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Investigating Localization and Activity-Dependent Translation of Astrocyte mRNA

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Investigating Localization and Activity-Dependent Translation of Astrocyte mRNA

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Investigating Localization and Activity-Dependent Translation of Astrocyte mRNA

<u>Acknowledgements</u>

Investigating Localization and Activity-Dependent Translation of Astrocyte mRNA

Abstract

Over the past two and a half years, I have studied fundamental aspects of astrocyte biology by investigating the existence and mechanism of astrocyte local translation peripherally around tripartite synapses consisting of pre- and post-synaptic neuron terminals and an associated astrocyte. Astrocytes are critical components of central nervous system synapses (which are predominately tripartite in nature[4]); thus, it is important to consider how astrocyte dysregulation and dysfunction could contribute to the pathogenesis of diseases of synaptic connectivity such as autism spectrum disorders, Alzheimer's disease, seizure disorders, and more. Overall, my projects involved the development of novel methods to identify astrocyte-specific mRNA targets and to visualize and measure local translation.

Chapter 1: Assessing Astrocyte-Specific mRNA Candidates via Fluorescent *In Situ* Hybridization

While it is well understood that synaptically-activated, rapid, local translation of new proteins mediates changes in neuronal plasticity and activity at the synapse[1], it has been unclear whether astrocytes also utilize subcellular translation. Hallmarks of astrocyte function include processing and responding to synaptic changes[2], so I hypothesized that, like neurons, astrocytes also utilize local protein synthesis as a response to synaptic changes. The lab's preliminary data supports this hypothesis by showing that ribosomes and ribosome-bound mRNAs exist in peripheral astrocyte processes (PAPs). In this chapter, I focus on validating the presence of PAP-enriched mRNAs, identified via a novel transcriptome profiling method coined "PAP TRAP" (translating ribosome affinity purification). I validated these transcripts with an independent *in vivo* method, fluorescent *in situ* hybridization (FISH), in which I create mRNA target probes and add them to coronal slices with sparsely labelled individual astrocytes, followed by probe detection and visualization via confocal microscopy. Through these experiments, I observed clear subcellular localization of multiple astrocyte-specific mRNAs at PAPs labelled by GFP.

Chapter 2: Investigating Activity-Dependent Regulation of Astrocyte Local Translation

Having demonstrated that PAP-enriched mRNAs are truly localized peripherally, I questioned what regulates local translation of these mRNAs. I conducted *ex vivo* experiments to test whether peripheral translation responds to treatment-induced synaptic modulation. I visualized protein translation in peripheral processes via puromycylation, a method to visualize newly made peptides. I treated acute mouse brain slices with KCl, brain-derived neurotrophic factor (BDNF), an mGluR5 antagonist (MTEP), an adenylate cyclase activator (forskolin), and a Na^+ channel blocker (tetrodotoxin, TTX) to modulate synaptic activity. Using a novel Sholl-like analysis to quantify puromycylation, I demonstrated that astrocytes modulate peripheral translation in response to treatment-induced synaptic changes.

All in all, this work contributes to ongoing research to better understand how astrocytes contribute to CNS functioning. Furthermore, novel methods developed through this work, including the PAP TRAP + RNA-seq transcriptome profiling technique and measurement of peripheral translation via quantification of puromycylation, can be applied more broadly to future neurobiological research. My thesis demonstrates that astrocytes can independently regulate activity in distinct subcellular regions, a novel finding that provides insight into fundamental astrocytic/synaptic regulation.

Chapter 1: Assessing Astrocyte-Specific mRNA Candidates via Fluorescent In Situ Hybridization

Introduction

Astrocytes can be broadly described as specialized glial cells critical for healthy synaptic transmission through maintenance of ion, fluid, pH, and transmitter homeostasis at the synapse[5]. They are the most numerous cell type in the central nervous system (CNS). Based on their now well-known functional roles, astrocytes require the ability to actively respond to synaptic imbalances to maintain homeostasis; however, the mechanism of astrocyte response is not well defined.

In neurons, subcellular mRNA localization and regulated translation underlie fundamental synaptic functions[6]. Until the end of the twentieth century, most of the field believed that the central dogma (that protein is translated from mRNA transcribed from nuclear DNA) was carried out in a centralized manner in neuronal nuclei and somata. However, a major shift in this paradigm occurred following the discovery that polyribosomes are also found in the bases of dendritic spines[1]. Over the past decade, it has become more clear that neurons localize RNA subcellularly to synaptic structures (signal-receiving dendrites and signal-transmitting axons) and respond to synaptic activity via regulated translation of these mRNAs[6]. This particularly impacts synaptic plasticity, as multiple papers have linked dendritic local translation to, for example, BDNF[7,8] and glutamate-induced[9] synaptic plasticity. Contrary to early hypotheses, dendrites are not the only substructures locally translating protein; it is important to note that axonal local protein synthesis is implicated in axon guidance signal-directed growth[10], regeneration[11], and maintenance[12]. Beyond the CNS, specific subcellular mRNA localization patterns have been broadly identified in mammals[13] and *Drosophila melanogaster*[14], suggesting that the local proteome may be significantly controlled by local mRNA translation rather than energy-demanding, time-intensive protein transport[15].

While a classical view of glia is that they passively maintain neuronal functioning and CNS structure, a growing body of evidence has demonstrated that glia, especially astrocytes, have an active role in information processing and central nervous system functioning[16]. Most neuronal synapses are tripartite in nature, consisting of pre and post-synaptic neuron tips and a dynamically-interacting astrocyte[4]. Interest in astrocytes stems from work in the early 90s when researchers demonstrated that elevated intracellular Ca^{2+} levels in cultured astrocytes are associated with increased astrocyte excitability[17,18]. This work showed for the first time that astrocytes are excitable cells, not just passive supports for other neural cell types. Furthermore, astrocyte Ca^{2+} elevations can also be triggered by neurotransmitter release during synaptic activity[19], providing the first evidence of neuron-astrocyte communication. This synaptic control of astrocyte Ca^{2+} level occurs through astrocytes expression of a wide variety of functional neurotransmitter transporters and receptors[20]. Ca^{2+} rises are associated with increased expression of these receptors[21].

Further evidence suggesting a dynamic and local mechanism of action in the astrocyte periphery is that astrocytes are highly polarized[23]. This polarization reflects the functional and biochemical compartmentalization of astrocytes. The astrocyte Ca^{2+} signal, and downstream responses, are modulated by the simultaneous activity of multiple varied synaptic inputs[22]. A single rodent astrocyte can contact up to 90,000 synapses[24] and contact multiple cell types (astrocyte-astrocyte, astrocyte-neuron, astrocyte-blood vessel)[5], so to be

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able to adapt to an enormous barrage of simultaneous chemical activity, a local response mechanism is necessary. For example, perisynaptic astrocyte processes have a molecular profile mirroring their roles in transmitter uptake[25] whereas astrocyte endfeet at the blood-brain barrier exhibit a much higher K^+ conductance than other parts of the endothelial membrane[26]. Evidence in the literature has demonstrated by *in situ* hybridization (ISH) that astrocyte-specific mRNAs, coding for proteins that transport glutamate and form the cytoskeleton, exist at peripheral astrocyte processes (PAPs)[27–29]. This data, coupled with my own experiments, led to the hypothesis that astrocytes utilize local translation in their distal, perisynaptic processes.

In this chapter, I demonstrate *in vivo* that PAPs contain ribosomes. Prior work in the lab identified astrocyte-specific mRNA candidates via a novel technique that applies translating ribosome affinity purification (TRAP)[30] to a crude synaptoneurosomal (SN) fraction from the cortex of a mouse with tagged astrocyte ribosomes. The lab coupled this technique, named PAP TRAP, with RNA-sequencing to reveal mRNA transcripts bound to ribosomes in PAPs. This result led to hypotheses about how local translation may be regulated: by subcellular RNA localization (explored in Chapter 1), by regulating translation in a localized manner (addressed in Chapter 2), or both (my eventual conclusion). To explore subcellular localization of identified mRNA targets, I utilized RNA *in situ* hybridization.

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Methods

<u>Animals</u>
I used C57BL/6J and Aldh1L1 bacTRAP mice, housed in standard conditions with food and water provided ad libitum. All procedures were performed in accordance with the Washington University Institutional Animal Care and Use Committee (IACUC) and the Animal Studies Committee (ASC).

