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Temporal gene expression during a critical time window following induction of LTP of mouse hippocampal CA3-CA1 synapses

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Temporal gene expression during a critical time window following induction of LTP of mouse hippocampal CA3-CA1 synapses

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by

Patrick Bon-Yung Chen

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ABSTRACT OF THE DISSERTATION

Temporal gene expression during a critical time window following induction of LTP of mouse hippocampal CA3-CA1 synapses

by

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University of California, Los Angeles, 2016

Professor Kelsey C. Martin, Chair

Long-lasting forms of synaptic plasticity—the ability of synaptic connections to change in strength and numbers—are thought to underlie behavioral learning and memory. Long-term potentiation (LTP) is a persistent increase in synaptic strength that relies on new gene transcription and translation during an early, critical time window following LTP induction. Although many genes have been implicated in LTP through single-gene studies, a comprehensive understanding of the molecular changes necessary for LTP persistence remains missing. Furthermore, the temporal component of gene expression within this early time window remains unexplored. To address these questions, I first tested parameters that would affect detectability of differential gene expression following stimulation in mouse acute hippocampal slices. Next, I utilized a cell-type specific and cell-type inclusive whole-genome approach to profile the temporal pattern of gene expression following LTP induction and found that gene

expression within excitatory neurons of CA1 neurons was bidirectional and increased over time. The changes in gene expression were enriched for regulatory features in important regulatory regions, pointing toward coordinated regulation. I also found that gene expression changes occurred in non-neuronal cell-types following LTP induction. Following these conclusions, I then laid out a conceptual framework to guide other researchers in interpreting and presenting whole-genome data. Through this work emerges a clearer perspective of gene expression during LTP and of whole-genome methodologies.

The dissertation of Patrick Bon-Yung Chen is approved.

Thomas J. O'Dell

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2016

This thesis is dedicated to all of my mentors, past and present

Table of Contents

Figure Table of Contents

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1. Chen PB, Kawaguchi R, Blum C, Coppola G, O'Dell TJ, Martin KC. "Mapping Gene Expression in Excitatory Neurons During Hippocampal Late-Phase Long-term Potentiation" *In preparation*
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3. Kim SM, Chen P, Martin KC. "Visualizing mRNA trafficking and local translation within individual neurons" Society for Neuroscience short course, 2010.

Chapter 1

Introduction

"At all levels, the systems of life—from sociopolitical systems to solar systems—are repugnant and should be negated as MALIGNANTLY USELESS."

- Thomas Ligotti, Conspiracy Against the Human Race[1]

The question of human consciousness, and where it comes from, has plagued humanity since its early infancy. Throughout the entirety of our short existence on this planet, philosophers have exhausted their lives debating whether ethereal elements or divine beings are responsible for our species' extreme self-awareness (dualism), or whether consciousness arises from nothing more than interactions within the physical realm (monism). With the refining of formal scientific disciplines (most notably the transition of philosophy of mind into neuroscience) the arguments in favor of dualism were convincingly refuted. In particular, destruction of brain tissue leads to profound changes in personality and memory—the very "soul" of an individual—indicating that the higher-order cognitive faculties unique to our species are nothing more than the specific arrangement of matter within the physical world.

The ability of the human race to create elaborate cultural constructions arising from and dedicated to our brain's capacity to process information is unique to our species. However, every function of our brains is a mere expansion of identical processes occurring within "lower" organisms such as worms and flies. Every day we succumb to conscious and unconscious complex biological impulses that masquerade as choices. Thus, it is important to remember that our species is still just a speck on the evolutionary continuum and that our own false imaginings of self-importance in this world should be tempered by the perspective that human life is no more important than the existence of rocks, sand, or other inanimate objects in the physical world.

Consciousness grants us the misfortune of being acutely aware of our own lack of importance in the universe; the universe will continue to exist quite nicely regardless of the fate of the human species. Most choose to delude themselves into positions of self-importance within the world, despite loud protests about our insignificance from our evolutionary history. The only release from the strings which consciousness dangles us is a separation from the mental constructs that tether us to our deluded self-importance. Things like God, family, and self must be recognized for what they are—a glamorous coat of paint slathered on the outside of a derelict vessel.

Understanding consciousness is a necessary first step in wrestling the reins of our thoughts from a foolishly unaware pilot and returning them to the equitable hands of nature. Although there is much debate as to all the considerations that truly define consciousness, one critical component of any conscious organism is its ability to self-reflect utilizing experiential memory. Thus, for our species' self-edification, a detailed understanding of the biological events underlying memory processes will be critical for deconstructing the mysticism surrounding consciousness.

The initial discovery of brain regions that might be responsible for memory processes came from the human patient H.M. in the 1950s. H.M. suffered from severe seizures that originated in the brain structure known as the hippocampus; this necessitated the bilateral removal of patient H.M.'s hippocampi. After the operation, Scoville and Milner found that H.M. could no longer form new declarative memories and had partial retrograde amnesia for the three years prior to the operation, but could still remember older memories formed before this period[2]. This, along with other patients they observed with extensive hippocampal removal, led them to

conclude that the hippocampal formation of the brain was important for new memory formation. Further studies in other model systems confirmed the importance of the hippocampus for new memory formation[3]. These findings complimented the work from Karl Lashley in the 1930s that found very long-term memories are diffusively localized to the cortex. Lashley had removed increasing amounts of cerebral cortex following a learning paradigm in rats and found a correlation between the area of cortex removed and the ability of the rat to remember, but that the precise region of cortex removal was not important[4]. Taken together, these results indicate that the hippocampus is important for new memory formation while the cortex is important for the long-term storage of long-lasting memories. One inference from this is that the representation of memory within the hippocampus must somehow be transferred into the cortex. How this process occurs, and whether the cellular and physiological representation is similar or distinct between cortical and hippocampal representations, remains unclear.

Although these studies implicated the hippocampus as an important structure for new memory formation, an understanding of the physiological changes within the hippocampus that might underlie memory formation remained unclear. In the 1970s, Bliss and Lømo became the first to demonstrate a long-lasting, activity-dependent increase in synaptic strength in the hippocampus occurring in the perforant pathway of anesthetized rabbits that lasted for hours[5]. This was accomplished by repeatedly stimulating the perforant pathway with a strong train of stimulations in rapid succession (15 stimulations per second for 10 seconds). The long-lasting increase in synaptic strength came to be known as long-term potentiation (LTP), a form of synaptic plasticity. Synaptic plasticity is defined as the ability of synaptic connections to increase or decrease their strength for short (on the order of milliseconds) and long (more than days) periods of time. As a result of the duration of LTP occurring within the hippocampus and the

length of memory retention, it arose as an attractive physiological explanation for behavioral learning with the idea that induction of LTP within hippocampal circuits results in new memory formation. Of note is that following the pioneering work done on synaptic plasticity in the hippocampus, different forms of synaptic plasticity in different circuits (utilizing different mechanisms) within the brain have also been shown to underlie behavioral learning that is dependent on these circuits[6,7].

The idea that memory could be further reduced beyond the level of physiological changes of synaptic plasticity was first convincingly demonstrated by the work from the laboratory of Eric Kandel. Using the model system of *Aplysia californica* to study a relatively simple learning event—gill-withdrawal sensitization following a tail shock[8]—in an intact preparation, the group first demonstrated that the temporal pairing of the firing of specific synaptic connections (which led to changes in synaptic strength) was responsible for the observed behavioral conditioning in the learning paradigm[9]. Following this, they demonstrated that the activity of cAMP signaling cascades and the cAMP response element-binding protein (CREB) transcription factor family was critical for the long-lasting synaptic changes that underlie learning by showing that blocking the activity of CREB-mediated transcription inhibited long (but not short) term plasticity[10]. In parallel, work in hippocampal LTP and hippocampal-dependent memory tasks in other systems demonstrated that new gene transcription and translation were required for the persistence of LTP and memory[3,11]. These results indicated that events at the molecular level were in fact able to explain the long-lasting changes in synaptic strength that underlie important memory processes. Interestingly, although the study of parallel molecular pathways underlying both LTP and memory supported the idea that the two were causally linked, only recently has this causality formally been demonstrated within the amygdala and hippocampus[12,13].

From this work emerged a consensus; new gene transcription and translation are required for the long-lasting (but not short-lasting) forms of both memory and LTP[14]. In particular, studies utilizing inhibitors of transcription and protein synthesis revealed that incubating hippocampal slices with inhibitors[15], or injecting inhibitors into animals following a learning paradigm[16,17], resulted in a loss of persistence of LTP or memory when these processes were inhibited within an early, roughly 2-3 hour early critical period. However, inhibition of transcription or translation at a later time point had no effect on LTP or memory[15–18]. This finding gave rise to the idea that an early, critical window of new gene expression was required for the persistence of LTP and memory. Later work has demonstrated that this dependency on new gene synthesis may not be limited to a strictly defined time window and instead depends upon stimulation strength and frequency[17,18], though the implications of this have not been fully explored.

What are the identities of these genes that are expressed within this critical window following induction of LTP and memory? To address this, many labs have used unbiased whole-genome approaches and candidate gene approaches to screen for genes that are functionally significant. Candidate gene approaches have identified immediate early genes such as Arc, c-Fos, Npas4, and Egr1 as being critical for LTP and memory[19–22]; of note is that many of these genes are transcriptional regulators. However, few genes have been rigorously, mechanistically tested in the context of LTP, with most genes being implicated through a knockout (loss of function) or overexpression (gain of function) experiment. Whole-genome studies have also been carried out (see Chapter 4) to identify novel candidates following LTP induction and to understand the scope of molecular changes following LTP induction, yet very few studies using this approach follow through with functional validation. This is almost certainly because these studies identify a large enough number of differentially-regulated genes where functional

implication would not be feasible. To date, there remains no simple way to transition between the overwhelming quantity of broad genetic information provided by whole-genome studies to the narrow and mechanistic approach of single-gene studies.

Despite all of the studies implicating over a hundred candidates in the persistence of LTP, a unified understanding of the molecular processes underlying LTP persistence still is lacking[23]. This is due (in part) to biological heterogeneity in studying LTP (*ie* different brain regions, different stimulation paradigms, different models systems of LTP), and technical heterogeneity in interpreting studies (*ie* whole-genome studies use different criteria for significance—see Chapter 4). Furthermore, one of the fundamental questions regarding gene expression following LTP induction remains unexplored: few studies have attempted to characterize the temporal pattern of gene expression within this early, critical time window of gene expression for LTP persistence using either candidate or whole-genome studies despite the high probability that gene expression regulation is not static.

The work presented in this thesis explores a fundamental question regarding LTP and memory: what is the temporal gene expression pattern following LTP induction within the hippocampus? First, I established critical parameters to develop an experimental paradigm that was appropriate for unbiased, whole-genome experiments within acute hippocampal slices. Second, I utilized a cell-type specific method of profiling the gene expression changes within excitatory neurons of the hippocampal slices alongside a cell-type inclusive method to identify temporal patterns of differential expression following LTP induction. Finally, I assessed the status of whole-genome research in the field of LTP and memory and provide guidelines for improving the interpretation and accessibility of large datasets for other researchers.

Chapter 2

Establishing the experimental parameters of the acute hippocampal slice preparation for gene expression studies

Introduction

Consistent detection of biological signal over background noise is an important consideration that can affect the sensitivity and the reliability of experimental results. In choosing an appropriate system to investigate the temporal gene expression pattern following induction of LTP, several factors need to be considered in parallel with the biological relevance of the system. The reliability and robustness of gene expression detection is strongly affected by both a highly-variable background expression level of genes between experiments, and a weak signal magnitude.

The acute hippocampal slice preparation is an appropriate system to study the molecular changes associated with long-lasting forms of synaptic plasticity, since this preparation preserves the physiological connections within the hippocampus while also allows induction of synaptic plasticity at a large number of synapses[24]. While this system has been used successfully to study molecular components of neural physiology, many variables[25,26] have been shown to affect the health of the slice preparation. These variables and variables affecting other parameters may therefore affect the detection of changes in gene expression following induction of LTP. The effect size of some of these variables on gene expression could be so large that they would occlude detection of LTP-induced changes. In less extreme cases, the experiment-to-experiment variation due to these uncontrolled variables would diminish the signal of differential gene expression following LTP induction and would prevent discovery of all but the most robust changes in gene expression. For these reasons, elimination of variables that contribute to fluctuations in basal gene expression and utilization of methods that maximize the extent of LTP

induction across all synapses are critical for assessing the program of gene expression that gives rise to long-lasting forms of LTP.

In the studies described in this chapter, I test the effect of different variables on the sensitivity of detecting differential gene expression in acute hippocampal slices following LTP induction. I tested the effect of slice-induced injury and recovery times, electrical and chemical forms of LTP induction, and age on gene expression following LTP induction using different metrics for gene expression and found that all three variables had an appreciable effect. These results demonstrate the need to carefully consider variables in experimental paradigms to maximize signal-to-noise in gene expression assays.

Slice-induced changes in phosphorylation of translational regulation pathways

The preparation of acute hippocampal slices involves physical injury to hippocampal tissue and the cells within. Following removal of the hippocampus, slices are prepared by cutting the hippocampus using a tissue chopper; this results in the severing of axonal and dendritic processes and, with enough time, leads to cell death. This treatment of the tissue has previously been shown to dramatically affect transcriptional upregulation of immediate early genes and receptors, without a corresponding change in protein levels[27]. Between the immediate effects of injury and the eventual death of the slice lies a period when the immediate injury-induced effects recover and cell death has not fully occurred. Thus, I set out to characterize the time-dependency of relevant gene expression signaling pathways following slicing of the hippocampus within an early window post-slicing.

To understand the effects of slicing on important gene expression regulation pathways, I measured the activation of the MAP kinase pathway, which is a key signaling pathway in cell

survival and gene expression[28], following slicing at 30 min, 60 min, and 120 min post-slicing. In conjunction, I also measured the activation of the translation factor eIF4E following slicing at the indicated time points because the planned experiments with whole-genome sequencing utilized a system that measured changes in the ribosome-associated population of RNA. Activation of both the MAP kinase pathway and phosphorylation of translation factors are important for processes related to synaptic plasticity[29], which makes establishing a low baseline for the activity of these pathways critical for maximizing the detectable signal from LTP induction.

Phosphorylation of the MAP kinases ERK1 and ERK2 leads to activation of these signaling pathways; activation of ERK1/2 in turn leads to activation of the translation initiation factor eIF4E through phosphorylation[29]. Immunoblotting with antibodies specific to phosphorylated forms of ERK1/2 (p-ERK1/2) and eIF4E (p-eFI4E) revealed a clear activation of both proteins (Fig 1a,b). ERK1/2 was most activated at 30 min, followed by a decrease at 60 min and 120 min. Although at 120 min post-slicing the activation was lower than at earlier time points post-slicing, it did not return to baseline. All three samples tested showed this pattern. A similar temporal trend was seen when immunoblotting for phospho-eIF4E in these same three samples, though there was greater variability in the time point at which peak activation occurred. Of the three samples tested, two showed peak activation at 60 min while one showed peak activation at 30 min. Nevertheless, all three replicates showed a return close to baseline levels of p-eIF4E activation at 120 min. One notable difference is that unlike p-ERK1/2 levels, p-eIF4E levels did approximate unsliced levels at this later time point. Taken together, these results confirmed slice-induced injury effects and revealed the time course for recovery.

Differential expression of immediate-early genes following different LTP stimulation paradigms

Widely-accepted induction protocols for LTP in the Schaffer collateral pathway connecting CA3 to CA1 of the hippocampus include both electrical stimulation patterns as well as chemical induction protocols[30,31]. However, different induction protocols may lead to different percentages of synapses being potentiated, which in turn would directly affect the detection sensitivity of differential gene expression. To understand whether an electrical or chemical induction protocol would lead to the greatest detectable increase in gene expression, I tested the upregulation of immediate early gene transcripts following LTP induction using an electrical LTP induction protocol consisting of 2x100 Hz stimulation and using a chemical LTP induction protocol (cLTP) involving elevation of cAMP followed by increased extracellular K^+ and Ca^{2+} in 0 Mg^{2+} artificial cerebral spinal fluid (ACSF).

To further increase the specificity of detecting gene expression within the relevant circuit, a number of different approaches were used. CA1 mini-slices, in which both CA3 and DG regions of the hippocampal slice were microdissected prior to resting, were used for electrical stimulation experiments to maximize detection of activity-dependent changes in gene expression by removing the contributions to RNA transcript levels from unpotentiated CA3 and DG cells. In contrast, cLTP was carried out on CA3/CA1 mini-slices, where only DG had been microdissected, because the selected cLTP protocol requires activity from CA3 neurons for LTP induction[32]. In conjunction with assaying differential gene expression from total RNA of hippocampal slices, I utilized a transgenic mouse system that allowed for purification of

ribosome-associated RNA from excitatory neurons (TRAP RNA)[33,34], which minimizes the influence of gene expression from multiple cell-types.

At 60 min post-LTP induction, quantitative reverse-transcription PCR (qPCR) of *Arc* and *c-Fos* transcripts demonstrated that the cLTP induction protocol resulted in a much larger increase in differential expression than the electrical induction protocol for both TRAP and total RNA populations (Fig 2a,c,d). From the electrical stimulation results in Fig 2a, I detected virtually no differential expression of *Arc* and *c-Fos* in the TRAP RNA and small increases in the total RNA. The long 3' UTR isoform of the *Bdnf* transcript did show a slight increase with electrical stimulation in both TRAP and total RNA populations.

Given the lack of detectible differential expression through electrical stimulation and the detection of upregulation of candidate genes at 60 min post-cLTP induction, I decided to focus on understanding the earliest time point when differential expression of *Arc* and *c-Fos* was possible following cLTP induction. I tested for differential expression of activity-dependent transcripts in the TRAP RNA population at a 15 min time point following cLTP induction and detected no differential expression at this time point (Fig 2b). However, at 30 min post-cLTP induction, I was able to detect changes in *Arc* and *c-Fos* in both the TRAP and total RNA populations (Fig 2c,d), indicating that 30 min was the earliest time point to consider collecting samples for whole-genome sequencing. Following this, I tested *Arc* and *c-Fos* differential expression at 120 min post-cLTP induction to confirm that differential expression was still detectable at this later time point. I found that, indeed, *Arc* and *c-Fos* levels were upregulated in both TRAP and total RNA populations at 120 min (Fig 2c,d), indicating this would also be suitable for whole-genome sequencing. Some of the samples shown here were used as samples

for TRAP and RNA sequencing in the following chapter. These results indicated that the chemical LTP induction protocol generated a much greater magnitude of differential expression, and that differential expression was detectable 30 min but not 15 min post-LTP induction.

Age-dependent effects on LTP-induced gene expression

Age is a critical variable that influences the robustness of both memory and plasticity, with hippocampal slices from older mice experiencing significantly reduced magnitudes of LTP[35]. This suggested that the effect of aging on gene expression regulation in an unstimulated state could in turn influence the degree of differential expression that follows LTP-inducing stimulation. Because of this age-dependence, I wanted to identify the age ranges that were appropriate for obtaining a robust upregulation of gene expression.

To address this, I tested the induction of *Arc* and *c-Fos* in hippocampal slices following cLTP stimulation from mice that were 8-12 weeks old and 10.5-12 months old. I found that induction of TRAP RNA *Arc* and *c-Fos* expression in younger animals was significantly greater than in older animals (Fig 3). This finding indicated that controlling for age—specifically, utilizing animals that were younger—was important for maximizing the differential expression magnitude of gene expression.

Discussion

Maximizing the signal of differential gene expression while minimizing background noise and variability is required for the accurate and reproducible detection of stimulus-induced changes in gene expression. In the context of whole-genome approaches, including RNA sequencing, the sensitivity of detection is critical for identifying subtle changes that may be

masked by a noisy, fluctuating background. The goal of these experiments was to minimize the variability between experimental replicates and to allow for a better-controlled experimental paradigm for studying the molecular changes following induction of LTP. Toward this end, I tested the effect of slice-induced injury recovery times, LTP induction protocols, and age on gene expression sensitivity in the context of LTP induction within acute hippocampal slices.

From studying the effects of slice-induced injury on phosphorylation of translation signaling pathways, I determined that a two-hour period of rest was optimal for the planned downstream experiments using RNA-seq. One limitation of these results is that p-ERK1/2 and p-eIF4E levels at later time points were not examined; allowing the slices more time to recover may allow p-ERK1/2 levels to fall even lower. However, given the challenges in maintaining slice health over extended periods of time (since in addition to the recovery period, I wanted to monitor gene expression at 120 min after LTP induction), I reasoned that 120 min post-slicing was sufficient recovery time to differentiate between injury-induced and LTP-induced changes in gene expression. Importantly, the phosphorylation levels of eIF4E roughly returned to baseline, which would arguably be the most important observation for the planned downstream experiments examining gene expression changes at the ribosome-associated level from RiboTag mice.

Despite the striking observation that total RNA levels of transcripts such as *Egr1*, *c-Fos*, and *GluA1* become and remain highly upregulated following slice-induced injury for hours (with no corresponding change in protein level)[27], I was still able to detect upregulation of these transcripts by qPCR in the total RNA population following LTP induction. This suggests that the capacity for transcriptional regulation did not reach a ceiling from slice-induced injury. This

raises questions about the biological effect of transcriptional upregulation following LTP induction; given that there is a > 20-fold increase in *c-Fos* following slicing even after 2 hours of rest, and there is a ~4-fold increase above that following LTP induction, this would suggest that there is an 80-fold upregulation of *c-Fos* in an LTP-stimulated slice compared to unstimulated, uncut hippocampus. It would be interesting to test whether different recovery times, which would result in different levels of slicing-induced transcriptional upregulation, affected the degree of upregulation of immediate early genes following LTP induction.

In the experiments testing the effect of electrical induction of LTP on gene expression, excitatory post-synaptic potentials (EPSPs) were only measured for 15 minutes post-stimulation for each mini-slice and served as a proxy for measuring persistent LTP. EPSP measurements for the full 60 minutes post-induction would have been experimentally intractable, as each experiment required ~15 slices.

Even despite the fact that the mini-slices for electrical stimulation only contained CA1—the site of LTP induction—while the cLTP mini-slices contained both CA3 and CA1, I detected a higher magnitude of differential expression by cLTP. This difference in magnitude detection is likely due to two factors. 1) Unlike cLTP induction where every synapse is potentiated, the electrical stimulation is limited by the dimensions of the stimulating electrode; the electrode I used may not have been able to stimulate every single Schaffer collateral fiber. 2) Despite the shared molecular pathways from cLTP and electrical LTP, the activation of signaling pathways may be greater by cLTP than by electrical stimulation, resulting in more robust changes. Further experiments examining the upregulation of activity markers in individual cells and counting the

fraction of activated cells over non-activated cells following these two LTP protocols would begin to separate the contribution from these two factors.

Age-related decline in hippocampal memory and plasticity is a well-established observation[36]. However, the ages tested in these experiments are younger than the age of animals where most age-related effects have been observed. The most striking effects at the level of both LTP induction and memory have been shown to occur starting at 1.5-2 years of age or older[37,38], whereas these experiments demonstrated a significantly decreased magnitude of gene expression of immediate early genes at even 10.5 months of age. Interestingly, previous studies have shown that the receptors and ion channels underlying LTP change with age. One of the basic requirements for LTP in younger animals is signaling through activation of NMDA receptors[39], but older animals can display normal levels of LTP induction through standard induction protocols despite requiring minimal NMDA receptor activation[40]. Instead, voltage-gated Ca2+ channels are upregulated in older animals[41] and have a larger role in LTP of aged mice[40]. These changes in the requirements for LTP likely underlie the differences I observed at a gene expression level between younger and older mice. It remains unclear whether the cLTP protocol used in these experiments would elicit the same degree of LTP as in younger animals (which could explain differences in differential gene expression magnitude), or whether LTP is faithfully induced despite the smaller changes in gene expression.

Concluding remarks

The variables tested here represent only a small fraction of the possible variables that affect gene expression following LTP in acute hippocampal slices. These variables were selected based on the assumption that they may have a significant impact on differential gene expression.

In theory, it would be possible to further test the effects of hundreds of other variables on gene expression during LTP induction in acute hippocampal slices and strenuously control for all of the ones that have an effect. Results from such a tightly-controlled system would be highly-reproducible and contain high signal-to-noise, but would also risk becoming biologically-meaningless. In practice, few studies are ever at risk for having too many controlled variables, or for having too detectable of a signal. However, in principle, this serves as a reminder that reproducibility and signal-to-noise are but some considerations—albeit important considerations—in designing experiments and interpreting scientific results.

Materials and Methods

Generation of RiboTag x Camk2a-Cre mice

Both RiboTag and T29/Camk2α-Cre mice were purchased from Jackson Laboratories (RRID:IMSR_JAX:011029, RRID:IMSR_JAX:005359). 8-12 week old Ribotag x Camk2α-Cre double heterozygotes were used to prepare hippocampal mini-slices. In the experiments with older-aged animals, 10.5-12 month old animals were used.

CA1 and CA3/CA1 mini-slice preparation

400 μm thick hippocampal CA3/CA1 mini-slices were prepared from hippocampi of RT x Camk2α-cre double heterozygotes. The DG was microdissected following slicing for CA3/CA1 mini-slices while both CA3 and DG were microdissected for CA1 mini-slices. Mini-slices were allowed to recover for 2 hours at 30°C in interface-type chambers with oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (ACSF) containing 124 mM NaCl, 4 mM KCl, 25 mM $NaHCO_3$, 1 mM NaH_2PO_4, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM glucose.

For slice-induced injury time point experiments, dissected but unsliced hippocampus was snap frozen in dry ice, while slices were allowed to recover on an interface chamber for 30 min, 60 min, or 120 min following slicing. Slices were then snap frozen and protein was purified using RIPA buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris) supplemented with protease and phosphatase inhibitors (Roche cOmplete protease inhibitor tablet and phosSTOP tablets).

Immunoblotting of ERK1/2 and eIF4E following slicing

Protein levels for each sample was quantitated using a BCA assay (BioRad), and ~20 μg was loaded onto SDS/PAGE gels containing 4% and 12% acrylamide for stacking and resolving gels respectively. Proteins were transferred to 0.2 μm nitrocellulose membranes using standard procedures. Membranes were blocked using Odyssey blocking buffer (LI-COR) for 1 hour and then incubated overnight in blocking buffer + antibody at 4^{o}C. Tris-buffered saline containing 0.05% Tween-20 (TBST) was used to wash antibody incubation for 3x 5 minutes. Fluorescent secondary antibody was incubated for 2-4 hours at room temperature, followed by another 3x 5 minute TBST wash. Immunoblots were imaged using an Odyssey scanner (LI-COR) and images were analyzed using Image Studio Software (LI-COR). Fluorescence intensities were first normalized against Tuj1 intensity for each sample before comparing between slice time-point and unsliced.

Primary antibodies used were: Rabbit anti ERK1/2 (1:1000, CST #9102), Mouse anti p-ERK1/2 (1:1000, CST # 9106S), Rabbit anti eIF4E (1:1000, CST # 9742S), Rabbit anti p-eIF4E (1:1000, CST # 9741), Mouse anti Tuj1 primary antibody (1:2000, MMS-435P). Secondary antibodies

used were from LI-COR: IRDye 800CW Goat anti Mouse (LI-COR #926-32210), IRDye 680LT Goat anti Rabbit (LI-COR #926-68021).

Electrical and chemical LTP induction protocols

For electrical LTP induction, mini-slices were allowed to recover for 2 hours following slicing. Field excitatory postsynaptic potentials (fEPSPs) were evoked by stimulating Schaffer Collateral fibers and recorded in stratum radiatum of the CA1 region using ACSF-filled glass microelectrodes. The maximum fEPSP was first calculated, with all subsequent stimulation at half-max (usually ~5 mV). A baseline of 5-10 minutes was first established before stimulating with 2x100 Hz (2 trains of 100 Hz stimulation for 1 second, 10 seconds apart) to induce LTP. fEPSPs were recorded for 15 minutes following stimulation to ensure potentiation. pClamp 10 (Molecular Devices) was used to analyze the recordings. Slices were then frozen at 60 min post-induction for RNA purification.

For chemical LTP induction, mini-slices were submerged in 50 µM forskolin (in normal ACSF containing 0.2% DMSO from addition of forskolin) for 5 minutes, followed by 30 mM KCl/10 mM Ca^{2+}/50 µM forskolin in 0 Mg^{2+} ACSF for 5 minutes. Control solution contained 0.2% DMSO in normal ACSF for 10 minutes. Slices were perfused with normal ACSF after LTP induction, with time 0 starting immediately after applying the K^{+}/Ca^{2+}/forskolin solution. Slices were snap frozen in crushed dry ice at the specified time points, and stored at -80°C until RNA purification.

Immunoprecipitation (IP) and RNA purification of ribosome-loaded/total RNA populations

Frozen slices were homogenized using a pestle in IP buffer which contained 50 mM Tris-HCl pH 7.4, 100 mM KCl, 12 mM $MgCl_2$, 1% NP-40, 1 mM DTT, 200 U/mL Protector RNAse inhibitor (Roche), 1 mg/mL heparin, 100 µg/mL cycloheximide, cOmplete protease inhibitor tablet (Roche). Pre-conjugated HA beads (EZview Red Anti-HA Affinity Gel, Sigma, RRID:AB_10109562) were washed twice with IP buffer before use. After homogenization, the homogenate was centrifuged twice at 10,000x g at 4°C. 1/10 of the homogenate at this step was immediately placed into Trizol (Invitrogen) for total RNA extraction. The remaining homogenate was incubated with washed HA beads overnight at 4°C. The following day the beads were washed 3x in a high salt buffer composed of 50 mM Tris, 300 mM KCl, 12 mM $MgCl_2$, 1% NP-40, 1 mM DTT, and 100 µg/mL cycloheximide. RNA was eluted from the beads using 350 µL Buffer RLT + BME supplementation from the RNeasy Micro kit (Qiagen). Eluted IP RNA was column purified according to the kit protocol. Total RNA was purified through a combined Trizol and RNeasy kit protocol: after the spin gradient step in the Trizol purification, the aqueous layer was taken and mixed 1:1 with 100% EtOH and purified on a spin column. RNA quantification was done with the Qubit RNA HS assay (Thermo Scientific).

Quantitative PCR for immediate early-gene quantitation following LTP induction

SuperScript III with random hexamer primers (Invitrogen) was used to reverse-transcribe 20-50 ng of TRAP or total RNA for basal and LTP samples. Technical triplicate reactions for each primer set using SYBR Green PCR Master Mix (Applied Biosystems) were prepared, with the cDNA being evenly divided into each reaction. Normalization of LTP and basal Ct values against Hprt1 was done before comparing fold-change of activity-dependent transcripts through relative Ct values. Basal/LTP samples where Hprt1 values were off by more than 0.75 Ct were

not considered. Some primer sequences were obtained from PrimerBank (http://pga.mgh.harvard.edu/primerbank) while others were designed to span exon-exon junctions.

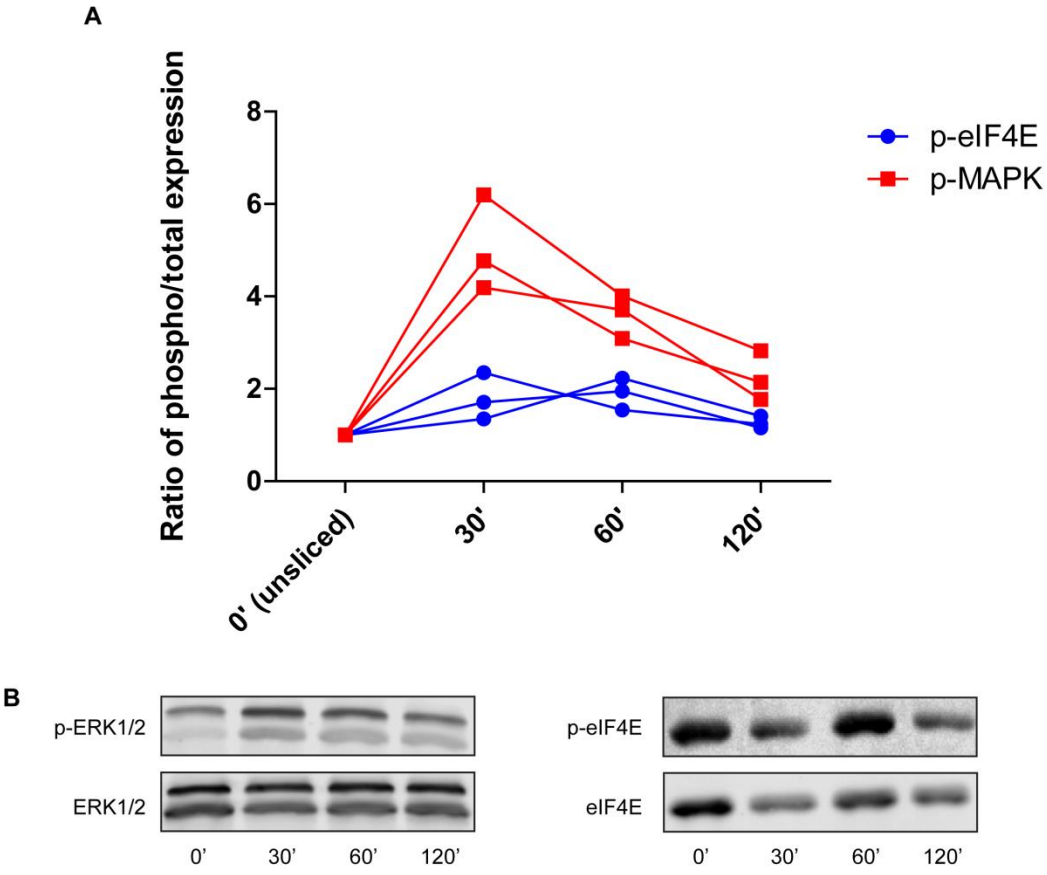
Figure legends

Figure 2-1. Time course of slice-induced injury and recovery measured through phosphorylation of MAPK and eIF4E. a) Phosphorylated levels of MAPK and eIF4E over time for three samples. Total signal intensity of MAPK and eIF4E levels were first normalized to TUJ1 levels for each sample/lane. Ratios of phosphorylated MAPK and eIF4E over total levels of MAPK and eIF4E were then calculated. Ratios at each time point were then normalized to the unsliced 0 minute time point. b) Representative immunoblots of p-MAPK and total MAPK (left), and p-eIF4E and total eIF4E (right).

Figure 2-2. Electrical and chemical LTP induction paradigms induce different magnitudes of upregulation of immediate-early genes. a) TRAP and total RNA changes at 60 minutes following 2x100 Hz stimulation of CA1 mini-slices. qPCR was carried out for transcripts at 60 min post-LTP and at 60 min in a time-matched DMSO-treated control; LTP levels were normalized to the basal control. b) TRAP RNA changes at 15 minutes following chemical LTP stimulation of CA3/CA1 mini-slices. c) TRAP RNA changes at 30, 60, and 120 minutes following chemical LTP stimulation of CA3/CA1 mini-slices. d) Total RNA changes at 30, 60, and 120 minutes following chemical LTP stimulation of CA3/CA1 mini-slices. All samples in a-d) were first normalized to *Hprt1*, an activity-independent transcript, before comparisons of basal and LTP. Error bars = 95% CI

Figure 2-3. *Arc* and *c-Fos* upregulation magnitude is significantly smaller in older mice at 60 minutes following cLTP induction. CA3/CA1 mini-slices from young (8-12 week) and old (10.5-12 month) mice were treated with cLTP and TRAP RNA was purified at 60 min post-LTP induction. Fold-change was calculated between *Arc* and *c-Fos* of LTP and basal time-matched controls, with normalization to *Hprt1* first. Error bars = 95% CI. * = $p < 0.05$

Figure 2-1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Ratio of phospho/total expression
8
6
4
2
0
0' (unsliced)
30'
60'
120'
p-eIF4E
p-MAPK
B
p-ERK1/2
ERK1/2
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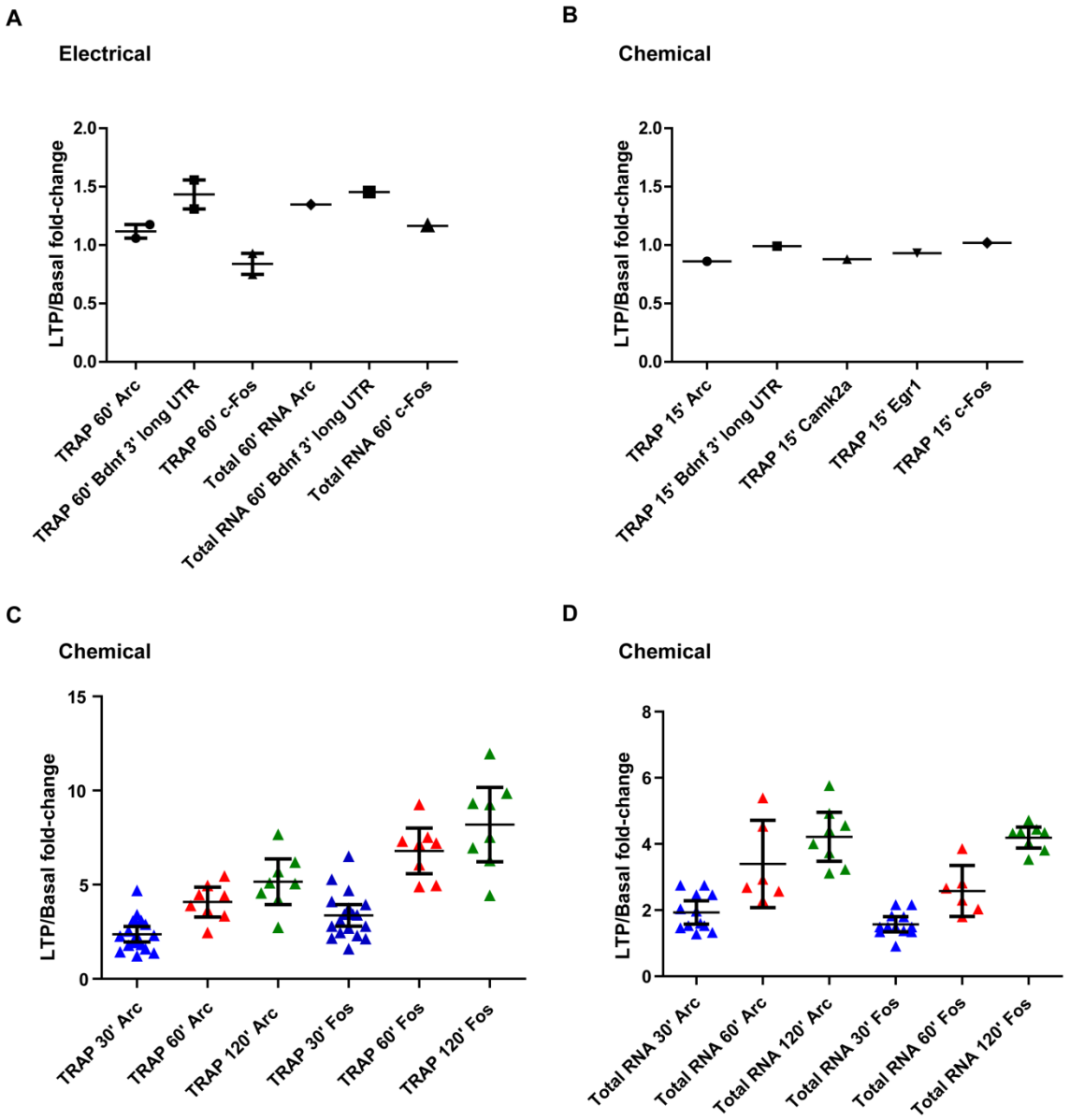
60'
120'
p-eIF4E
eIF4E
0'
30'
60'
120'

Figure 2-2


A
Electrical
LTP/Basal fold-change
2.0
1.5
1.0
0.5
0.0
TRAP 60' Arc
TRAP 60' Bdnf 3' long UTR
TRAP 60' c-Fos
Total 60' RNA Arc
Total RNA 60' Bdnf 3' long UTR
Total RNA 60' c-Fos
B
Chemical
LTP/Basal fold-change
2.0
1.5
1.0
0.5
0.0
TRAP 15' Arc
TRAP 15' Bdnf 3' long UTR
TRAP 15' Camk2a
TRAP 15' Egr1
TRAP 15' c-Fos
C
Chemical
LTP/Basal fold-change
15
10
5
0
TRAP 30' Arc
TRAP 60' Arc
TRAP 120' Arc
TRAP 30' Fos
TRAP 60' Fos
TRAP 120' Fos
D
Chemical
LTP/Basal fold-change
8
6
4
2
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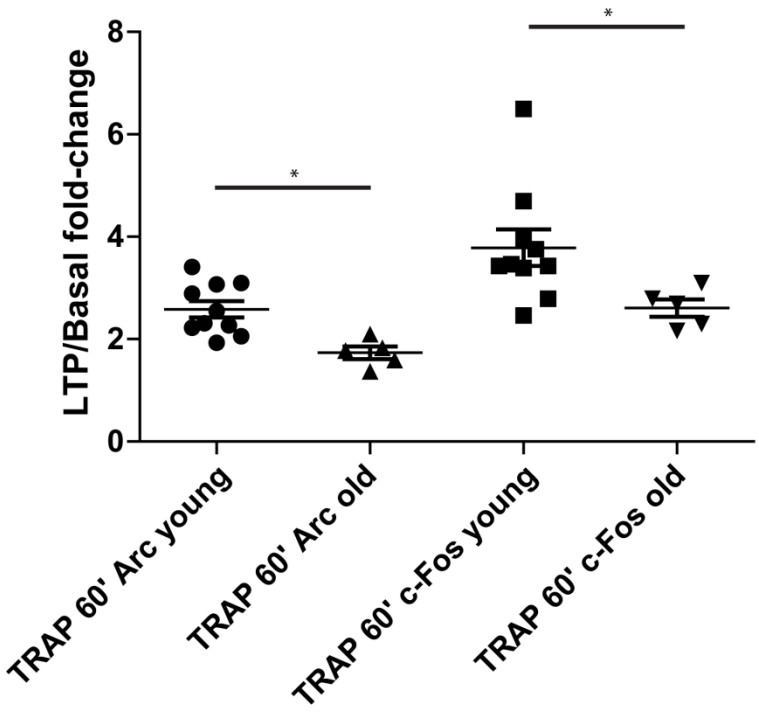
Total RNA 30' Arc
Total RNA 60' Arc
Total RNA 120' Arc
Total RNA 30' Fos
Total RNA 60' Fos
Total RNA 120' Fos

Figure 2-3


LTP/Basal fold-change
0
2
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TRAP 60' Arc young
TRAP 60' Arc old
TRAP 60' c-Fos young
TRAP 60' c-Fos old

Chapter 3

Mapping Gene Expression in Excitatory Neurons During Hippocampal Late-Phase Long-Term Potentiation

Introduction

Synaptic plasticity, the experience-dependent remodeling of neuronal connectivity, provides a means of storing memories in the brain[42]. Long-term potentiation (LTP) of hippocampal synapses, an activity-dependent, long-lasting increase in synaptic strength, provides an experimental model for investigating the cellular and molecular mechanisms underlying the formation of long-term hippocampal-dependent memories[43]. The late phase of LTP (L-LTP) can be differentiated from early LTP (E-LTP) by its requirement for RNA and protein synthesis [44,45]. Previous studies have shown that pharmacological inhibition of transcription and translation within an early time window ($\leq$ ~2 hours) after induction of LTP inhibits the persistence of LTP, but inhibition of transcription and translation at a later time point (> ~2 hours) has no effect on the persistence of L-LTP[18,45–47]. These observations are consistent with the idea that a critical early temporal window of new transcription and translation underlies the persistence of stimulus-induced plasticity and memory[48]. Many activity-dependent and LTP-induced transcripts have been identified through candidate and whole-transcriptome approaches, with demonstrated functions for some of these genes during both LTP and memory[49–51]. In this study, we profiled the temporal pattern of gene expression specifically within excitatory pyramidal neurons following induction of Schaffer collateral (CA3 to CA1) hippocampal LTP.

The long-lasting changes in synaptic strength that occur after a learning event require the coordinated effort of multiple cell populations. Thus, excitatory neurons, astrocytes, and inhibitory neurons have all been shown to contribute to hippocampal LTP[52,53]. Single-cell RNA sequencing of individual neurons[54] and cell-type specific RNA-seq[55] have revealed large differences in the expression of transcripts between cell types within the brain. Previous studies

of hippocampal gene expression following LTP induction[56–60] have not, however, differentiated between changes occurring in one cell population versus another. The presence of multiple cell types in the hippocampus diminishes the true extent of differential expression following LTP induction, as many transcripts are expressed in multiple cell types, yet may be regulated only in subsets of these cells. New methodologies have been developed in recent years for cell-type specific analysis of genome-wide changes. These include the promoter-dependent tagging of ribosomal proteins with a small protein tag[61], which allows for downstream purification of the ribosome-associated RNA population within a genetically defined cell population from tissue composed of multiple cell types.

Here, we take advantage of Translating Ribosome Affinity-Purification Sequencing (TRAP-Seq[34,62]) and transcriptome profiling technologies (RNA-seq) to determine the time course of differential expression following LTP induction in a cell-type specific manner. We performed TRAP-seq of the ribosome-associated population of RNA purified from excitatory neurons in hippocampal CA3/CA1 mini-slices 30, 60, and 120 minutes following chemical induction of LTP. We identified 899 differentially-expressed (DE) transcripts by TRAP-seq across these time points. We found that upregulated and downregulated transcripts differed in their enrichment of biological functions, and that upregulated transcripts had significantly longer untranslated regions (UTRs) than downregulated transcripts. Furthermore, we detected an enrichment of specific RNA binding protein (RBP) motifs in the 3' UTRs of upregulated transcripts. We found that different ensembles of transcripts were DE at each time point, with further temporal profiling of DE transcripts revealing clusters of temporally-regulated transcripts that were most prominently enriched in transcription-associated genes. While there was some overlap in the DE transcripts identified at each time point by TRAP-seq (from pyramidal

neurons) and RNA-seq (from all cell types), TRAP-seq detected both greater numbers of DE transcripts and greater magnitudes of differential expression at all time points. Transcripts DE exclusively in TRAP-seq were enriched for cell adhesion and cytoskeletal genes, while transcripts DE exclusively in RNA-seq were enriched for cytokine genes. Bioinformatic analyses of the RNA-seq DE transcripts identified some DE transcripts that were enriched in microglial and astrocytic cell types. Taken together, our results highlight the complexity and diversity of gene expression that occurs following LTP induction, and underscore the importance of considering both cell-type specificity and time after stimulation in determining the program of gene expression that gives rise to long-lasting brain plasticity and memory.

Results

To monitor the temporal pattern of gene expression within excitatory pyramidal neurons following induction of Schaffer collateral LTP, we prepared acute hippocampal mini-slices (which only contain the CA3/CA1 region) from adult RiboTag mice[34] (Fig. 3-1a). The RiboTag mouse expresses floxed HA-tagged ribosomal protein L22 (HA-L22) in cells expressing Cre recombinase, which allows for immunoprecipitation of ribosome-associated transcripts in a cell-type specific manner[34]. We crossed the RiboTag mouse line with a transgenic mouse line expressing Camk2α-cre[33], resulting in endogenous levels of expression of HA-tagged ribosomes exclusively within excitatory pyramidal neurons of our hippocampal mini-slices (Fig. 3-1b). We first tested the cell-type specificity of HA-L22 expression by immunoprecipitating ribosome-associated RNAs from hippocampal tissue and measuring the expression of cell-type specific transcripts by quantitative RT-PCR (qPCR). As shown in Figure 1c, the excitatory neuron-specific transcripts *Arc* and *Camk2a* were present in the affinity-purified RNA, but the inhibitory

neuron-specific transcript *Gad1*, astrocyte-specific transcript *Gfap*, and oligodendrocyte-specific transcript *Cnpase* were heavily de-enriched. These results indicated that TRAP-seq from hippocampal slices from these mice would be enriched for transcripts expressed in excitatory neurons.

To optimize identification of activity-dependent changes in gene expression, we also systematically reduced the variables in our experimental protocol. We first determined the optimal recovery time following slice preparation (2 hours) for induction of LTP, using qPCR to monitor injury-induced up-regulation of a set of positive control immediate early transcripts (*Arc* and *c-Fos*) and phosphorylation levels of ERK1/2 and eIF4E (Fig. 3-S1a). We next examined the effect of age on stimulus-induced gene expression, and found that the magnitude of LTP-induced upregulation of *Arc* and *c-Fos* expression was significantly greater in 2 month-old mice than in 10.5 to 12-month old mice (Fig. 3-S1b). While the possibility of age-related decline in activity-dependent gene regulation is of great interest for future studies, we chose to focus our efforts in this study on the LTP induced changes in ~2.5 month old mice to maximize signal to noise in RNA-seq and TRAP-seq experiments.

The use of mini-slices that only contained CA3/CA2/CA1 regions allowed us to identify changes in gene expression occurring specifically within the circuit undergoing plasticity. We tested a variety of stimulation paradigms to induce L-LTP of CA3 to CA1 synapses. We found that electrical stimulation of the CA3-CA1 synapses using 2x100Hz produced significantly lower amplitude changes in *Arc* and *c-Fos* expression as measured by qPCR than did chemical induction of LTP (data not shown). We chose a cLTP induction protocol that has been shown to be transcription- and translation-dependent (modified from Chotiner et al., 2003; see Materials

and Methods) and that produces LTP by triggering bursting of CA3 neurons, and thus involves synaptic mechanisms of LTP induction since removal of CA3 prevents LTP induction[31]. Slices were prepared from 10.5-12 week-old RiboTag mice. Immunohistochemistry using anti-HA antibodies revealed that ~95% of pyramidal neurons within CA1 stratum pyramidale expressed HA-tagged L22 (data not shown). Slices were allowed to recover for 2 hours before stimulation with the 10 minute cLTP induction protocol. Perfusion with artificial cerebrospinal fluid was then resumed for 30, 60 or 120 minutes before the slices were snap frozen for RNA immunoprecipitation/purification, library preparation, and RNA sequencing. We isolated both ribosome-associated RNA and total RNA from each set of mini-slices, and performed TRAP-seq to monitor changes in RNA association with ribosomes specifically in excitatory pyramidal neurons and total RNA sequencing (RNA-seq) to monitor changes in the whole mini-slice transcriptome.

TRAP-seq: LTP-induced changes in ribosome-associated transcripts in CA3 and CA1 pyramidal neurons

TRAP-seq results from the three biological replicates per time point were highly correlated (Pearson correlation coefficients of 0.99 for all time points; Fig. 3-S2). We assessed differential expression of transcripts using the Bioconductor package edgeR[63]. We considered a transcript significantly DE if the false discovery rate (FDR) was ≤ 0.1 and absolute $\log_2$ fold change was ≥ 0.4. We validated both differential expression and the fold-change cut-off by qPCR for 12 genes in an independently-generated biological replicate for the indicated time points (Fig. 3-S3). The number of DE transcripts identified by TRAP-seq was: 90 at 30 minutes,

353 at 60 minutes, and 592 at 120 minutes (Fig. 3-2a, Table S1), indicating a gradual increase in DE transcript numbers over time following LTP induction. A large number and fraction of DE transcripts were downregulated at the two later time points (20%, 50% and 40% at 30, 60, and 120 minutes respectively; Fig. 3-2a). Comparison of the magnitude of the fold-change of transcripts over time revealed that many individual transcripts underwent a time-dependent increase in amplitude of down- or upregulation (more intense green and red signals in the heat map in Fig. 3-2b).

To better understand the biological implications of the directionality of differential expression, we examined the upregulated and downregulated transcripts showing the most significant changes by FDR. The most significant upregulated and downregulated transcripts encoded genes involved in diverse and distinct cellular and molecular processes, with upregulated transcripts encoding transcription factors and phosphatases, and downregulated transcripts encoding G protein coupled receptors, kinases, and metabolic pathway enzymes (Fig. 3-2c). To further explore the possibility that transcripts whose association with ribosomes was upregulated and downregulated following LTP induction are functionally distinct, we tested for enrichment of functional gene pathways using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (Table S2). We found that upregulated transcripts were enriched for genes involved in MAP kinase signaling, extracellular matrix interactions, cell adhesion, cytoskeletal regulation and RNA degradation (Fig. 3-3a, top), whereas downregulated transcripts were enriched for genes involved in metabolic pathways, neuroactive ligand-receptor interactions and calcium signaling (Fig. 3-3a, bottom).

We next wanted to understand whether the differential expression of these groups of ribosome-associated transcripts could be in part explained by differences within key regulatory elements of the transcripts. Direct binding of specific classes of RNA binding proteins (RBP) in the 5' and 3' untranslated regions (UTRs) of mRNAs has been shown to regulate translation of transcripts[64,65]. Both the 5' and 3' UTRs of mRNAs are enriched for RBP binding sites compared to the coding region[66], with longer UTRs likely containing greater numbers of binding sites for RBPs. We first asked whether UTR length was correlated with down or upregulated transcripts by comparing the lengths of UTRs between transcripts that were significantly down- or upregulated. We detected significantly longer 5' and 3' UTRs in upregulated transcripts as compared to downregulated transcripts (Fig. 3-3b). 5' and 3' UTRs were also significantly longer for upregulated transcripts as compared to previously-published brain-expressed transcript 5' and 3' UTRs (from Kang et al., 2011; see Materials and Methods). Since a direct interaction between RBPs, ribosomes and the 3' UTRs of mRNAs has previously been shown to affect translation of associated transcripts following neuronal depolarization[68], we sought to determine whether specific RBPs play a role in the regulation of gene expression following LTP induction. To address this, we used a previously-published compendium of RBP motifs[69] to test whether there was enrichment of specific RBP motifs in the 3' UTRs of upregulated and downregulated transcripts compared to brain-expressed transcripts. As shown in Fig. 3-3c and Table S3, we found that U-rich motifs were significantly overrepresented in the 3'UTRs of upregulated transcripts, as were the binding motifs for several RBPs known to regulate RNA metabolism in neurons, including CPEB2 and 4 and Pum (Darnell and Richter 2012), Hu/Elavl proteins (Lee, Lee and Kaang, 2015), and Fus (Ling, Polymenidou and Cleveland 2013). Binding motifs for only two RNA binding proteins, Vts1p (yeast, mammalian Smaug or Samd4) and Fmr1, were

underrepresented in the 3' UTRs of upregulated transcripts. A smaller number of significantly overrepresented motifs were present in downregulated transcripts (Table S3). Many of the RBPs in Table S3 were expressed within excitatory neurons from our TRAP-seq data, suggesting that RBP binding may serve as one mechanism of regulating transcript expression following LTP induction.

Our finding that the number of DE transcripts increased over time (Fig. 3-2a) led us to more carefully examine the temporal characteristics of differential expression following LTP induction. Although the general direction of change was consistent across time for most transcripts (Fig. 3-2b), there was only marginal overlap of significant DE transcripts between time points (Fig. 3-4a). Despite significant overlap of DE transcripts between time points ($p < 1.0^{-30}$ for paired time point overlap), the proportion of exclusively DE transcripts at their respective time points was 48% percent of DE transcripts at 30 minutes, 73% of DE at 60 minutes, and 83% of DE transcripts at 120 min. To more rigorously characterize the temporal dynamics of differential expression following LTP induction, we utilized Short Term Expression Miner (STEM[70]) to group DE transcripts based on the similarity of their temporal expression patterns. Previous studies have shown that temporally co-regulated transcripts encode genes with related biological functions[71,72]; this approach would therefore increase the likelihood of detecting biologically significant processes related to LTP. STEM analysis revealed that most transcripts clustered into a temporal profile representing a general upregulation over time (cluster 1, Fig. 3-4b, Table S4). The other two clusters contained transcripts that were largely downregulated over time (clusters 2 and 3, Fig. 3-4b, Table S4). We next tested for functional enrichment within the groups of transcripts associated with each cluster. To further increase the specificity of this analysis for processes related to LTP, we utilized a background gene list

derived from our TRAP-seq data that included transcripts with a minimal expression level of an average of 20 normalized reads across all samples. Gene ontology (GO) analysis revealed that cluster 1 was heavily enriched for processes involved in transcription and transcriptional regulation (Fig. 3-4c, Table S5), while GO analysis of clusters 2 and 3 revealed no significant enrichment. Genes within a selected enriched transcriptional regulation GO category (Fig. 3-4d) demonstrated a clear increase in $\log_2$ fold change over time; only a few of these transcription factors have been previously implicated in plasticity. Importantly, some of these genes fell below our significance criteria at individual time points but are included within significant STEM profiles, confirming that analysis of DE at multiple time points is more sensitive than single time point experiments in identifying co-regulated transcripts sharing biological functions.

In addition to TRAP-seq, we also purified and sequenced total RNA (composed of RNA from all cell types) from the same set of mini-slice homogenates used to generate RNA for TRAP-seq, allowing a direct comparison between the two techniques from a single biological sample (Table S6). Overall, more DE transcripts were identified by TRAP-seq compared to RNA-seq at every time point following LTP induction (Fig. 3-5a), though the time-dependent increase in DE transcript numbers and magnitude of differential expression we observed by TRAP-seq was also observed by RNA-seq (Fig. 3-5a, Fig. 3-S4). There was significant overlap of DE transcripts in TRAP-seq versus RNA-seq at each time point ($p < 1.0^{-20}$ for overlap at each time point); however, the overlap was modest, with a smaller fraction of TRAP-seq DE transcripts being detected by RNA-seq at all time points. Another difference we observed was that unlike TRAP-seq, a majority of DE transcripts detected by RNA-seq at earlier time points was also DE at later time points (Fig. 3-S4, Fig. 3-4a). Differences in fold-change between RNA-seq and TRAP-seq, and between time points, were validated by qPCR of a separate biological

replicate for both larger and smaller fold-change magnitudes close to our fold-change cutoff (Fig. 3-S3).

When collapsed across all time points, a total of 740 transcripts were DE only by TRAP-seq, while 176 transcripts were DE only by RNA-seq and 159 were DE in both (Fig. 3-5b, Table S7). This indicated that only a subset of TRAP-seq and RNA-seq DE transcripts were shared, and that each technique identified different sets of transcripts. Interestingly, the three categories of genes—DE in both TRAP-seq and RNA-seq, DE in only TRAP-seq, or DE in only RNA-seq—were enriched in distinct biological functions. DE transcripts in both TRAP-seq and RNA-seq were enriched for transcription associated genes (Fig. 3-S5), DE transcripts in only RNA-seq were enriched for cytokine signaling pathways (Fig. 3-S5), while DE transcripts in only TRAP-seq were enriched for cell adhesion and cytoskeletal genes (Fig. 3-5c). Of note, members of the cadherin and protocadherin families as well as members of several different classes of microtubule-binding and motor proteins (e.g. MAP2 and MAP1a/1b, Myo6 and Dnah2) were DE only within the TRAP-seq data. These families of genes are involved in biological processes important in LTP[73,74], and certain DE transcripts in these enriched categories have been shown to be critical for excitatory transmission and LTP[75,76].

Interestingly, transcripts known to be neuron-specific, such as *Map2* and *Map1b*, were DE by TRAP-seq but virtually unchanged by RNA-seq. This raised the possibility that transcripts could be regulated differently at ribosome-associated and total RNA steady states; specifically, changes in the ribosome-associated population would not necessarily require corresponding changes in the total RNA concentration. To address this possibility using a more unbiased approach, we used a bioinformatics approach to identify neuron-enriched transcripts by

looking for transcripts whose basal expression was overrepresented by TRAP-seq as compared to RNA-seq (see Materials and Methods for details). We confirmed the neuron-enriched expression of these transcripts using the Allen Brain Atlas (http://mouse.brain-map.org/); of the 30 DE neuron-enriched transcripts that had detectable expression by the Allen Brain Atlas, only 1 was excluded for being non neuron-enriched. We then compared differential expression of this set of transcripts in the TRAP-seq and RNA-seq data. From this, we identified 16 transcripts that were DE at comparable levels by both RNA-seq and TRAP-seq (mainly comprised of immediate-early genes that are rapidly transcriptionally/translationally upregulated[49]) and 43 transcripts that were only DE by TRAP-seq (Fig. 3-6, Table S8). Taken together, the magnitude of differential expression of these transcripts by TRAP-seq was significantly greater than RNA-seq, (Fig. 3-6, $p < 0.0001$; Wilcoxon signed rank test). This greater magnitude of differential expression was observed for both upregulated and downregulated transcripts.

Given that specific transcription factors have been shown to coordinate the transcription of multiple important transcripts for LTP and memory[47], we next sought to determine whether DE transcripts identified through RNA-seq were coordinately regulated by distinct transcription factors. Using two separate bioinformatic approaches to assess enrichment of known targets or binding motif sequence enrichment—oPOSSUM-3[77] (Fig. S7a, Table S9A) and TRANSFAC[78] (Table S9B)—we identified CREB1 as the transcription factor with the most enriched binding sites, consistent with previous literature on the critical role of CREB1-mediated transcription following LTP induction[79]. Other transcription factors with highly enriched targets within our DE transcripts included STAT1 and EGR1, which have previously been implicated in plasticity[80,81], and NFAT1 and MZF1 (Table S9A,B), transcription factors that have not been

reported to be involved in neuronal plasticity but that are expressed in hippocampal tissue as determined by our RNA-seq data.

To our surprise, the GO categories of DE transcripts identified only through RNA-seq included processes not generally associated with neurons (Fig. 3-S5). Specifically, we detected an enrichment of biological processes involved in immune functions, such as those involved in cytokine signaling pathways. This raised the possibility that the induction of LTP triggered DE of transcripts not only in neurons, but also in other cell types. To explore this possibility, we utilized published transcriptional profiles from individual cell types in the mouse brain[55] to generate a list of transcripts that were enriched in neurons, astrocytes, myelinating oligodendrocytes, or microglia (see methods). We then determined the number of DE transcripts that were enriched in each cell type (Fig. 3-7b, Table S10). While most DE genes could not be attributed to a specific cell type, microglia had the highest number of cell-enriched DE transcripts (46 transcripts), including chemokine ligands and receptors. To further validate the cell-type specificity of gene expression following LTP induction, we stained for the significantly DE microglial gene Il1b by immunohistochemistry at 120 minutes post-LTP induction and detected significantly increased immunoreactivity of Il1b protein in microglia following LTP induction (Fig. 3-7c,d). These results suggest that a portion of the observed changes in total RNA were due to LTP-induced changes in the transcriptome of specific non-neuronal cell types. Taken together, these results underscore the need for cell-type specific studies of gene expression to understand the changes in gene expression that give rise to persistent forms of learning-related plasticity.

Discussion

Decades of research have aimed to identify the specific activity-dependent genes whose expression gives rise to long-term plasticity and long-term memory. Many activity-dependent immediate early genes such as *Arc, c-Fos,* and *Egr1*, were initially identified using subtraction-cloning approaches comparing stimulated versus unstimulated hippocampi[82]. The development of microarray and next-generation sequencing technologies has permitted systematic and quantitative genome-wide analysis of the changes in gene expression that occur at various time points following the induction of LTP[56–58,60]. Our intent in this study was to use sensitive, genome-wide approaches to optimize the discovery of activity-dependent genes and to thereby provide a comprehensive picture of the changes in gene expression that occur in excitatory neurons following induction of plasticity. We utilized a cLTP induction protocol to ensure that the maximal number of synapses would be potentiated for downstream gene expression analysis, and used CA3/CA1 mini-slices to focus our transcriptome analyses specifically on the pathway undergoing plasticity. Cell-type specific TRAP-seq was used to focus on gene regulation specifically within excitatory neurons. Although analysis of gene expression following learning in the behaving animal might be considered more relevant to identifying the genes whose expression lead to long-term memory formation, the sparse encoding of memory within neural circuits[83,84] reduces signal to noise and thus impedes the identification of activity-dependent alterations in gene expression. For example, we identified a total of 335 DE transcripts by RNA-seq across all time points (Table S6), but a recent study utilizing RNA-seq to assay gene expression in hippocampus following fear conditioning [57] only identified 112 DE transcripts with more time points included and even less stringent criteria for differential expression than our study. In another recent publication using TRAP-seq to identify changes in ribosome-association of mRNAs in pyramidal cell dendrites following contextual fear conditioning, the variation

between the biological replicates within the control and fear-conditioned animals was as large as the variation between the control and fear-conditioned animals (Ainsley et al., 2014; also E.M. Schuman comment in PubMed Comments). We believe that using TRAP-seq to systematically detect DE genes following LTP induction in a reduced slice preparation provides a fruitful first step in identifying specific genes that can subsequently be studied during learning and memory in the animal.

Temporal regulation of gene expression following LTP induction

Studies using transcriptional and translational inhibitors have given rise to the idea that changes in gene expression only during the first two hours following stimulation are critical to the persistence of hippocampal LTP (Fonseca et al., 2006; Frey et al., 1988; Nguyen and Kandel, 1997; Nguyen et al., 1994). The results of our TRAP-seq and RNA-seq experiments call this idea into question. We observe a time-dependent increases in the number of DE transcripts, with the greatest number of DE transcripts detected at 120 minutes, a time point when LTP persistence has been reported to lose sensitivity to translational and transcriptional inhibitors. Our results add to a growing list of studies[17,86–89] that challenge the idea of a relatively short and early time window in which gene expression is exclusively occurring. Transcripts DE only at 120 minutes could serve as novel markers for late-phase LTP, as current proxies for synaptic plasticity such as *c-Fos* and *Arc* are transcribed immediately following non-LTP inducing levels of activity, and are not always associated with long-lasting plasticity and memory (unpublished data, Kim et al., 2010). Further studies examining the temporal patterns of gene expression during even later time points may provide greater insights into the temporal dependence of gene expression following LTP and learning.

One unexpected aspect of temporal regulation that emerged from our TRAP-seq data was that a relatively small (but significant) fraction of transcripts was DE across multiple time points (Fig. 3-4a). This finding indicates that regulated ribosome association of mRNAs at later time points is not simply a continuation of regulated ribosome association at earlier time points, but rather that there are discrete rounds of differential ribosome association of transcripts following LTP induction. In contrast, RNA-seq showed a much greater proportion of temporal overlap between DE transcripts than TRAP-seq (Fig 3-4a, Fig. 3-S4), consistent with a more continuous pattern of transcriptional than translational regulation following LTP stimulation. This may be expected given the more dynamic kinetics of ribosome-association compared to total RNA regulation.

Cell-type specificity of gene regulation underlying LTP

In this study, we measured differential expression occurring specifically within excitatory neurons following LTP induction using TRAP-seq, which revealed a large number of upregulated and downregulated DE transcripts that were not detected by RNA-seq from all cell types (Fig. 3-2, Fig. 3-5a). This difference can in part be explained by differences in cell-type composition, with RNA-seq detecting fewer DE transcripts due to a dilution effect from other cell types. For example, transcripts such as *c-Fos* are DE in neurons but are also highly expressed within astrocytes[91] (and unpublished data), which would diminish the detectability of DE by RNA-seq relative to excitatory neuron-specific TRAP-seq. It is unclear whether DE of transcripts expressed within neurons also occurs within other cell types. Interestingly, our bioinformatic identification of cell-type enriched transcripts that were DE by RNA-seq suggests that differential expression occurs in non-neuronal cell types following LTP induction, though

most DE transcripts could not be ascribed to a single cell type using this approach. We detected 46 microglia and 26 astrocyte-enriched transcripts that were differentially expressed in our RNA-seq experiments, and demonstrated a corresponding microglial protein level expression change for the microglial-enriched transcript *Il1b* (Fig. 3-7b-d). Astrocytic functions have been shown to be important for LTP and memory[52] and microglia have been shown to regulate activity-dependent synaptic pruning[92]. In this context, our findings highlight the importance of analyzing gene expression in non-neuronal cell types during forms of synaptic plasticity such as hippocampal LTP.

Gene expression regulation following LTP induction

Gene expression is a coordinated process involving multiple layers of regulation, including transcriptional and post-transcriptional, with changes in total RNA following LTP induction traditionally thought to be required for changes in protein level. RNA-seq measures the concentration of total RNA, which reflects transcriptional regulation as well as RNA stability, while TRAP-seq measures the concentration of ribosome-associated mRNAs, which reflects not only mRNA concentration but also post-transcriptional regulation at the level of ribosome association. The difference in 5' and 3' UTR lengths between upregulated and downregulated transcripts of the ribosome-associated mRNAs, and enrichment of specific RBP motifs within their 3' UTR sequences (Fig. 3-3b,c), provide clues into the types of post-transcriptional regulation mechanisms that may underlie the upregulation and downregulation of these transcripts. Further analysis taking into consideration RNA secondary structural motifs, which are known to play critical roles in regulatory mechanisms such as RBP binding[93], will be

important for identifying other mechanisms of post-transcriptional regulation following LTP induction.

We note that the fold-change magnitude of most DE transcripts was modest, with most transcripts not changing more than an absolute log_2FC of 1. While transcripts undergoing larger fold-change differential expression are better targets for some types of downstream experimental analyses, and are easier to confirm using less quantitative methodologies, such as immunoblotting or immunohistochemistry, DE transcripts with smaller fold-changes may still play a critical role in LTP. Smaller fold-changes may in fact be expected for the fine-tuning of biological systems, such as during synaptic plasticity, rather than a complete overhaul, such as during developmental changes in cell fate. Importantly, despite the low magnitude of fold change, differential expression was highly reproducible, not only between biological replicates used for RNA-seq and TRAP-seq, but also in the biological replicates used for qPCR confirmation (Fig. 3-S2, 3-S3).

Local protein synthesis has also been shown to be critical for LTP, which can occur in a transcription-independent manner[94]. A number of the DE transcripts that were identified only by TRAP-seq have also previously been shown to be localized to dendrites[95] (Table S11), raising the possibility that their differential expression occurs at the ribosome-associated level in dendrites as well as in cell somata. Thus, the magnitude of differential expression we detected in many of these DE transcripts may in fact be changing at greater levels specifically within subcellular compartments; this has been demonstrated with translation of Camk2α protein only occurring locally within dendrites following stimulation[96]. We also note that TRAP-seq may also incorporate measurements of ribosomal stalling—a mechanism used by FMRP to decrease rates

of translation[97]—which would affect the correlation of TRAP-seq changes and protein level changes for some of these transcripts. All of these issues highlight the necessity of better tools to dissect the different layers of regulation during LTP. For example, combining TRAP-seq with ribosome profiling provides a means of differentiation between ribosome stalling and procession, while methods like BONCAT allow for cell-type specific proteomic measurements of activity-dependent changes in protein expression[98].

When comparing the differential expression magnitude of neuron-enriched genes, we found that DE transcripts had significantly larger fold-change magnitudes at the ribosome-associated level than the total RNA level. This may result from 1) the faster kinetics of ribosome-associated regulation compared to regulation of total RNA steady states; 2) the lower basal copy number of ribosome-associated transcript compared to total RNA copies; and/ or 3) distinct stimulus-induced post-transcriptional (and transcription-independent) regulation. Indeed, the fact that we detected such a large number of downregulated transcripts at all time points by TRAP-seq but not by RNA-seq (Fig. 3-2a and Fig. 3-S4) is consistent with the faster kinetics of ribosome association (as compared to regulation of total RNA levels). These results suggest that neuronal stimulation can regulate transcript translation independently of changes in transcript concentration, and focuses attention on activity-dependent translational regulation. This is consistent with studies of stimulus-induced gene expression in a homogenous cell population that revealed significantly greater changes in polysome-associated RNA than total RNA[99]. Although we were very conservative in our criteria of neuron-enrichment, we cannot fully rule out the possibility that there may be expression of these transcripts in non-neuronal cells. To systematically test the extent of differential regulation at the total RNA and ribosome-loaded level, cell-type specific total RNA analysis is needed for a direct global comparison of the

kinetics between the two RNA populations[100]. This type of analysis will likely require the development of new technologies that reduce the amount of starting material required for cell-type specific RNA-seq. Thus, performing TRAP-seq without amplification required 20 hippocampal slices per condition per replicate (40 per experiment per replicate), and adding cell-type specific RNA sequencing using methods such as 4TU labeling (which labels only a fraction of RNA), would require an even greater number of animals per replicate, greatly reducing the feasibility of these experiments.

Concluding remarks

The design and scope of this study was not to hand-select candidates and assay their function in LTP. Previous studies utilizing this approach have proven invaluable in implicating different molecules important for LTP, but a unified model of the molecular requirements for LTP is lacking[23,101]. The large degree of time-dependent differential expression of hundreds of transcripts encoding genes involved in diverse functions raises questions about the interpretation of single-gene studies in understanding mechanisms of LTP and memory. The results presented here paint a clear picture of the sprawling complexity of gene regulation, even within only a single cell type, following LTP induction. When studying a single gene within this large web of interconnected genetic pathways, manipulations of a single gene may have widespread consequences—even altering the entire molecular network that is involved in plasticity. Thus, experimentation with individual candidates should involve more rigorous follow-up profiling of the downstream consequences on gene expression within the cell. Our results would argue that detailed, naturalistic studies are necessary to first provide an important molecular blueprint by which candidate-based studies can be interpreted.

Materials and Methods

Generation of RiboTag x Camk2a-Cre mice

Both RiboTag and T29/Camk2α-Cre mice were purchased from Jackson Laboratories (RRID:IMSR_JAX:011029, RRID:IMSR_JAX:005359). 8-12 week old Ribotag x Camk2α-Cre double heterozygotes were used to prepare hippocampal mini-slices.

CA3/CA1 mini-slice preparation and cLTP induction protocol

400 µm thick hippocampal CA3/CA1 mini-slices were prepared from hippocampi of RT x Camk2α-cre double heterozygotes. The dentate gyrus was microdissected following slicing and mini-slices were allowed to recover for 2 hours at 30°C in interface-type chambers with oxygenated (95% O_2/5% CO_2) ACSF containing 124 mM NaCl, 4 mM KCl, 25 mM $NaHCO_3$, 1 mM NaH_2PO_4, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM glucose. LTP was induced by submerging slices in 50 µM forskolin (in normal ACSF containing 0.2% DMSO from addition of forskolin) for 5 minutes, followed by 30 mM KCl/10 mM Ca2+/50 µM forskolin in 0 Mg2+ ACSF for 5 minutes. Control solution contained 0.2% DMSO in normal ACSF for 10 minutes. Slices were perfused with normal ACSF after LTP induction, with time 0 starting immediately after applying the K^+/Ca^{2+}/forskolin solution. Slices were snap frozen in crushed dry ice at the specified time points, and stored at -80°C until RNA purification.

Immunoprecipitation (IP) and RNA purification of ribosome-loaded/total RNA populations

Frozen slices were homogenized using a pestle in IP buffer which contained 50 mM Tris-HCl pH 7.4, 100 mM KCl, 12 mM $MgCl_2$, 1% NP-40, 1 mM DTT, 200 U/mL Protector RNAse inhibitor

(Roche), 1 mg/mL heparin, 100 µg/mL cycloheximide, cOmplete protease inhibitor tablet (Roche). Pre-conjugated HA beads (EZview Red Anti-HA Affinity Gel, Sigma, RRID:AB_10109562) were washed twice with IP buffer before use. After homogenization, the homogenate was centrifuged twice at 10,000x g at 4°C. 1/10 of the homogenate at this step was immediately placed into Trizol (Invitrogen) for total RNA extraction. The remaining homogenate was incubated with washed HA beads overnight at 4°C. The following day the beads were washed 3x in a high salt buffer composed of 50 mM Tris, 300 mM KCl, 12 mM $MgCl_2$, 1% NP-40, 1 mM DTT, and 100 µg/mL cycloheximide. RNA was eluted from the beads using 350 µL Buffer RLT + BME supplementation from the RNeasy Micro kit (Qiagen). Eluted IP RNA was column purified according to the kit protocol. Total RNA was purified through a combined Trizol and RNeasy kit protocol: after the spin gradient step in the Trizol purification, the aqueous layer was taken and mixed 1:1 with 100% EtOH and purified on a spin column. For each biological replicate, four mice were used, with ~20 slices per treatment condition. TRAP purification yielded ~200 ng RNA per 20 mini-slices, and total RNA from 1/10 of this homogenate yielded ~400 ng RNA. RNA quantification was done with the Qubit RNA HS assay (Thermo Scientific).

Immunohistochemistry of RiboTag x Camk2a-Cre mice

Hippocampal slices were fixed in 4% PFA for 2 hours at room temperature, rinsed 2x with PBS, covered in HistoGel (Thermo Scientific), then paraffin-embedded. 4 µM thick paraffin sections were deparaffinized and underwent heat-induced antigen retrieval. Sections were permeabilized with 0.1% TX-100 at room temperature for 30 minutes, then blocked in 10% goat serum at room temperature for 60 minutes. Slices were incubated with mouse anti-HA antibody (Covance,

1:1000, RRID:AB_291263) and rabbit anti-MAP2 (Millipore, 1:2000, RRID:AB_91939) overnight at 4°C, and with secondary antibodies (1:2000) at 2 hours at room temperature and counterstained with Hoechst (1:1000).

Rabbit anti-Il1b (Abcam, 1:200, RRID:AB_308765) was used to confirm a microglial DE transcript. Images were taken from control- and LTP-treated slices from two animals at the 120 minute time point, with 3-4 slices per animal per condition. ROIs were drawn around staining from cell bodies and processes, and the mean pixel intensity was taken for each ROI using ImageJ software. Each data point represents one slice. Mann-Whitney nonparametric test was used to determine significance.

Quantitative PCR for non-specific transcript depletion, cLTP induction, validation

To assay depletion of non-specific transcripts, 50 ng of total RNA and TRAP RNA were reverse transcribed into cDNA using SuperScript III with random hexamer primers (Invitrogen). Technical triplicate reactions for each primer set (Gfap, Gad1, Cnpase) using SYBR Green PCR Master Mix (Applied Biosystems) were prepared, with the cDNA being evenly divided into each reaction. Percent de-enrichment was calculated by comparing relative Ct values between total and TRAP RNA samples for each set of primer. To assay LTP induction, 20 ng of total RNA and TRAP RNA for both basal and LTP conditions were reverse transcribed. Normalization of basal/LTP Ct values with HPRT1 was done before comparing fold-change of activity-dependent transcripts (*Arc*, *c-Fos*) through relative Ct values. To validate sequencing results, we collected a separate biological sample for both 60 and 120 minute time points and performed RT-qPCR for the indicated transcripts. Some primer sequences were obtained from PrimerBank

(http://pga.mgh.harvard.edu/primerbank) while others were designed to span exon-exon junctions.

RNA sequencing library preparation and sequencing

~150 ng of TRAP RNA and total RNA were used in the library preparation, with a total of 12 samples per time point (biological triplicates for total basal/LTP and TRAP basal/LTP). RNA libraries were made using Illumina TruSeq RNA library preparation v2 kit. rRNA was first depleted from all samples using the Ribo-Zero Gold kit. Libraries were prepared according to manufacturer's instructions, skipping the oligodT purification step. Single-end (30 minute time point) and paired-end sequencing (60 and 120 minute time points) was performed using the Illumina Hiseq2500 system. Both library preparation and sequencing were done at the UCLA Neuroscience Genomics Core. Read lengths were 64bp for single-end reads and 69bp for paired-end reads, with an average of 45 million mapped reads per sample.

RNA-seq, time-series, GO enrichment analysis

RNA sequencing reads were mapped to the mm10 annotation of the mouse genome using STAR aligner [102] and differential expression analysis was done with edgeR [63]. Reads were normalized by the trimmed-mean method for each time point before differential expression analysis. Significant differential expression used a cutoff of FDR $\leq$ 0.1 and $\log_2$ fold-change of at least $\pm$0.4. Hypergeometric tests were used to determine significance of overlap between DE transcripts of different variables.

Heatmaps were generated with gplots in R displaying all significant transcripts across all time points. The bottom 5% expressed transcripts (ie lowly expressed significant transcripts) were excluded as to minimize visual artifacts.

KEGG pathway analysis of up and downregulated transcripts was done using WebGestalt with a whole genome background list. All functional category enrichment analyses considered an adjusted p-value ≤ 0.05 after BH correction to be significant, with a minimum of 4 genes per category. Up and downregulated transcripts from all time points were included.

Time series analysis was done with Short Time-series Expression Miner (STEM) (http://www.cs.cmu.edu/~jernst/stem/) using the fold-changes for transcripts that were significant in at least one time point. Gene ontology analysis was done using WebGestalt (http://bioinfo.vanderbilt.edu/webgestalt/) on the temporal profiles derived from STEM analysis. All transcripts that had at least an average read number of 20 normalized reads by TRAP-seq across all time points was used as the background transcript list. For enrichment of TRAP-seq, RNA-seq, or both TRAP-seq and RNA-seq DE transcript function, PANTHER (http://pantherdb.org/) was also used to identify enriched categories and the genes associated with them with a background list derived from RNA-seq expression levels of 20 normalized reads on average across all samples and time points.

UTR length and RBP motif enrichment analysis

Annotated 5' and 3' UTR lengths were obtained from BioMart using the Ensembl GRCm38 build. In cases of multiple annotated UTRs for a given gene, the average was taken and used for resampling. The annotated UTRs of brain-expressed transcripts from Kang et al., 2011 were compared against downregulated and upregulated transcripts using a Wilcox ranked test. This

was also completed with a mouse-specific brain-expressed transcript list[103] that yielded similar results but is not shown here. A large majority of transcripts overlapped between the two brain-expressed lists.

RBP motif enrichment analysis was completed with a resampling approach using 3' UTR sequences of brain expressed transcripts because RBP motifs were underrepresented in simple scrambling of UTR sequences. A total of 244 mouse RNA binding protein motifs (taken from Ray et al., 2013) were tested on the 3' UTR of upregulated and downregulated transcript sets, against a background of brain-expressed transcripts. We identified the numbers of RBP binding sites for each RBP within all 3' UTRs of our up and downregulated genes, and generated a distribution of chance RBP motif numbers by resampling 3' UTRs from the brain-expressed list 5000 times. We then compared the observed value against the distribution of expected numbers of RBP motif occurrences to generate a p-value. UTR length was accounted for during the resampling by fixing the total combined nucleotide length of all sampled UTRs to equal the total nucleotide length of all UTRs in up or downregulated transcript sets, as length variability had a measurable effect on the results. Significance criteria was $p < 0.05$ and a fold-enrichment $> \pm 0.2$

Cell-type specific enrichment analysis

We used the RNA-seq dataset from Zhang et al., 2014 to calculate cell type-enriched transcripts. It is important to note that the Zhang et al study was done in P7 mice, while our study was done in 10-12 week old mice. Using FPKM numbers for neurons, myelinating oligodendrocytes, astrocytes, microglia, and endothelial cells, we calculated a neuronal enrichment factor by comparing FPKM for a given cell type against all other cell types that are expressed in the hippocampus. For example, for astrocytes we used a formula of (astrocyte FPKM) / (neuron +

oligodendrocyte + microglia + endothelial cell FPKM) as a conservative way to determine which transcripts were astrocyte-enriched. We used an enrichment factor cutoff of 1.5 for all cell types. Transcripts we considered to be significantly DE in astrocytes had to be 1) astrocyte-enriched and 2) differentially expressed by a cutoff of FDR $\leq$ 0.1 and a log2 fold-change of at least $\pm$ 0.4.

To determine neuron-enriched genes using TRAP-seq/RNA-seq data, we took the average of normalized basal read counts across all samples from all time points for each gene and found the ratio of TRAP-seq reads over RNA-seq reads. We considered a ratio of 1.5 to be neuron-enriched; this was empirically a stringent cutoff for neuron-enrichment because many known neuron-specific genes such as MAP2 and Arc were excluded from our neuron-enriched list. This was done to avoid including transcripts that may be expressed at significant levels in other cell types. From this list, we included a 10-read minimum cutoff to ensure genes with inflated enrichment ratios were excluded. Significant DE transcripts by TRAP-seq were then identified from this list and included in the analysis. For transcripts that were DE across multiple time points (as was the case for many transcripts DE by both TRAP-seq and RNA-seq), we took the average fold-change across all significant DE time points. Significance testing between $\log_2$FC of TRAP-seq and RNA-seq was calculated using a Wilcoxon signed rank test.

Transcription-factor binding analysis

A list of enriched transcription factor targets and significantly enriched binding motifs was identified using oPOSSUM 3.0 (http://opossum.cisreg.ca/oPOSSUM3/) and TRANSFAC on BioBase respectively. Significant transcripts identified through total RNA sequencing at any time point were included in the analysis, with a background list of $\geq$20 average reads transcripts

at 120’ for oPOSSUM and >10 average reads for TRANSFAC. Graphical representation is for oPOSSUM results only, while results from both analyses are included in Table S9A,B.

Author Contributions

PBC and KCM designed experiments; TOJ prepared hippocampal mini-slices; PBC bred and characterized Ribotag-CamKIIα mice, performed cLTP stimulations, prepared samples and performed immunohistochemistry and qPCR; PBC, RK, CB and GC performed bioinformatics analysis; PBC and KCM wrote manuscript, with edits from TOJ, RK and GC.

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Figure legends

Figure 3-1. Expression and RNA purification of ribosome-associated transcripts is specific for excitatory neurons. a) Diagram of the experimental design and immunohistochemistry demonstrating mini-slice preparation. Red = HA, blue = Hoechst. Scale bar = 200 μm. b) HA-tagged ribosomes are expressed in excitatory neurons (labeled with Map2 antibodies, top) but not astrocytes (labeled with Gfap antibodies, bottom) within hippocampal mini-slices. Scale bar = 20 μm c) Immunoprecipitation and RNA purification of ribosome-associated populations from

hippocampal mini-slices de-enriches for astrocytic (Gfap), oligodendrocytic (Cnpase) and inhibitory neuron-specific (Gad1) transcripts, but captures excitatory neuron specific transcripts (Arc and Camk2a). IP RNA values were normalized against input RNA values to generate de-enrichment ratios. n = 2 biological replicates, error bars = SEM.

Figure 3-2. TRAP-seq of transcripts from excitatory neurons following LTP induction at 30, 60, and 120 minutes reveals increasing bidirectional differential expression over time. a) Increasing numbers of both upregulated and downregulated DE transcripts over time. b) Heat map of significant transcripts at all time points. Color key = log2 fold change. c) List of selected significantly downregulated and upregulated transcripts at 120' post-induction with their respective log2 fold-changes. Transcripts selected by lowest FDR.

Figure 3-3. Upregulated and downregulated transcripts differ in biological functions and in 5' and 3' UTR lengths, with upregulated transcripts enriched for specific RNA binding protein motifs. a) KEGG pathway enrichment analysis of upregulated and downregulated transcripts. The most significant pathways are shown. Red = upregulated, green = downregulated. Numbers represent number of DE transcripts per category. b) Distribution of 5' UTR lengths and 3' UTR lengths for upregulated (red) and downregulated (green) transcripts, along with UTRs of brain-expressed transcripts and UTRs across the whole genome. p-values for comparison between up and downregulated 5' UTRs = 6.996e-05, 3'UTRs = 0.001748. Brain-expressed transcripts have significantly longer 3'UTRs than the rest of the genome (Ramsköld et al., 2009). c) RBP motif enrichment in 3' UTRs of upregulated transcripts when compared to 3' UTRs of brain-expressed transcripts. Selected RBP motifs with highest or lowest enrichment and distinct motif sequences are shown. Gene symbols were converted to mouse homologs.

Figure 3-4. Minimal overlap of DE transcripts between time points and enrichment of transcription-associated genes within temporally co-regulated transcripts. a) Numbers of significant transcripts at each time point and shared transcripts between time points. b) STEM analysis grouping of significant differentially-expressed transcripts into three temporal profile clusters. Black line represents model temporal profile. c) Selected GO terms enriched in cluster 1 transcripts. Numbers represent number of DE transcripts per category. Clusters 2 and 3 did not show enrichment for any GO terms when taken separately or combined. d) The log2 fold-changes at each time point for genes belonging to the GO category "Regulation of transcription, DNA-dependent".

Figure 3-5. Marginal overlap of DE transcripts between TRAP-seq and RNA-seq at each time point, with a specific enrichment of cytoskeletal and cell adhesion genes identified in transcripts only DE in the ribosome-associated population. a) Overlap of significant transcripts at each time point between RNA populations. b) Numbers of transcripts that were identified in one or both populations, collapsed across time. c) Enriched functional categories within exclusively ribosome-associated DE transcripts, with genes corresponding to each category. Genes were assigned based on PANTHER annotation in conjunction with WebGestalt results.

Figure 3-6. Neuron-enriched transcripts demonstrate significantly greater fold-change magnitudes by TRAP-seq than RNA-seq. Each point represents one transcript that was both neuron-enriched and DE by TRAP-seq.

Figure 3-7. Enrichment of transcription factor binding in RNA-seq DE transcripts suggests potential regulators of differential expression, and some DE transcripts are enriched in non-neuronal cell types. **a)** Transcription factor enrichment ordered by Fisher score, which represents

overrepresentation of transcripts containing motif over background levels. TRANSFAC analysis identifying motif overrepresentation within our significant transcripts was also performed (Table S9B). **b)** Numbers of DE transcripts that were enriched in each cell type (see methods). **c)** