo-Gal4 x UAS-HA:UPRT 3.2 progeny there is more expression of the enzyme compared to repo-Gal4 x UAS-HA:UPRT 2.1.

3.2.1.2. Immunohistochemistry

Immunohistochemistry analysis was done in order to visualize the absence and the presence of the UPRT in the adult fly brain cells. The same antibodies were used: repo antibody (Alexa Fluor 555, red channel) and HA-tag antibody (Alexa Fluor 488, green channel).

As explained before, in the case of crosses with the repo Gal-4 driver, the UPRT should be expressed in the glia cells. Thus, the repo and the HA-tag should be co-localized in the glia cells (Fig. 16 and 17 A-C). On the other hand, in the case of crosses with the nsyb-Gal4 driver, the UPRT should be expressed in the neurons, meaning the repo and the HA-tag should not be co-localized (Figure 18 and 19 A-C).

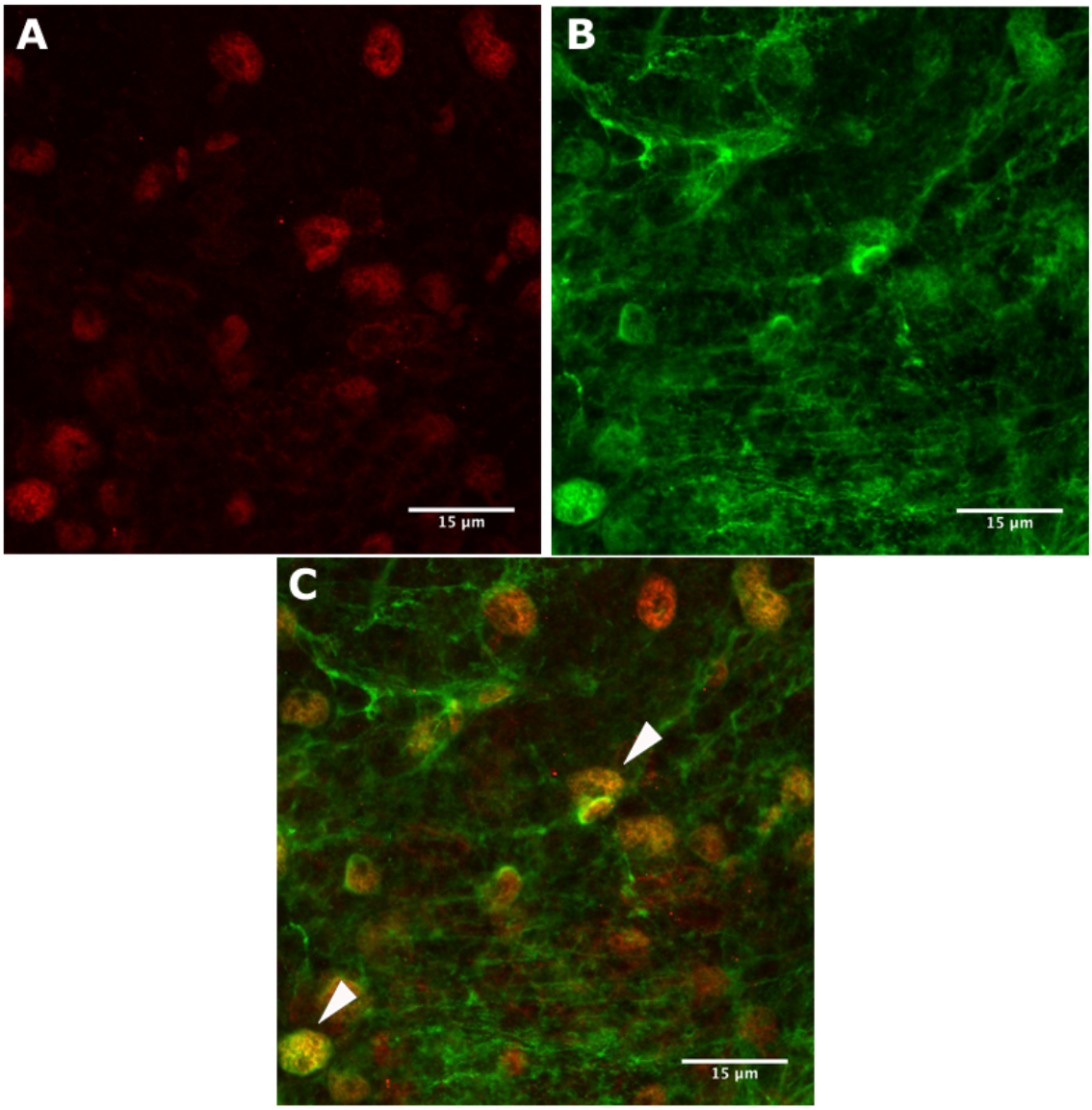

Figure 16: Repo-Gal4 x UAS-HA:UPRT 3.2 progeny staining, mid brain. **(A)** Repo staining; Alexa Flour 555. **(B)** HA staining; Alexa Flour 488. **(C)** Merged version of A and B. White arrows show the co-localization.