<u>Peripheral Astrocyte Process Translating Ribosome Affinity Purification (PAP TRAP)</u>
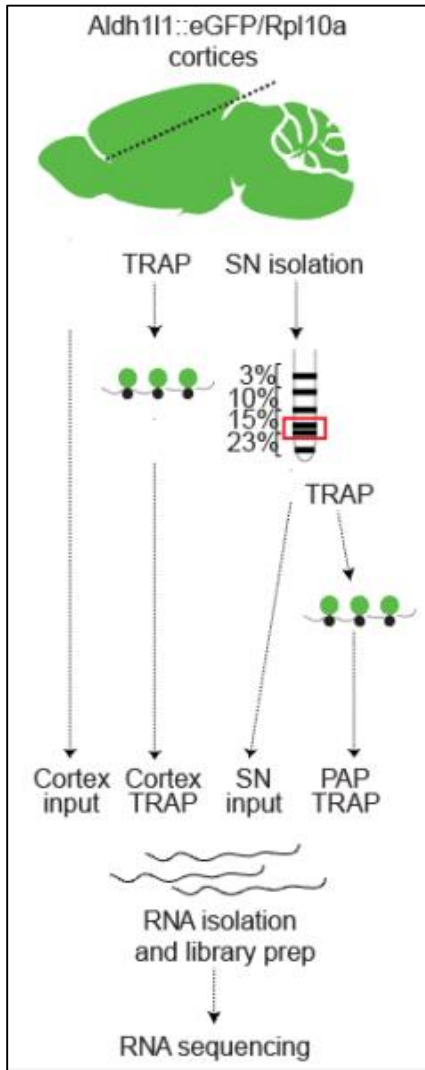
Figure 1: Overview of PAP TRAP + RNA-sequencing methodology used to identify PAP-enriched transcripts. Cartoon depiction of gradient used to isolate synaptoneurosomal (SN) fraction and TRAP methodology. Aldh1L1 promotor drives expression of eGFP/Rpl10a fusion protein, allowing capture of ribosomes by GFP IP. The lab applied TRAP to a PAP SN fraction (bottom right). Adapted from figures created by Kristina Sakers[31].

The lab bred wild-type C57BL/6J mice to mice expressing the TRAP transgene (eGFP/Rpl10a) under an astrocyte-specific promotor (Aldh1L1). This produced GFP+ and GFP- offspring. We removed GFP+ brains from these mice under sterile conditions via decapitation at age P21. To isolate peripheral astrocyte ribosomes and their associated mRNAs, we homogenized the cortices of the dissected brains and separated a synaptoneurosome (SN) fraction via a sucrose percoll gradient, in addition to collecting an overall cortex homogenate from the supernatant. We then performed TRAP by immunoprecipitating GFP-tagged ribosomes from the tissue homogenates; we named this process 'PAP TRAP' when applied to the SN fraction in Aldh1L1:eGFP/Rpl10a mice since it is TRAP specifically applied to peripheral astrocyte processes present at synapses. RNA purification was performed using Qiagen RNEasy MinElute kit following manufacturer's instructions. We performed RNA-sequencing cDNA library preparation from 2ng of RNA using the Nugen Ovation RNAseq System V2 and the NEBNext Ultra DNA Library. We then sequenced the libraries on an Illumina HiSeq 2500.

<u>AAV9-GFP Sparse Astrocyte Labeling</u>
For sparse labeling of astrocytes for microscopy, I injected 2µl of AAV9-CBA-IRES-GFP virus (concentration: 10^{12} vector genome (vg) / mL, obtained from the Hope Center Viral Vectors Core at Washington University) in P2 pups bilaterally in the cortex, 1.5mm from the midline in two regions: 1mm caudal to bregma and 2mm rostral to lambda. I performed injections using a 33g needle (Hamilton #7803-05) with a 50µl Hamilton syringe (#7655-01). At a postnatal age of 3-4 weeks, I performed CO_2 euthanasia and transcardial perfusion with PBS, followed by tissue fixation with 4% paraformaldehyde in PBS and subsequent

decapitation and removal of the brains. I prepared tissue for IF or FISH via cryosectioning and slide mounting as described below.

Fluorescent *In Situ* Hybridization (FISH)
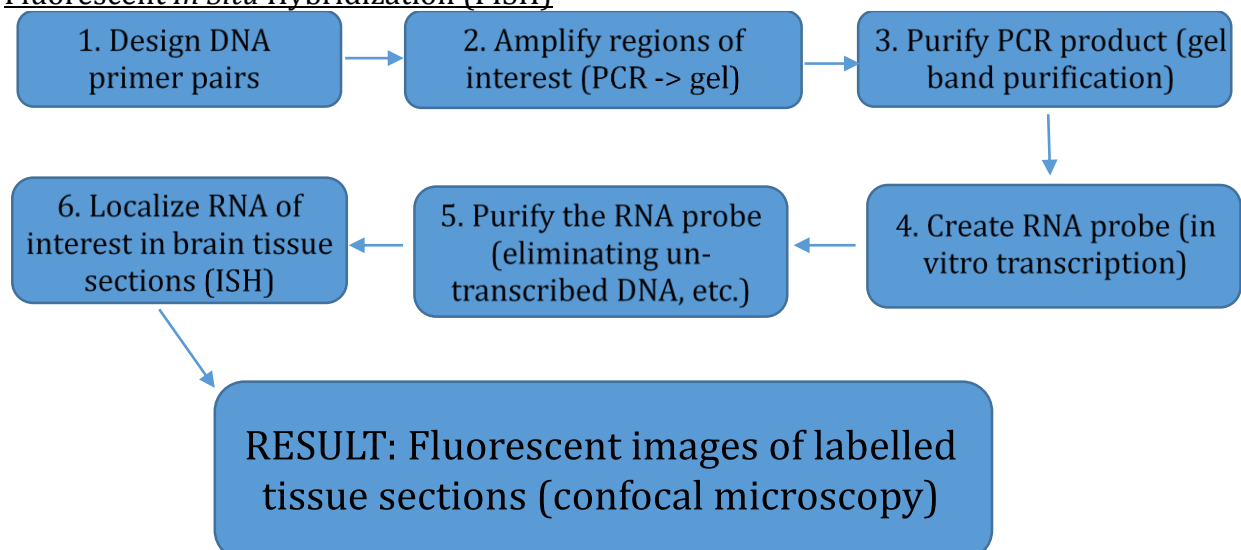
Figure 2: FISH experimental scheme. This is a step-by-step schematic of experimental preparation for detecting RNA probes labeling specific mRNA targets *in situ*.

I performed FISH on 14μm slide-mounted cryosections after 4% paraformaldehyde fixation of either Aldh1L1:eGFP/Rpl10a or AAV9-CBA-IRES-GFP tissue. I utilized Digoxigenin-labeled antisense probes from purified PCR products containing a T7 promoter site in the reverse primer. Using T7 polymerase (Promega #P2075) and Dig RNA labeling Mix (Roche #11277073910), I performed in vitro transcription according to manufacturer's instructions. I designed primers for probe templates as follows:

Gene	Forward Primer (5' -> 3')	Reverse Primer (5' -> 3')
Slc1a2	AAGCGTGTGACCAGATTCGT	TAATACGACTCACTATAGGG TCTCCTCAACACTGCAGTCAGC
Slc1a3	GTTCCCTCCCAATCTGGTAGA	TAATACGACTCACTATAGGGC CTCCAGGGCAATGTACTTGT
Sparc	GCCTGGATTCCGAGCTG	TAATACGACTCACTATAGGGC CTCCAGGGCAATGTACTTGT
Glul	CGGGCCTGCTTGTATGCTG	TAATACGACTCACTATAGGG TCTTCTCCTGGCCGACAGTCC
CamKiiα	AGTCTCCAAGCCAACCCC	TAATACGACTCACTATAGGG CCTGGTGTGCGCTCTAT

Table 1: Primers for FISH probe templates.

I hybridized the probes to the fixed tissue at 65°C overnight in a humid chamber, followed by SSC buffer washes and H_2O_2 blocking at room temperature.

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I detected the probes using Sheep Anti-Dig-POD (Roche #11207733910) followed by Tyramide Signal Amplification Cyanine 3 Tyramide (PerkinElmer #NEL704A001KT) as per manufacturer's instructions. I then incubated the slides with antibodies against additional specific proteins (see IF below) for dual FISH/IF.

Immunofluorescence (IF)
For dual FISH/IF, I incubated sections with Rabbit Anti-GFAP (Dako, 1:1000) and Chicken Anti-GFP (Aves, 1:1000) at room temperature, followed by detection with appropriate Alexa conjugated secondary antibodies (Invitrogen). I counterstained nuclei with DAPI. For IF alone, I cryosectioned 40μm sections into PBS from post-fixed brains frozen in OCT. Sections were stained as described for dual FISH/IF. After removing excess buffer, edges of the coverslip were sealed with nail polish before imaging.

Microscopy
I performed confocal microscopy on an UltraVIEW VoX spinning disk microscope (PerkinElmer) or an AxioImager Z2 (Zeiss). Representative images are from at least triplicate experiments.

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Results

Astrocytes contained ribosomes in their distal tips *in vivo*

Dendritic protein synthesis was founded following the discovery of synapse-associated polyribosome complexes (SPRCs), defined as polyribosomes selectively localized beneath pre- and post-synaptic sites on the dendrites of CNS neurons[1]. Thus, I began my investigation of local translation by testing whether astrocytes, too, ribosomes co-localized with their distal processes (Fig 3A). I used BAC transgenic mice in which, within astrocytes, large ribosomal subunit protein Rpl10a is fused with green fluorescent protein (Aldh1L1:eGFP/Rpl10a) to localize ribosomes *in vivo*[30]. The specificity of this labelling to astrocytes has been confirmed previously via IF co-labeling of common cell-specific markers in Alhd1L1:eGFP/Rpl10a tissue[32]. Small ribosomal subunit proteins are also co-localized with the eGFP/Rpl10a fusion protein throughout the processes (Fig 3B), consistent with my hypothesis that there are distal, intact ribosomes co-localized with the fusion protein in our animal model.
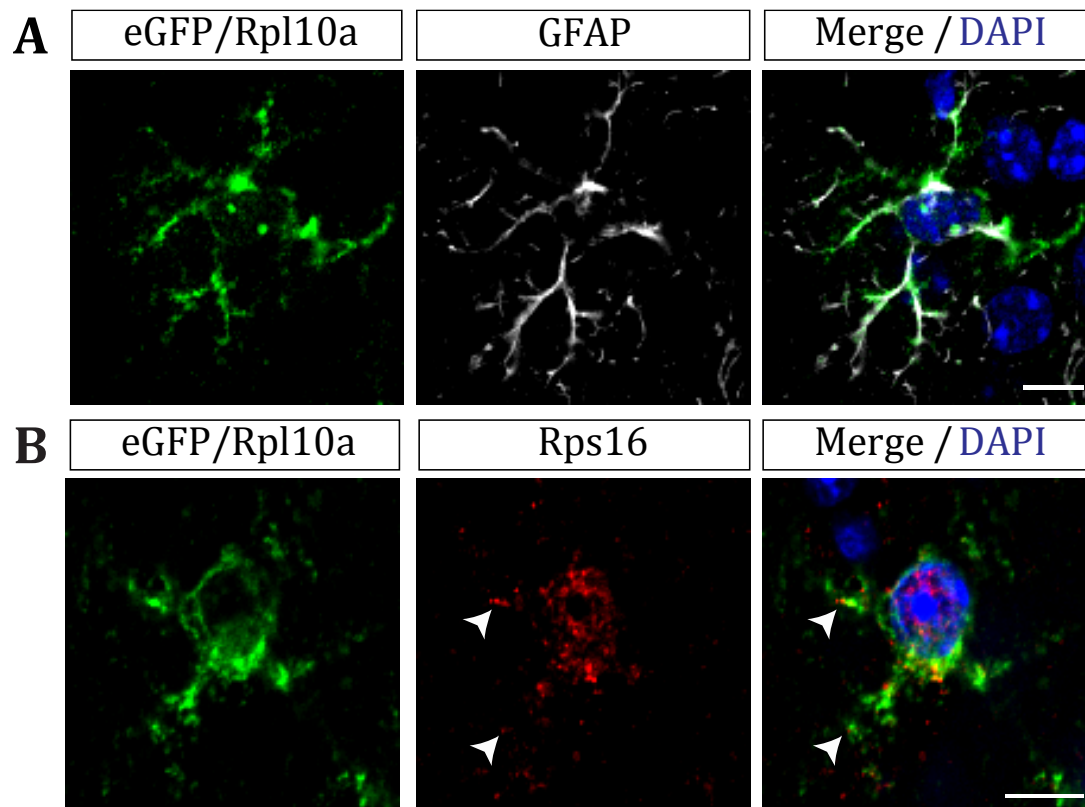
Figure 3: Astrocyte ribosomes extend past GFAP+ astrocyte processes. (A) Confocal IF of an Aldh1L1:eGFP/Rpl10a cortical astrocyte *in vivo*, P21, stained for glial fibrillary acidic protein (GFAP), a marker of astrocyte processes[5]. EGFP/Rpl10a (green) shows ribosomal tags extending throughout and past GFAP+ processes, scale bar = 10μm. **(B)** Confocal IF of an Ald1L1:eGFP/Rpl10a cortical astrocyte *in vivo*, P21, stained for Rps16, a small ribosomal subunit protein. Rps16 overlaps with eGFP/Rpl10a labelling throughout processes, scale bar = 10μm.

This data confirms previous work that showed free polyribosome-like structures existing in PAPs via electron microscopy[33].

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PAP TRAP identified numerous ribosome-bound, astrocyte-specific transcripts

Local translation in neurons is often transcript-specific, meaning proteins with designated synaptic roles are synthesized at pre and post-synaptic tips[34]. Thus, I hypothesized that astrocytes likely have their own subset of specific mRNAs localized to the PAP. To identify these transcripts, Kristina Sakers, a Dougherty Lab member, pulled down translating ribosomes in astrocytes by applying translating ribosome affinity purification (TRAP) to a synaptoneurosome (SN) fraction from the aforementioned Aldh1L1:eGFP/Rpl10a transgenic mice (see Methods, Figure 1 for full procedure). She then performed RNA-sequencing on RNA purified from PAP TRAP samples to identify astrocyte-specific candidates for further investigation by comparing Cortex TRAP and PAP TRAP RNA-seq readouts (see Figure 1). Our analysis identified 224 transcripts enriched on PAP ribosomes and 116 that are depleted ('Soma' transcripts). The full transcript list is submitted for publication[35]. In particular, PAP-enriched transcripts included transcripts encoding proteins for glutamate transport (*Slc1a2, Slc1a3, Glul*), biosynthesis of unsaturated fatty acids (*Scd1, Scd2, Fads1, Fads2, Elovl5, Hadha*), motor/cytoskeletal support (*Kiflc, Myo10*), and intercellular signaling (*Mertk, Sparc, Thbs4*). There were few PAP-enriched transcripts that were also soma-enriched, and transcripts enriched in the soma were depleted in PAPs.

Astrocyte-specific transcripts are localized to peripheral processes *in vivo*
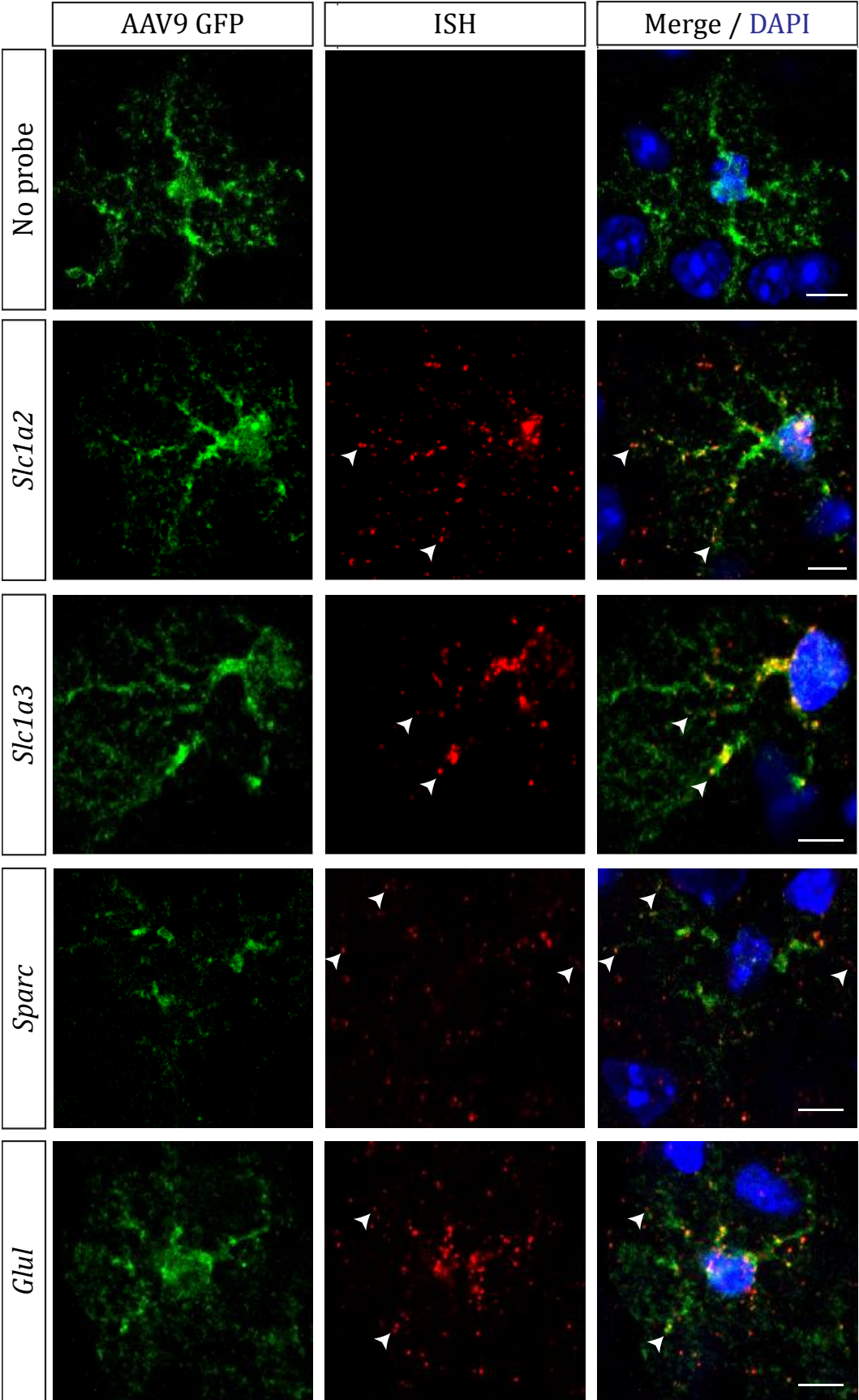
To validate astrocyte-specific candidates predicted by PAP TRAP and RNA-seq, I decided to utilize an independent method *in vivo*, fluorescent *in situ* hybridization (FISH). While PAP TRAP determined the sequence-specific targets enriched on astrocyte ribosomes, PAPs do not have a stable structure of any sort (such as axon terminals, easily identifiable by their synaptic vesicles) that can be used to confirm their presence in SN fractions via electron microscopy or other high-resolution methods. Therefore, utilizing FISH independent of PAP TRAP allows us to verify the predicted targets identified by RNA-seq.

An additional modification in the FISH experiments was the use of an AAV9 virus expressing GFP. This method was utilized to address the issue of densely populated astrocytes all being labelled by the lab's Aldh1L1:eGFP/Rpl10a labelling method. I injected pups at P2 with this virus so that when euthanized and perfused at P21, the mouse brains had sparse, widespread GFP labelling of cortical astrocytes. Sparse labelling allows us to properly quantify individual astrocytes rather than having to worry about inadvertently quantifying IF from neighboring astrocytes.

Using FISH, I successfully confirmed the peripheral localization of several PAP TRAP candidates (*Slc1a2, Slc1a3, Sparc, Glul*) and validated the FISH methodology by testing a no probe control (Figure 4). *Slc1a2, Slc1a3,* and *Glul* were also qualitatively localized to astrocyte cell bodies, whereas *Sparc* was predominately localized along processes.

Figure 4 (below): Sparse astrocyte labeling and fluorescent *in situ* hybridization of astrocyte-specific targets reveal their peripheral localization. Early postnatal pups were injected transcranially with AAV9 expressing GFP, resulting in sparse cortical astrocyte labeling around P21. Confocal IF of a GFP-labelled astrocyte shows extension of *Slc1a2*, *Slc1a3*, *Sparc*, and *Glul* FISH(red) into peripheral processes. Confocal IF of a GFP-labelled astrocyte also shows no FISH signal for no probe control. Scale bar = 10μm.

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Discussion

In this chapter, I showed the subcellular co-localization of the main molecular machinery required for protein translation, ribosomes and mRNAs, in peripheral processes of astrocytes. This evidence represents a novel first step supporting my main hypothesis about the presence of local translation in astrocytes at the tripartite synapse. Furthermore, the astrocyte-specific mRNAs the lab identified via the novel PAP TRAP method encode proteins with interesting functional roles with implications in neural health and disease. I validated the localization of these mRNAs *in vivo*, confirming both the phenomenon of subcellular localization and the biochemical method of localizing particular mRNAs to assist with the aforementioned astrocyte functions. Broadly speaking, the advantages of subcellular RNA localization to an astrocyte could be twofold: *(a)* an energy advantage since multiple proteins can be synthesized from a single mRNA and energy does not need to be expended trafficking protein to/from the synapse, and *(b)* a kinetic advantage since stimulus response can happen on-site rather than following the delay associated with signal transduction to the nucleus and protein trafficking. In these respects, mRNA localization can be thought of as a method for spatial regulation of gene expression.

Specifically, glutamate is the predominant excitatory neurotransmitter in the CNS and is involved in numerous key brain functions including learning and memory. However, excessive synaptic accumulation of glutamate is associated with neuronal death and brain damage[36]. Furthermore, glutamate accumulation and associated excitotoxicity has been implicated in multiple neurological diseases including Alzheimer's disease, Huntington's disease, seizure disorders, and more[36,37]. Astrocytes play a major role in synaptic clearance of glutamate, limiting activation of post-synaptic glutamate receptors via expression of astrocyte-specific glutamate transporters GLT-1 and GLAST[38,39]. These transporters are encoded by *Slc1a2* and *Slc1a3*, respectively. Abnormal *Slc1a2* mRNAs[40] and a selective loss of GLT-1[41] have been identified in the motor cortex of ALS patients, leading to hypotheses that RNA processing dysregulation contributes to the pathophysiology of neurodegenerative disease. Human *Slc1a3* mutations have been associated with episodic ataxia, hemiplegia, and seizures as reported in a 2005 case study[42], and GLAST-deficient mice show optic nerve degeneration similar to that observed in glaucoma[43]. I also visualized peripherally localized *Glul* mRNA, which encodes glutamine synthetase (GS; a.k.a. glutamate-ammonia ligase, or GLUL), a key enzyme also involved in synaptic glutamate metabolism by catalyzing glutamate conversion to glutamine. Astrocyte GS levels increase rapidly after ischemia in response to ischemic glutamate excitotoxicity[44], and GS levels are also significantly decreased in the prefrontal cortex of an Alzheimer's disease mouse model[45]. My results demonstrating localization of *Slc1a2*, *Slc1a3*, and *Glul* mRNA at peripheral processes suggests that aberrant translation of these three major astrocyte-specific glutamate regulators could greatly contribute to glutamate accumulation and associated neurodegeneration.

In addition to functional roles in glutamate clearance, in the past decade, astrocytes have emerged as important regulators of synaptic connectivity[46]. The *Sparc* mRNA I demonstrated as peripherally localized encodes the secreted protein acidic and rich in cysteine (SPARC). This protein allows for astrocytes to dynamically regulate excitatory synaptogenesis by antagonizing hevin, a positive regulator of synapse formation[47]. Based on the

aforementioned functional roles, local translation to produce SPARC could be a molecular mechanism underlying temporal and spatial regulation of synaptic plasticity in the CNS.

All in all, my work investigates a molecular mechanism for previously characterized astrocyte functions. Under the assumption that form implies function, the co-localization of ribosomes and astrocyte-specific mRNAs in astrocyte distal processes suggests that: *(a)* ribosomes may synthesize molecular constituents of the synapse, and *(b)* translation could be regulated by synaptic activity at the localized sites. However, even though the discussed functional roles of the astrocyte-specific mRNAs I targeted seem to corroborate these proposed mechanisms, the presence of translational machinery does not biologically equate to the translation and expression of protein. It is plausible that astrocyte mRNA could be trafficked to or from peripheral processes prior to translation. Thus, it is important that I independently examine local translation itself given that translation has not yet been established as the definitive mechanism of astrocyte peripheral responses. Peripheral ribosome and mRNA localization serves as the groundwork for Chapter 2 of this work, describing the peripheral translation of protein and the effects of synaptic activity modulation on translation levels.

Chapter 2: Investigating Activity-Dependent Regulation of Astrocyte Local Translation

Introduction

A fundamental aspect of astrocyte function in the CNS is sensing and responding to neuronal activity[2,17,19,48,49]. However, as has been discussed, even though the response itself has been defined as calcium influxes or regulation of synaptic coverage (see recent review by Kacerovsky & Murai[50]), the activity-dependent astrocyte response is not well-characterized.

Astrocytes exhibit numerous functional roles that are mediated by neuronal activity. Takano *et al.* demonstrated *in vivo* that astrocytes translate neural activity into vasodilation to assist the brain in increasing blood flow to active brain regions ("functional hyperemia")[51]. Neuronal activity blockades with TTX have been shown to reduce astrocyte perisynaptic density and localization of GLT-1 clusters, while enhanced neuronal activity increased both parameters, suggesting that astrocyte-specific glutamate transport is regulated by neurons throughout development[52]. Furthermore, it has been shown that PAPs dynamically remodel morphologically following neuronal activity[39]. These and the following functional roles suggest the need for a local synaptic response mechanism.

Since not much is known in the field, I set up a screen to investigate the regulation of astrocyte translation using well-studied drugs. To measure translation, I utilized puromycin (PMY), a Tyr-tRNA analog that terminates translation via ribosome-catalyzed covalent incorporation into the C-terminus of the nascent polypeptide chain[3,53,54]. I then detected puromycylated nascent chains via immunofluorescence (IF) of an anti-PMY monoclonal antibody and an Alexa-conjugated secondary. This is a particularly efficacious way to detect translation; rather than detecting translated protein that may have been trafficked post-translation to the periphery, I detect actively-translating peripheral proteins. To block PMY action and confirm that puromycylation is translation-dependent, I utilized anisomycin, an inhibitor of translation that blocks the enzyme allowing amino acids to bond together[53].

I selected the following drug treatments based on their potential regulatory influences on astrocytes, as described in previous studies:

Drug	Treatment concentration	Function
KCl	5mM	Stimulates neuronal activity
Forskolin	20μM	Adenylate cyclase agonist
MTEP	50nM	mGluR5 selective antagonist
BDNF	50ng/μL	Brain-derived neurotrophic factor

Table 2: Proposed translational modifiers and their demonstrated effects on synaptic components.

Potassium chloride (KCl) stimulates neuronal activity by promoting neuronal depolarization. While neurons are robustly activated by KCl, astrocytes do not 'fire' or propagate action potentials along their processes[5] and are only slightly depolarized by KCl[55]. Furthermore, astrocyte activation via increases in intracellular Ca^{2+} is triggered by transmission of signaling molecules from neurons or neighboring astrocytes (see Chapter 1). Thus, I hypothesized that applying a treatment with KCl would increase astrocyte local translation secondary to (and predominately due to) neuronal firing.

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Forskolin is an activator of adenylate cyclase (AC) in membranes and intact cells. In a rapid and reversible manner, forskolin treatment causes a 35-fold elevation of cAMP in rat cerebral cortical slices (and has been shown to have similar effects on astrocytes[56]), demonstrating its efficacy as a method to modulate physiological roles of cAMP *in vivo*[57]. cAMP promotes the normal state of differentiated astrocytes by upregulating genes involved in maturation, communication, and homeostatic control[58]. Furthermore, forskolin induces changes in the synthesis of a few proteins and drastically changes astrocyte morphology *in vitro*[59], including increases in amyloid precursor protein that accumulates into amyloid-β plaques in Alzheimer's disease[60]. Since astrocyte cAMP production is endogenously regulated, by β-adrenergic stimulation at least in part from neurons *in vitro*[63], I posited that I could test neuronal regulation of astrocyte translation by upregulating AC-cAMP astrocyte pathways with forskolin.

Metabotropic glutamate receptor subtype 5 (mGluR5) is an astrocyte-specific glutamate GPCR that is selectively blocked by the mGluR5 antagonist 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP)[64]. Down-regulation of mGluR5, associated with the mouse model of Fragile X Syndrome (*Fmrp*^{-/-}), underlies GLT-1 down-regulation in this model[65]. Thus, I utilized MTEP to test if neuronal regulation of translating proteins like GLT-1 is mGluR5-dependent.

Brain-derived neurotrophic factor (BDNF), a growth factor involved in synaptic plasticity, stimulates local dendritic protein synthesis[7]. In a prior investigation by members of the lab, proposed PAP-enriched genes were subjected to an upstream analysis pathway that identified upstream regulators of those transcripts. The results of this query identified BDNF as a significant regulator (Figure 5). Since astrocytes are involved in the BDNF recycling process[66], I hypothesized that treating with recombinant BDNF (an un-recycled form similar to endogenous mouse neuronal BDNF) would increase translation levels due to BDNF's role as a PAP transcript regulator.

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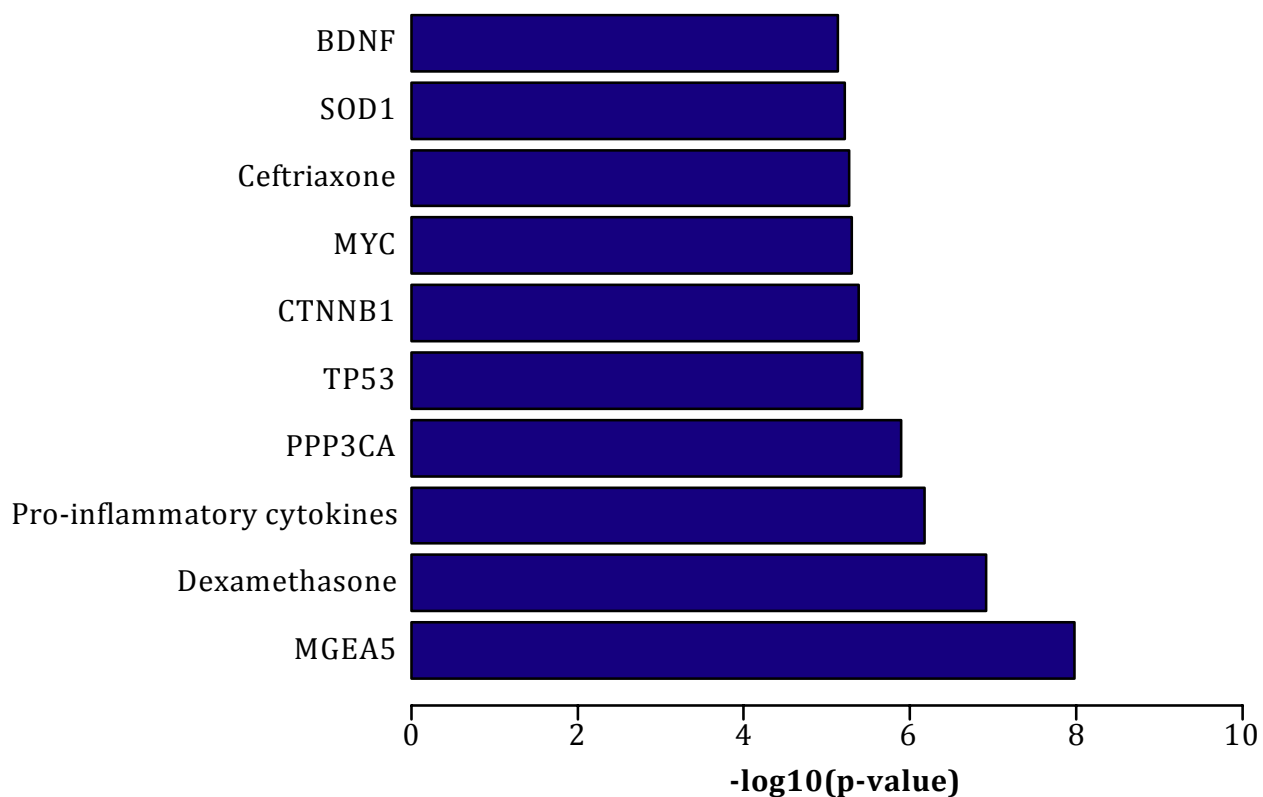
Figure 5: Ten top upstream regulators of PAP-enriched genes. I analyzed astrocyte-specific transcripts enriched on PAP ribosomes (as determined by PAP TRAP + RNA-seq described in Chapter 1). I utilized Ingenuity Pathway Analysis[65] to identify upstsream regulators of these genes, with the top ten most significant results displayed above.

In this chapter, we utilize a new method to quantify the abundance of nascent peptides throughout *ex vivo* astrocytes in acute mouse brain slices labelled with AAV9-GFP. I also confirm that PMY is a viable label of ongoing translation by blocking PMY binding with a competitive inhibitor, anisomycin. I then treat acute slices with various drugs and synaptic activity inducers/suppressers, demonstrating that I can up and downregulate astrocyte translation levels. I use a novel metric of translation by quantifying puromyclation immunofluorescence via a Sholl-like analysis around the nucleus of labelled astrocytes that allows us to identify differences in measured translation based on distance from the nucleus and treatment condition. Overall, I show that astrocyte local translation does indeed appear to be modulated in a synaptic activity-dependent manner.

Chapter 2: Investigating Activity-Dependent Regulation of Astrocyte Local Translation

Methods

Animals
See Chapter 1.

Acute slice preparation and drug treatment
Using a vibratome, I prepared acute cortical slices (300µm) from AAV9-CBA-IRES-GFP (see Chapter 1) P21 mice in artificial cerebrospinal fluid (aCSF, in mM: 125 NaCl, 25 glucose, 25 NaHCO$_3$, 2.5 KCl, 1.25 NaH$_2$PO$_4$, equilibrated with 95% oxygen-5% CO$_2$ plus 0.5 CaCl$_2$, 3 MgCl$_2$; 320 mosmol). 5mM KCl, 20µM forskolin (Sigma #F6886), 50nM MTEP (Sigma #M4699), and/or 50ng/mL recombinant human/murine/rat BDNF (PeproTech, #450-02) were added to slices with 3µM Puromycin (Tocris #40-895-0) in aCSF and allowed to incubate for 10 minutes at 37°C. In Anisomycin conditions only, 1mM Anisomycin (Sigma #A9789) in aCSF was added to slices 30 minutes before Puromycin at 37°C. I fixed slices with 4% paraformaldehyde in PBS for 30 minutes, followed by 30 minutes in 30% sucrose and freezing in OCT for cryosectioning.

Immunofluorescence (IF)
I cryosectioned 40µm sections into PBS from 300µm acute slices frozen in OCT. I incubated sections with Rabbit Anti-GFAP (Dako, 1:1000), Chicken Anti-GFP (Aves, 1:1000), and Mouse Anti-Puromycin (Kerafast, 1:1000) at room temperature, followed by detection with appropriate Alexa conjugated secondary antibodies (Invitrogen). I counterstained nuclei with DAPI. After removing excess buffer, edges of the coverslip were sealed with nail polish before imaging.

Microscopy
I performed confocal microscopy on an AxioImager Z2 (Zeiss). Representative images are from at least triplicate experiments.

Image quantification
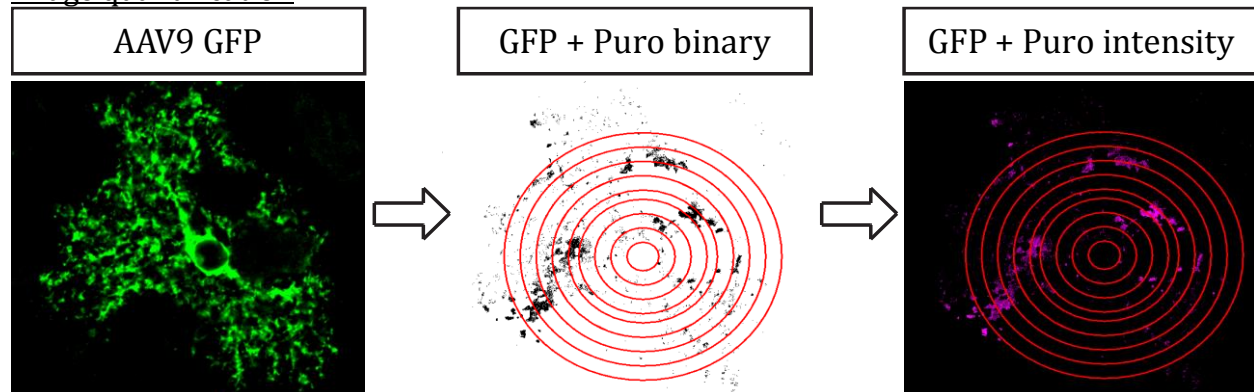
Figure 6: Overview of puromycin quantification methodology used to measure local translation in GFP+ astrocytes. A GFP+ astrocyte is imaged, puromycin IF that is co-localized with GFP IF is selected, and pixel intensity of both GFP and puromycin IF are measured.

To determine the distribution of translation occurring in the astrocyte, I drew Sholl-like concentric rings around the nucleus with increasing radii of 3µm. I empirically determined that a 30µm radius encapsulates most of an astrocyte without overlapping with neighboring astrocytes, which is similar to the literature value of mean recorded rodent astrocyte

diameter (56μm)[66]. I then masked all PMY-IF outside of GFP+ areas and measured the PMY-IF and GFP+ intensity within each ring (see above, adapted from literature[35]).

Statistical analysis

I used R 3.3.2 software for all statistical analyses of quantification data. Most data were analyzed (after log transformation) by repeated measures ANOVA for main effects of condition and distance followed by *post hoc* Wilcoxon rank sum tests for pairwise comparisons. I adjusted p-values using Bonferroni multiple test corrections.

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Results

Astrocytes synthesize proteins at PAPs *ex vivo*

As outlined in Chapter 1, I demonstrated the presence of both ribosomes and mRNA in PAPs, so my logical next step was to test whether this machinery produces protein. I did so using PMY to label actively translating nascent polypeptides in acute brain slices (Figure 7A). Using a PMY antibody for IF in astrocytes labelled sparsely with AAV9-GFP, I was able to detect translation within PAPs. I also demonstrated blockage of translation with anisomycin, indicating that PMY-IF labelling requires active translation (Figure 7B). I quantified the total astrocyte translation from cell body to distal process by measuring PMY-IF intensity at all points co-localized with GFP (Figure 7C). This data revealed that anisomycin significantly blocks PMY labelling.
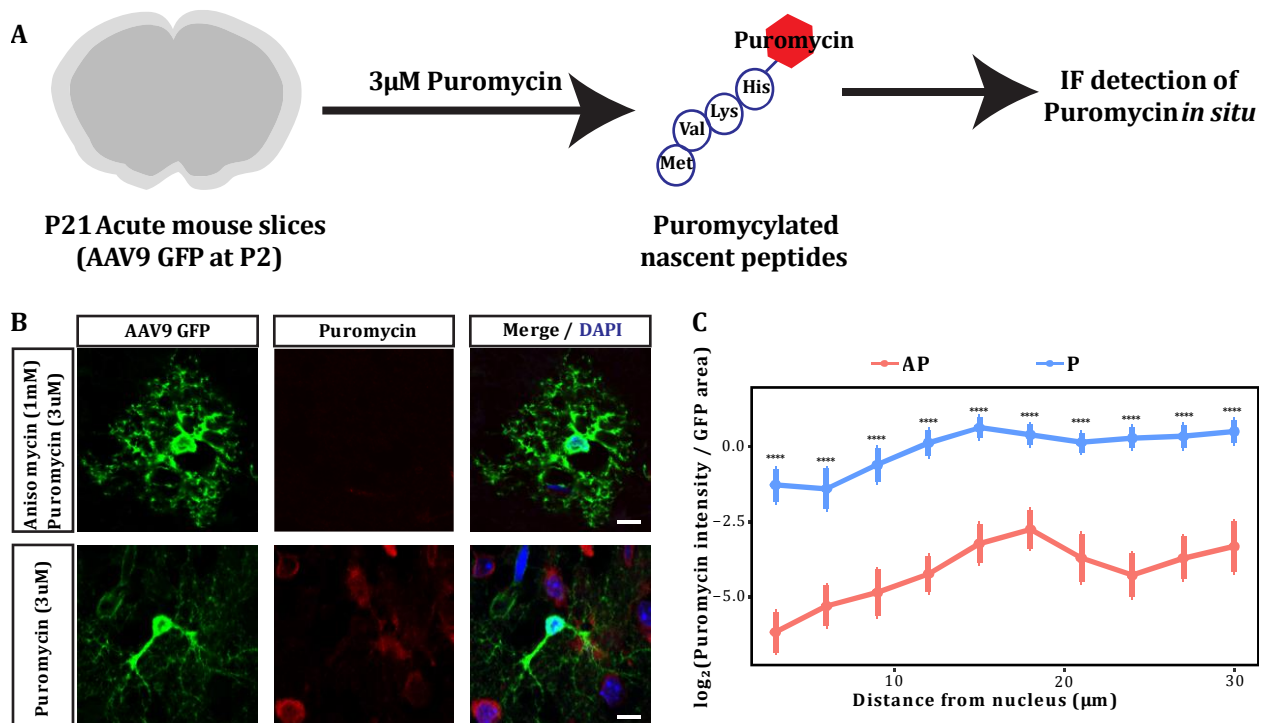
Figure 7: Actively-translating proteins are present in PAPs. (A) Acute slices (300μM) from P21 mice with sparse astrocyte labeling (generated as in Figure 3A) are treated for 10 minutes with puromycin with or without 30 minutes of anisomycin pretreatment and are then fixed, sectioned, and processed for IF against puromycin(red), and GFP(green). **(B)** IF of puromycylation (PMY) of nascent proteins in a GFP-labelled astrocyte shows that translation occurs in peripheral processes, and is blocked by pretreatment with anisomycin, scale bar = 10μm. **(C)** Quantification of percent of GFP pixels co-localized with PMY pixels at increasing distances from nucleus indicates that translation occurs robustly across the astrocyte periphery. No puromycin control had no PMY pixels (data not shown). Repeated measures ANOVA revealed main effects of condition $F(2, 138) = 9.694$, $p = 0.0001$ and distance $F(8, 1104) = 19.023$, $p < 2E-16$ with a significant interaction between condition and distance $F(16, 1104) = 2.019$, $p = 0.01$. Data represented as mean ± SEM. Asterisks represent Wilcoxon rank sums test with Bonferroni correction, *post hoc*. ****$p < 0.001$. N = 40 and 37 cells for PMY and AP conditions, respectively.
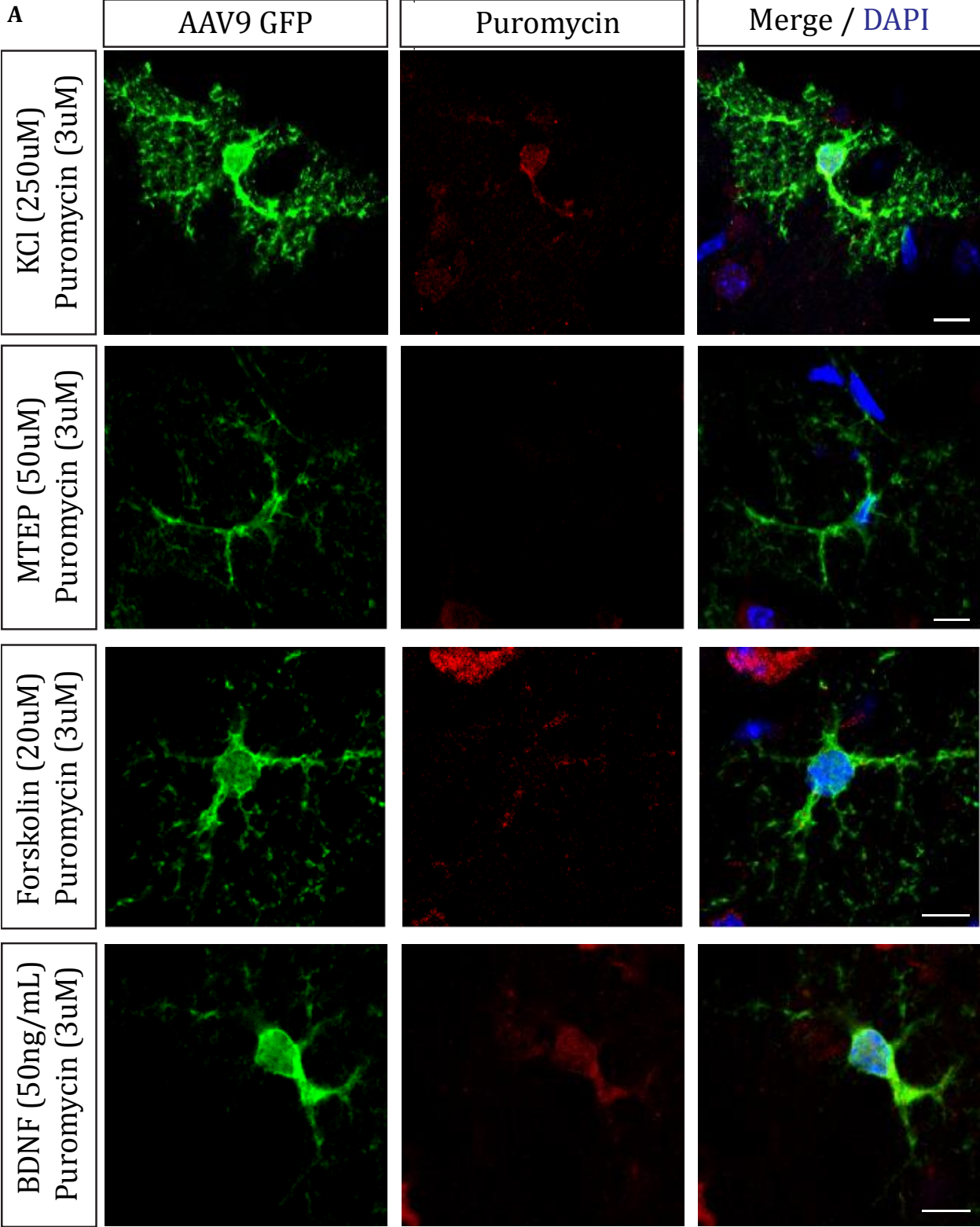
Chapter 2: Investigating Activity-Dependent Regulation of Astrocyte Local Translation

Synaptic activity regulates astrocyte local translation levels *ex vivo*

After detecting PMY and quantifying PMY-IF to measure translation levels across astrocyte processes, I utilized the newly validated methodology to test astrocyte response to synaptic modulation. I treated *ex vivo* astrocytes (labelled with AAV9-GFP) in acute brain slices with the drugs listed in Table 2, and simultaneously with PMY for 10 minutes as described in the methods. Once again, I used a PMY antibody for IF (Figure 8A) and found that treating with BDNF, forskolin, and KCl significantly increased the PMY-IF signal within astrocytes, whereas MTEP and anisomycin decreased the PMY-IF signal (Figure 8B). These data indicate that BDNF, forskolin, and KCl significantly upregulate translation in astrocytes, whereas MTEP and anisomycin significantly downregulate translation.

I also examined the dependence of KCl-stimulated increases in translation on mGluR5. This experiment was designed to test whether I could stimulate an acute slice with KCl, but block or abrogate local translation increases by inhibiting mGluR5 with MTEP (Figure 8C). I pre-treated slices with MTEP for 10 minutes prior to treating with KCl and PMY for 10 minutes as usual. Based on a comparison between PMY-IF signal from KCl treated and KCl + MTEP treated slices, MTEP significantly ($p < 0.001$) blocks KCl-induced local translation increases outside of a 6μm radius around the astrocyte cell body (see Table 3).

A

	AAV9 GFP	Puromycin	Merge / DAPI
KCl (250uM) Puromycin (3uM)			
MTEP (50uM) Puromycin (3uM)			
Forskolin (20uM) Puromycin (3uM)			
BDNF (50ng/mL) Puromycin (3uM)			
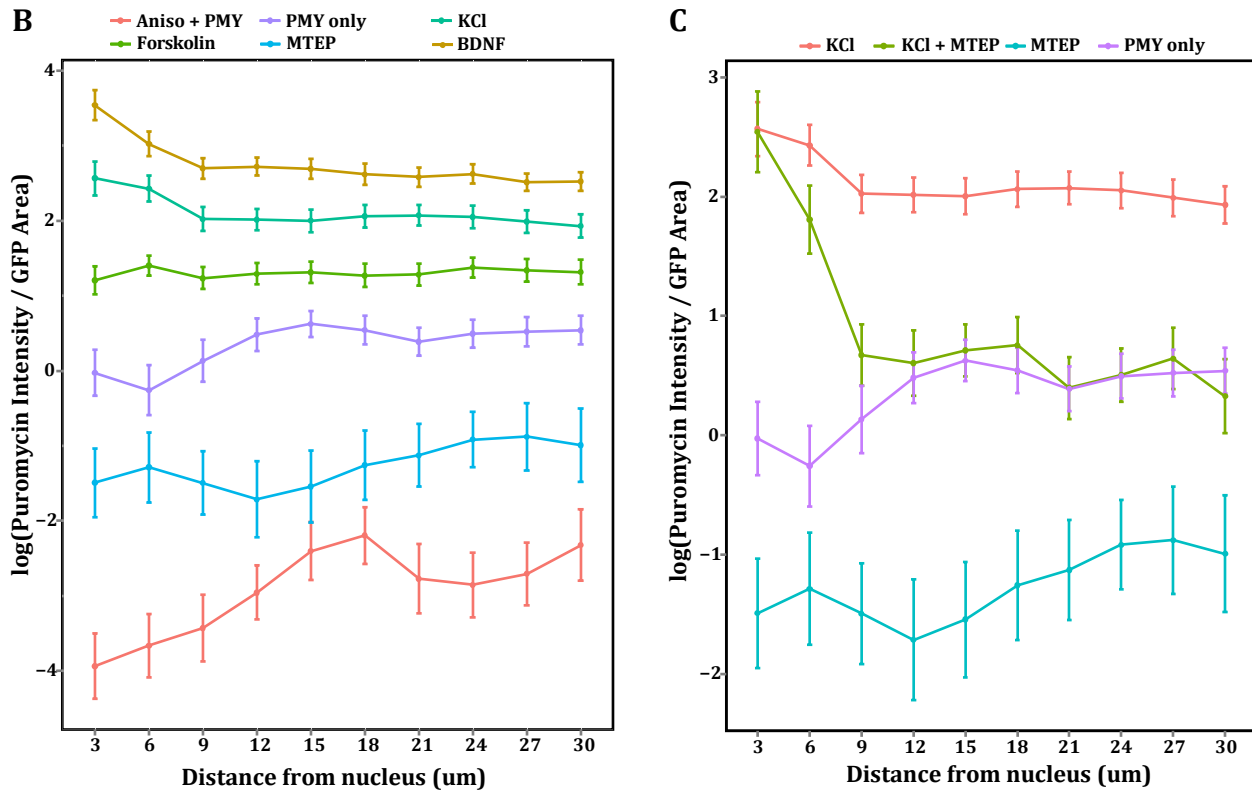
Figure 8: Peripheral astrocyte translation levels change in response to synaptic modulators *in vivo*. Slice/IF preparation as described in Figure 4. Drug treatments are applied with puromycin for 10 minutes. **(A)** IF of puromycylation (PMY) of nascent proteins in a GFP-labelled astrocyte shows that translation is up-regulated by KCl, BDNF, and SR 141716A and down-regulated by MTEP. **(B)** Quantification of percent of GFP pixels co-localized with PMY pixels at increasing distances from nucleus indicates that translation is regulated as observed in Figure 5A. Repeated measures ANOVA across all data revealed main effect of condition $F_{(8, 258)}$ = 40.02, p < 2.2E-16, but not distance $F_{(9, 2322)}$ = 1.055, p = 0.393, with a significant interaction between condition and distance $F_{(8,258)}$ = 5.458, p < 2.2E-16. Data presented are mean ± SEM. For *post hoc* statistics, see Table 3 (below). N(A+PMY) = 37 cells, N(BDNF) = 13, N(Forskolin) = 45, N(KCl) = 44, N(MTEP) = 39, N(PMY only) = 40. **(C)** MTEP abrogates KCl-stimulated increases in local translation throughout the periphery. Acute slices were pre-treated for 10 minutes with MTEP followed by 10 minutes with KCl and PMY. Data presented are mean ± SEM. For *post hoc* statistics, see Table 3 (below). N(KCl) = 44 cells, N(KCl + MTEP) = 10, N(MTEP) = 39, N(PMY only) = 40.

| Distance (μm)	Comparison	BonfP value	Significance level
30	Kcl > P	1.21E-05	****
27	Kcl > P	1.68E-05	****
24	Kcl > P	3.10E-07	****
21	Kcl > P	1.56E-08	****
18	Kcl > P	2.73E-06	****
15	Kcl > P	4.12E-06	****
12	Kcl > P	4.12E-06	****
9	Kcl > P	4.12E-06	****
6	Kcl > P	5.16E-10	****
3	Kcl > P	2.09E-07	****
30	AP < P	5.93E-05	****
27	AP < P	6.00E-10	****
24	AP < P	8.08E-10	****
21	AP < P	1.56E-08	****
18	AP < P	6.55E-08	****
15	AP < P	8.44E-09	****
12	AP < P	5.43E-10	****
9	AP < P	3.67E-08	****
6	AP < P	5.12E-08	****
3	AP < P	1.10E-07	****
18	MTEP < P	0.03901	*
15	MTEP < P	0.00414	***
12	MTEP < P	0.03366	*
30	BDNF > P	1.14E-05	****
27	BDNF > P	1.12E-06	****
24	BDNF > P	9.97E-08	****
21	BDNF > P	3.59E-07	****
18	BDNF > P	5.75E-07	****
15	BDNF > P	1.70E-07	****
12	BDNF > P	9.97E-08	****
9	BDNF > P	9.02E-07	****
6	BDNF > P	5.57E-09	****
3	BDNF > P	8.07E-09	****
30	KCl > KclMTEP	1.98E-09	****
27	KCl > KclMTEP	4.18E-09	****
24	KCl > KclMTEP	3.37E-10	****
21	KCl > KclMTEP	2.06E-11	****
18	KCl > KclMTEP	3.11E-09	****
15	KCl > KclMTEP	3.61E-09	****
12	KCl > KclMTEP	3.11E-09	****
9	KCl > KclMTEP	8.53E-09	****
6	KCl > KclMTEP	2.65E-05	****
3	KCl > KclMTEP	0.00370	***

Table 3: Significant *post hoc* Wilcoxon rank sum test statistics for Figures 8B and 8C. ****$p < 0.001$, ***$p < 0.005$, **$p < 0.01$, *$p < 0.05$. Comparisons/distances not shown did not meet statistical significance criteria.

Chapter 2: Investigating Activity-Dependent Regulation of Astrocyte Local Translation

Discussion

Overall, my data shows that, as hypothesized, astrocytes do have shifts in local translation levels in response to various synaptic modulators. In particular, it appears astrocytic activation via KCl-induced neuronal depolarization is dampened by mGluR5 inhibition, suggesting the possibility of an mGluR5-dependent mechanism of astrocyte activation. This is consistent with literature hypotheses about activity-dependent mGluR5 regulation of glutamate transport via GLT-1[52].
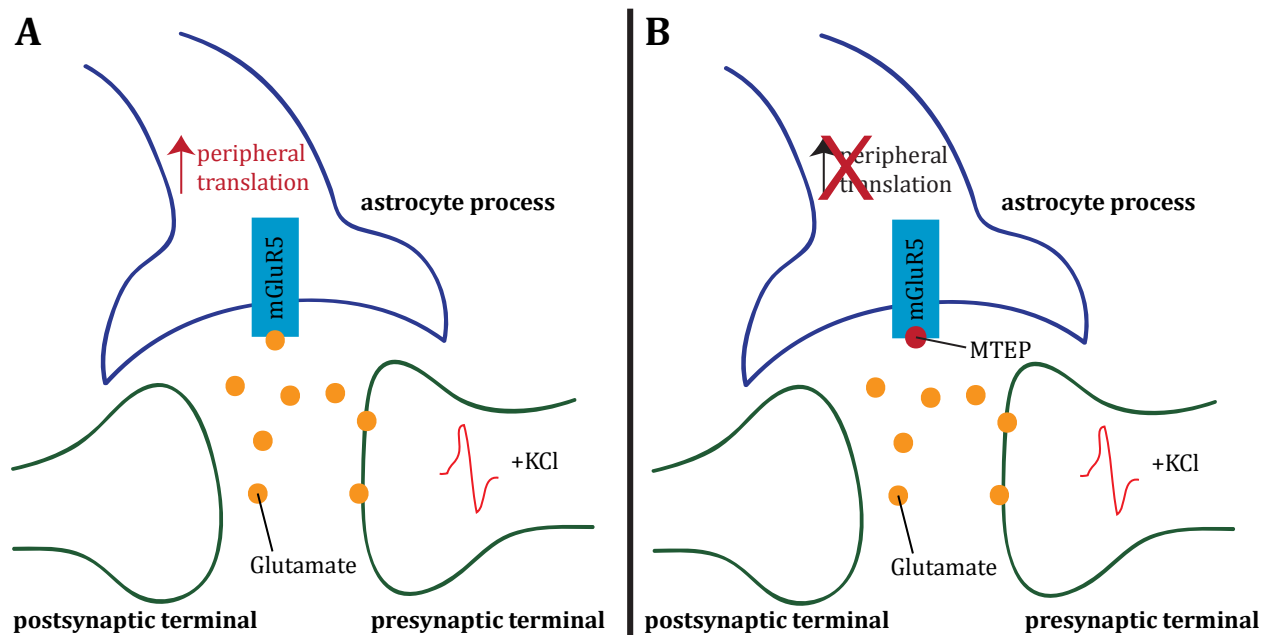
Figure 9: Cartoon of proposed mGluR5-dependent mechanism of astrocyte activation via KCl. (A) KCl induces action potential firing in the presynaptic neuron, leading to glutamate release. Glutamate binds astrocytic mGluR5, resulting in increased local translation at perisynaptic processes. **(B)** MTEP treatment inhibits glutamate binding to mGluR5, blocking local translation increases in astrocyte perisynaptic processes.

While my model (Figure 9) shows that KCl treatment leads to astrocyte stimulation via neuronal depolarization and subsequent neurotransmitter release, it is important to also acknowledge that KCl does stimulate and mildly depolarize astrocytes, leading to Ca^{2+} influx and gliotransmitter release[55]. Furthermore, neuron-astrocyte direct communication is likely not the only mechanism at work given the *ex vivo* model; very recent findings demonstrate that neuroinflammatory microglia induce astrocyte reactivity and cause neurotoxic phenotypes including the induction of neuron and oligodendrocyte death[67]. These findings emphasize the importance of the lab's future experiments to block neuronal activity with tetradotoxin prior to KCl treatment *ex vivo*, and to test cell-autonomous astrocyte functioning in response to treatment with KCl and drugs via an *in vitro* primary cortical astrocyte culture model.

mGluR5 down-regulation occurs in a Fragile X Syndrome (FXS) mouse and underlies astrocytic GLT-1 dysregulation, contributing to enhanced neuronal excitability that is hallmark of the FXS mouse model[65]. Furthermore, mGluR5 dysregulation is implicated in long-term depression (LTD) effects on synaptic plasticity/working memory impairment in FXS[68]. Thus, the fact that MTEP abrogates a KCl-induced, activity-dependent translational

increase suggests that perhaps the dysregulation of mGluR5 relates to neurotoxicity induced by GLT-1 dysregulation in FXS and other models of asotrocyte-related neurotoxicity. Further investigation of this mechanism could elucidate the biochemical mechanism of astrocyte-neuron communication disruption in FXS and other diseases of synaptic dysfunction.

Prior to these activity-dependence studies, I proposed a protein-specific method of assessing local translation through use of the newly developed proximity ligation assay (PLA)[69]. My goal was to have single protein resolution for greater specificity in measuring the proteomic responses characterized in this work. In this method, acute brain slices are incubated briefly with puromycin (PMY) to stop peptide elongation. Anti-PMY and Anti-N-terminal protein of interest antibodies are used and then detected with specific PLA secondaries. These secondaries interact by their conjugated, linked oligonucleotides and allow detection by a complementary fluorescent probe (protocol summarized from Dieck *et al.* 2015)[70]. While this protocol has not yet been successful in our lab, the development of a sequence-specific translational measurement method of this nature would be an optimal way to delve deeper into local translation mechanisms. For example, I could test the hypothesis generated from my results that neuronal activity-dependent activation of astrocytes occurs in an mGluR5-dependent manner, as I hypothesize would be reflected by GLT-1 downregulation when mGluR5 is blocked with MTEP. Furthermore, with the ability to test any individual protein, I could test for local translation of any of the PAP-enriched transcripts the lab identified by PAP TRAP. These studies would establish local translation as an astrocyte response mechanism with specific functional implications.

While my initial data is promising, the lab's future plans include attempting to recapitulate my puromycin immunofluorescence results *in vitro* with cultured primary cortical astrocytes to test whether or not astrocyte local translation is a cell-autonomous function. Furthermore, I am considering experiments to block neuronal action potentials via tetradotoxin (TTX), which has been shown to reduce GLT-1 perisynaptic density *in vivo*[52]. Combining this blockade with KCl, BDNF, or forskolin treatment would be a good way to examine whether astrocyte local translation regulation is indeed due to neuronal synaptic activity rather than cell-autonomous astrocyte functions. Another proposed experiment is utilizing PAP TRAP + RNA-seq to characterize the PAP transcriptome after drug or ion-induced synaptic activity. This would be a high-throughput method and would utilize functional genomics to identify the roles of PAP-enriched mRNAs that are increasingly localized and loaded onto ribosomes under, for example, *in vivo* KCl-induced neuronal depolarization conditions.

All in all, my thesis summarizes data backing up a few hypotheses about astrocyte functional mechanisms. Firstly, as explored in Chapter 1, astrocytes regulate local responses by subcellular RNA localization; mRNAs specific to perisynaptic astrocyte function are co-localized with astrocyte ribosomes in peripheral astrocyte processes. Furthermore, as assessed in Chapter 2, astrocyte translation is regulated locally and modulates in response to changes at the synapse. Local regulation of translation and subcellular localization of mRNAs are two mechanisms by which astrocytes sustain their role maintaining synaptic health and facilitating healthy neurotransmission, and continued exploration of these mechanisms can provide insight into how astrocytes impact diseases of synaptic dysfunction.

Investigating Localization and Activity-Dependent Translation of Astrocyte mRNA

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