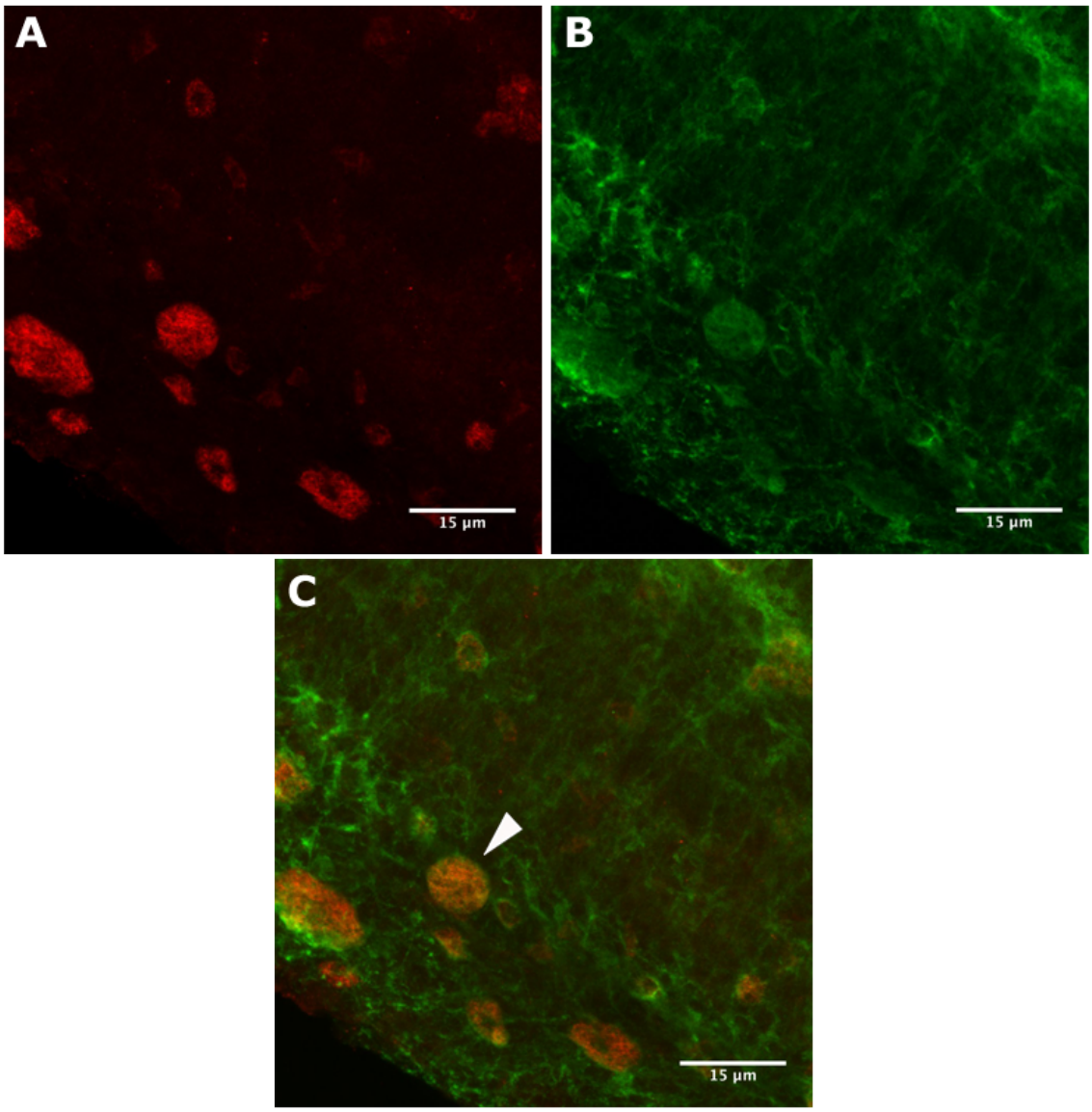

Figure 17: Repo-Gal4 x UAS-HA:UPRT 3.2 progeny staining, optic lobe of the brain. **(A)** Repo staining; Alexa Flour 555. **(B)** HA staining; Alexa Flour 488. **(C)** Merged version of A and B. White arrows show the co-localization.

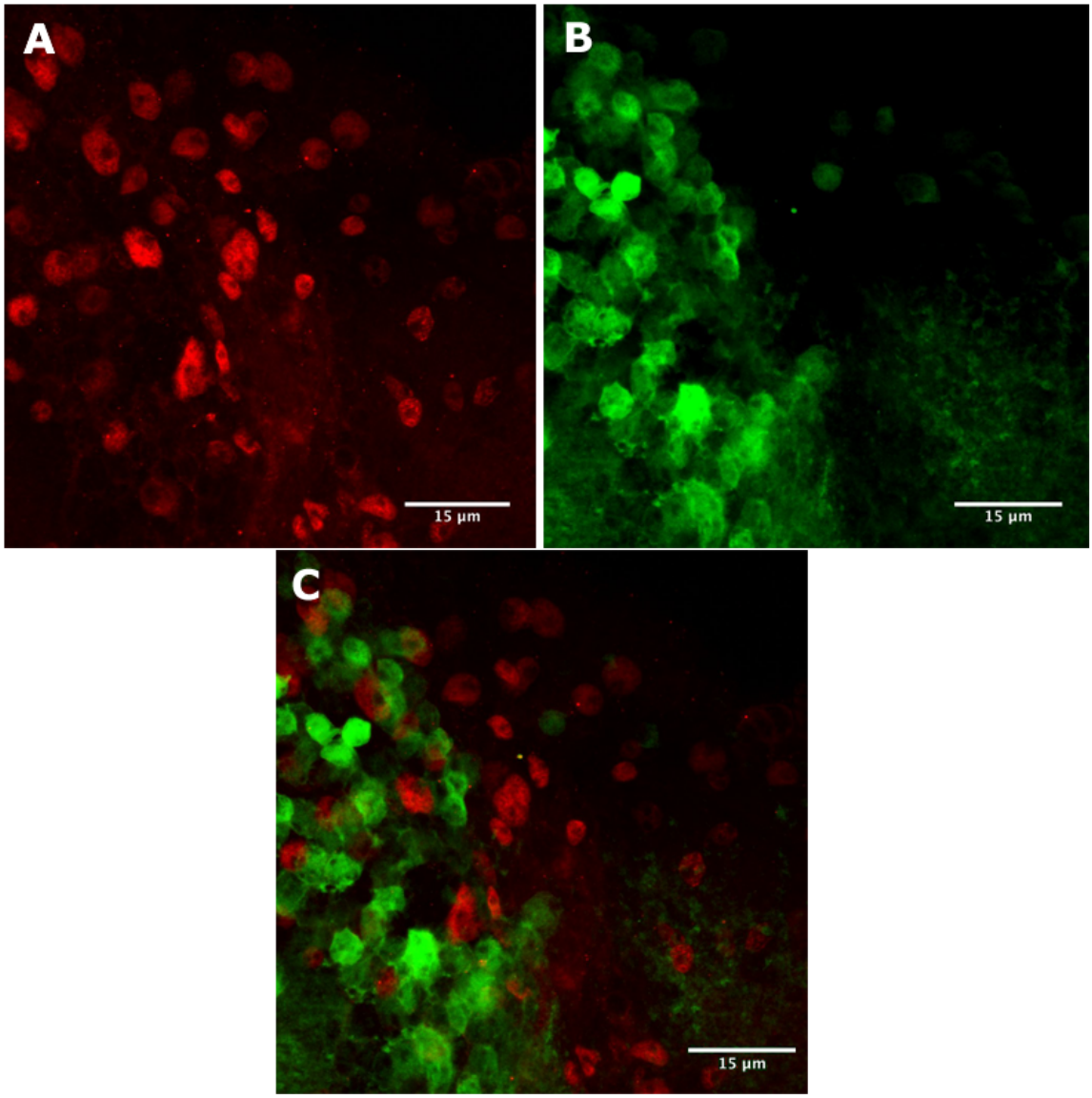

Figure 18: Nsyb-Gal4 x UAS-HA:UPRT 3.2 progeny staining, mid brain. **(A)** Repo staining; Alexa Flour 555. **(B)** HA staining; Alexa Flour 488. **(C)** Merged version of A and B.

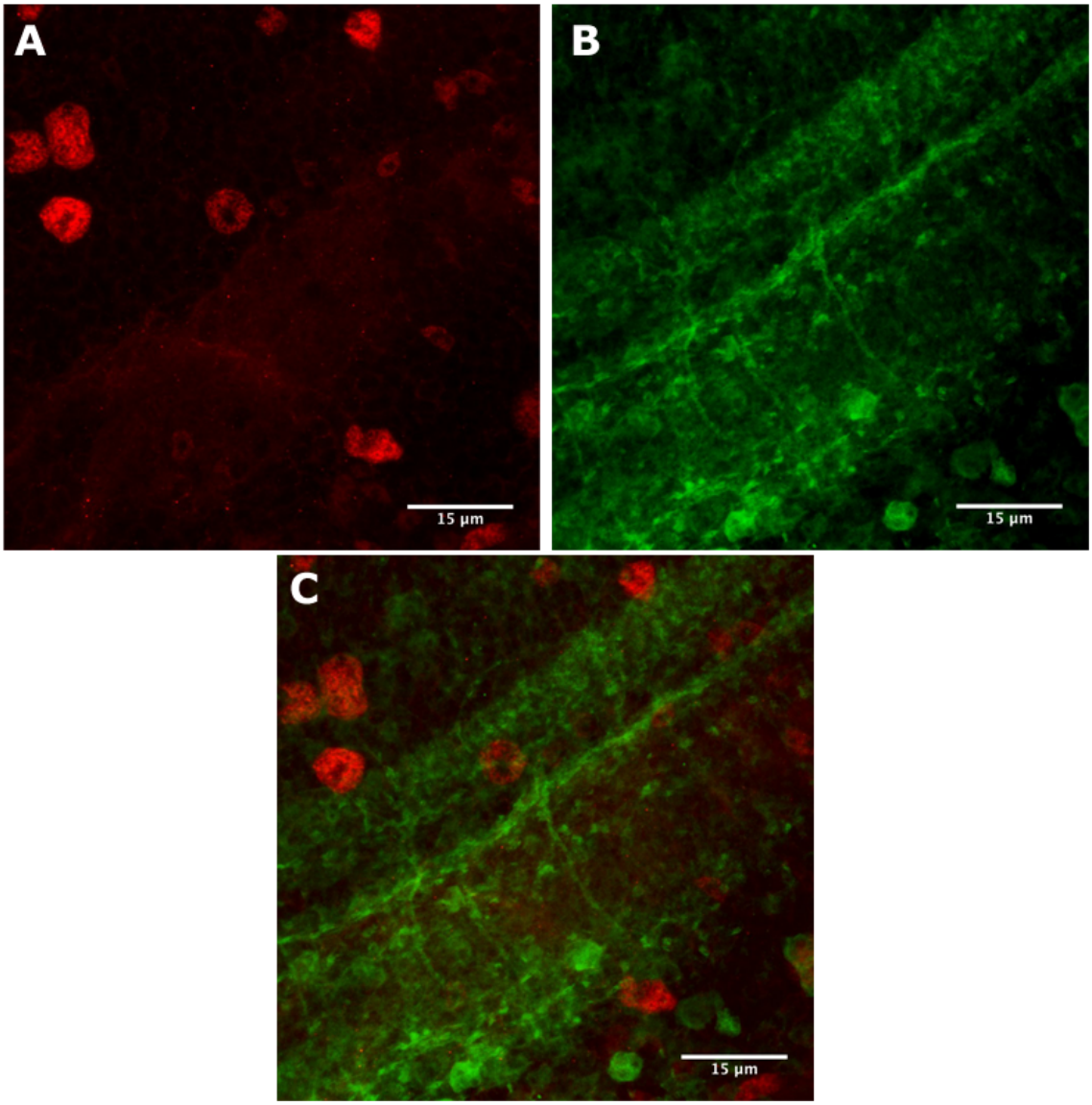

Figure 19: Nsyb-Gal4 x UAS-HA:UPRT 3.2 progeny staining, optic lobe of the brain. **(A)** Repo staining; Alexa Flour 555. **(B)** HA staining; Alexa Flour 488. **(C)** Merged version of A and B.

Double immunohistochemical labeling revealed that UPRT is localized to glia cells in repo-Gal4 X UAS-HA:UPRT 3.2 crosses, while there is no co-localization of glia cells and UPRT in nsyb-Gal4 X UPRT 3.2 cross as anticipated. Additionally, the UAS-HA:UPRT 3.2 line was stained in order to prove there is no expression of the UPRT without the driver (Fig. 20 A-C).

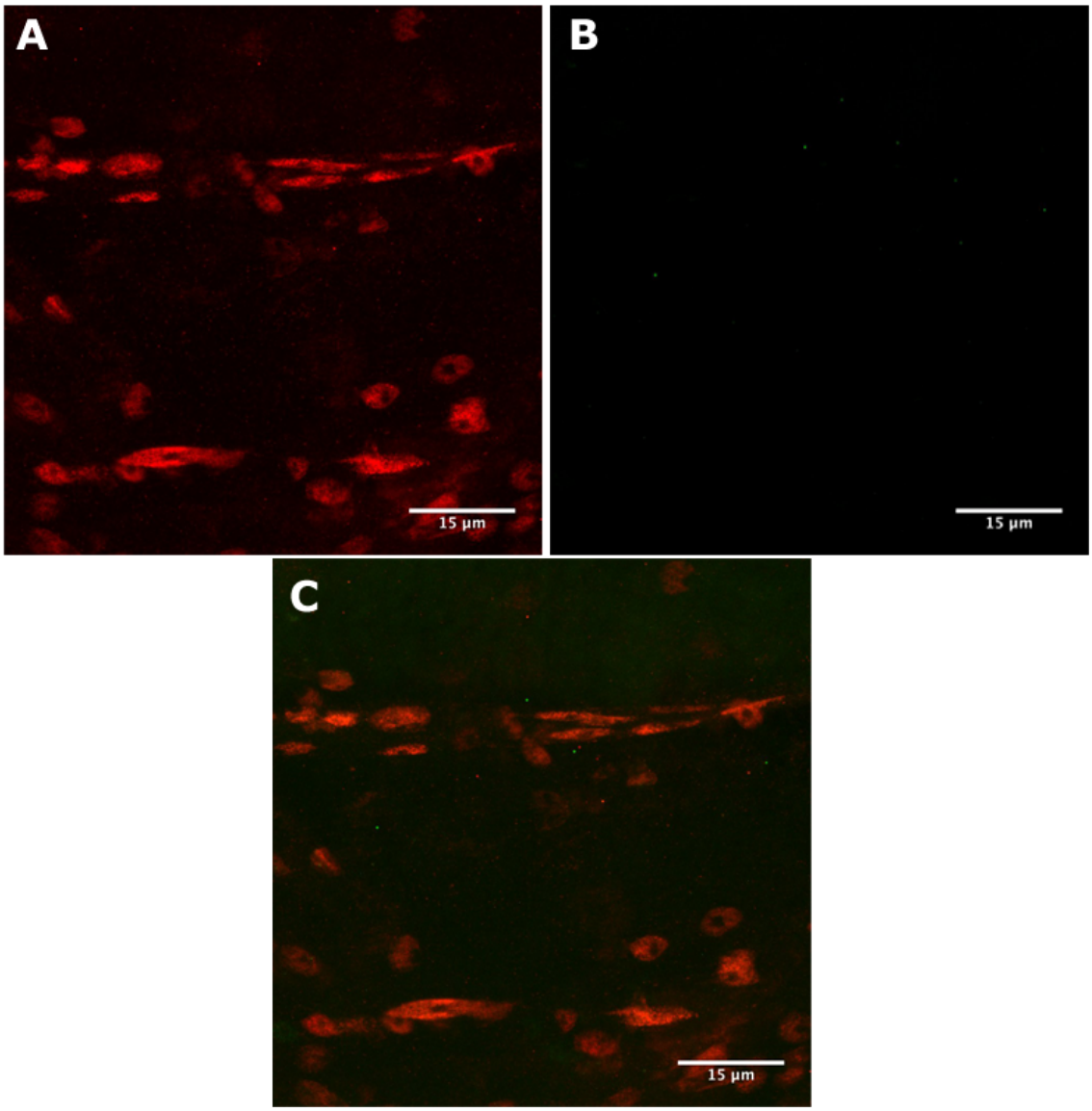

Figure 20: UAS-HA:UPRT 3.2 staining. **(A)** Repo staining; Alexa Flour 555. **(B)** HA staining; Alexa Flour 488. **(C)** Merged version of A and B.

As it is clear from the figure 20, there is no expression of the UPRT while repo is presented and these results were also in an agreement with the western blot result.

3.2.2. TU-Tagging Experiments

TU-Tagging experiments were repeated with the new UAS-HA:UPRT 3.2 line. Additionally, RNA fragmentation step was included (before biotinylation step) since it was reported as reducing background labeling (13). This step fragments the RNA into small pieces (~200 base pairs) using divalent cations under elevated temperature. In order to demonstrate the effect of fragmentation on fold enrichment, Two identical TU-Tagging experiments (one with fragmentation while the other one without fragmentation) were compared by using nsyb-Gal4 X UAS-HA:UPRT 3.2 cross (Fig. 21). Furthermore, 2 more positive control markers were added to experiments: UPRT and Gal4 in order to make sure of the validity of the method. As Gal4 induces the expression of the UPRT, they both need to be presented in the same cell type and they served as a control of cell type specificity.

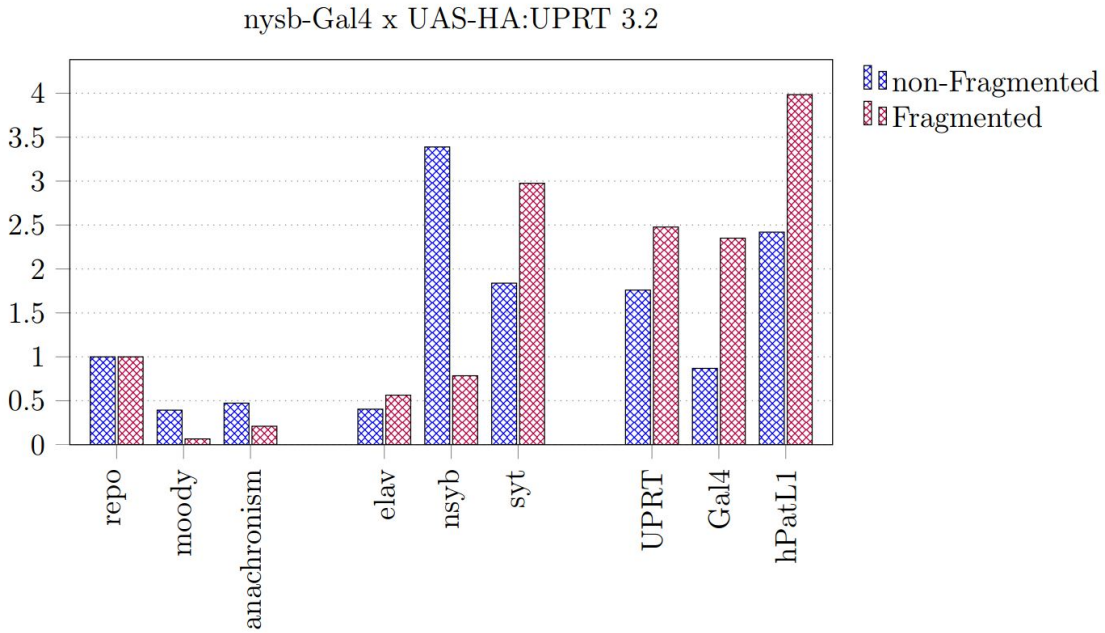

Figure 21: Comparison of fragmented and non-fragmented RNA. Bars represent fold enrichment. First 3 markers are for glia while the other 6 markers are for neurons and controls, respectively.

According to the graph, the enrichment of neuronal markers and controls were increased (except nsyb), while glial markers were reduced with fragmentation. It is logical as the cross was based on nsyb-Gal4 (neuronal markers' enrichment were increased while glial markers' enrichment were decreased). As a conclusion, fragmentation appeared as a helpful step and it was included to following experiments. Next, TU-Tagging was performed with repo-Gal4 X UAS-HA:UPRT 3.2 (Fig.22) and nsyb-Gal4 X UAS-HA:UPRT 3.2 (Fig. 23) crosses.

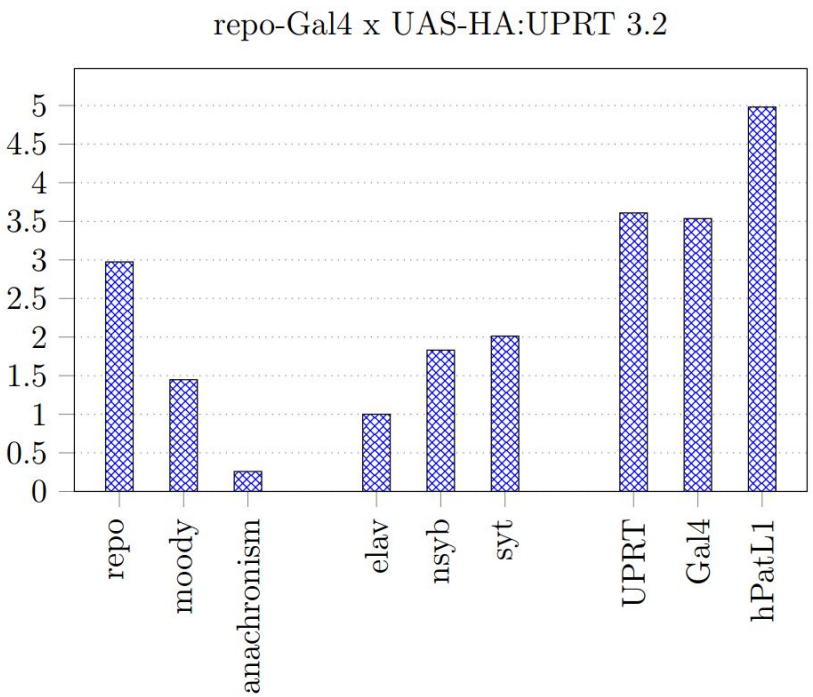

Figure 22: Bars represent the fold enrichment of RNA from larvae. First 3 markers are for glia while the other 6 markers are for neurons and controls, respectively.

According to the controls, TU-Tagging worked, UPRT is also highly expressed in the cross. However, both glial and neuronal markers were presented in TU-Tagged RNA. Note that the fold enrichment of anachronism was even lower than the neuronal markers.

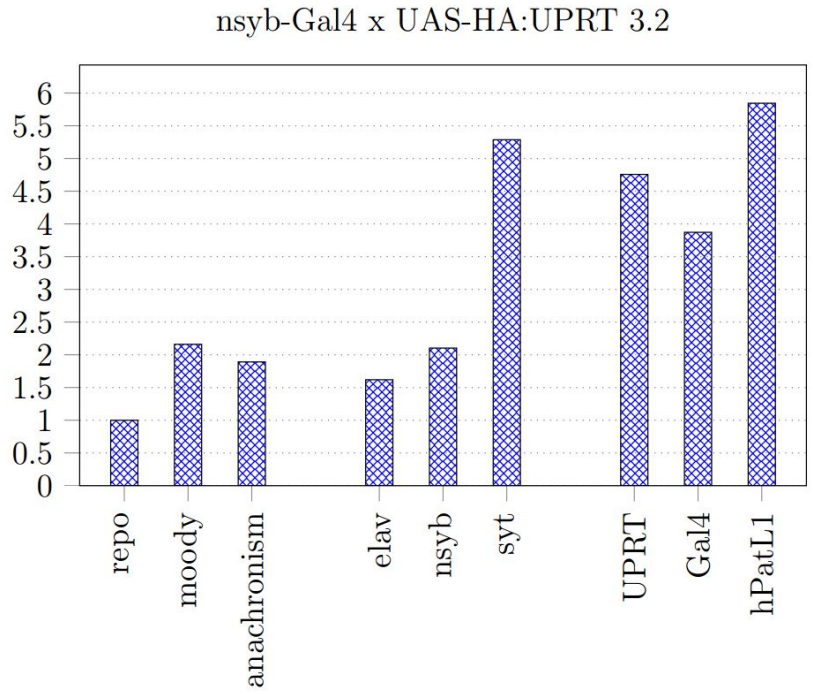

Figure 23: Bars represent the fold enrichment of RNA from larvae. First 3 markers are for glia while the other 6 markers are for neurons and controls, respectively.

As in the previous experiment, according to the controls TU-Tagging worked. However, even though this is a nsyb based cross, anachronism and moody were highly enriched. In a short conclusion, according to the hPatL1 control, TU-Tagging chemistry works. On the other hand, both neuronal and glial markers were presented in TU-Tagged RNA, which might be indicate a problem about the cell type specificity of the markers and will be discussed in detail in the next section.

4. Discussion & Conclusion

The purpose of this thesis was optimization and validation of TU-Tagging by using *Drosophila* CNS. In order to validate the chemistry of the method, a powerful external control was needed. hPatL1 mRNA was transcribed *in vitro* by using 4-TU and as it is already labeled with 4-TU served as the control. It was added to each TU-Tagging experiment as a fixed amount and the enrichment level was highly reproducible between the experiments. hPatL1 mRNA proved that once 4-TU in incorporated into RNA, the isolation part of the TU-Tagging (biotinylation and streptavidin isolation) works extremely well.

On the other hand, even though the method works, there was a problem in respect to cell type specific markers. In the all TU-Tagging experiments (both for glial and neuronal RNA isolation), cell type specific markers were presented together. There are several possible explanations of this issue, however the reason(s) remains unknown and demands further investigation.

First explanation might be that Gal4 is leaky and drives the expression of the UPRT in undesired cell types (Fig. 5-6 C). However, according to the staining (Fig. 16,17,18 and 19 C) this possible leakage problem was fixed with the new UPRT line (3.2). Furthermore, it is possible to test this idea by a double staining of repo-Gal4 X UAS-GFP cross with repo and HRP antibodies. This experiment would provide valuable information about where the glia cells are and where Gal4 triggers the GFP expression.

The second explanation might be the cell type specific markers are not cell type specific as proposed. For instance, in this study elav was used as a well-known neuron specific marker (41). However, a recent study demonstrated that elav is transiently expressed in glia cells of the embryonic CNS of *Drosophila* (42). Additionally, anachronism is a inhibitor of neuroblast proliferation and specific to glia cells (43), however, a recent study showed expression of anachronism in neurons (44). These examples discredit the cell type specificity of well-known markers and open them to questioning.

Lastly, glia cells are derived from neuronal progenitor cells (45) and Gal4 might be activated in early developmental stages of *Drosophila*. It might be result in expression of UPRT in the early developmental stage and UPRT might be persistent during the development. Temporal expression of Gal4 can be controlled by temperature sensitive Gal80 protein (inhibitor of Gal40) (46). Gal80 binds to Gal4 in order to inhibit its expression and by shifting the temperature Gal4 expression can be triggered (47) (hence the UPRT expression in this case).

In conclusion, in this thesis, TU-Tagging chemistry was proven to be working but the issue with this method lies in the cell type specificity of the markers being used. Despite the problem with the markers specificity in flies, TU-Tagging provides a powerful approach for

cell type specific RNA isolation. For instance, as TU-Tagging is applicable to mouse, with the appropriate mouse lines it would be possible to study the different aspects of the CNS such as learning and memory formation (contribution of the glia cells to this processes), as well as diseases such as cancer. Hence, TU-Tagging promises to be applicable to different questions in biology.

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Supplementary Information

A. Methods

A.1. Lowry Protein Determination

In order to obtain a BSA standard curve, BSA (Thermo Scientific, 23209) was diluted in ddH_2O in a range of 0-40 µg. The samples from the fly head extraction were thawed and subsequently kept on ice during the procedure. Each of the unknown samples was diluted 200 and 400 times in 1 mL ddH_2O. 100 µL of 0.15% DOC was added to the samples and standards and vortexed and incubated at room temperature for 10 minutes. Subsequently, samples and standards were put on ice and 100 µL of 72% TCA was added to precipitate total protein. Samples were mixed and kept on ice for 10 minutes to allow complete precipitation of protein. This was followed by a centrifugation at 21100 g for 10 minutes at 4°C to recover the precipitate. Supernatant were discarded and a mix of 250 µL of ddH_2O and 750 µL of solution III were added to each tube. All tubes were then vortexed and kept for 30 minutes at room temperature to completely solubilize the recovered proteins. Finally, 75 µL of solution IV was added to each tube. After vortexing and incubating the solutions for 45 minutes at room temperature, absorbance was measured with a spectrophotometer (Amersham Biosciences Ultraspec 2100 pro) at 750 nm. Using the BSA measurements, standard curve was drawn. Subsequently, the protein content was calculated by linear regression analysis and corrected for any dilution factor using Excel (Microsoft). Accurate concentration of the total protein was calculated in µg for per µL.

A.2. Schägger Gel Preparation

Schägger Gel System Recipe for 2 mini gels

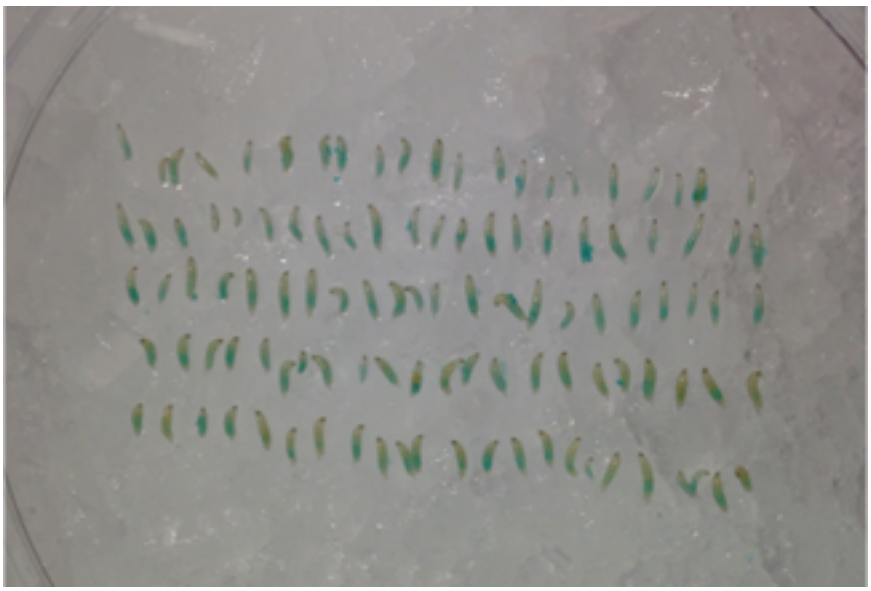
	Resolving Gel	Stacking Gel
30% Acrylamide	3.32 mL	400 µL
Gel Buffer	3.35 mL	750 µL
ddH_2O	1.14 mL	1.85 mL
50% Glycerol	2.12 mL	-
TEMED	6 µL	4 µL
10% APS	50 µL	20 µL

TEMED (Sigma, T9281) and APS were added first to the resolving gel since it starts to get polymerize. Subsequently, resolving gel was poured into casting frame (around 3.5 mL). Pure ethanol was put on the top of the gel to make the surface flatten. After leaving the gel 30 minutes to polymerize, the ethanol was discarded in order to avoid dehydration. After that, APS and TEMED were added to stacking gel. Subsequently, gel mix was poured above the resolving gel and the well comb was placed. Gels were kept 4 °C for overnight

in PBS soaked paper towels in orders to avoid dehydration and ensure complete polymerization.

B. Results

Picture showing larvae fed with blue dye containing food. As larvae were transparent, food intake were observed in larvae's intestines.



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TU-Tagging: a method for studying cell type specific gene expression

Richting: **master in de biomedische wetenschappen-klinische moleculaire wetenschappen**
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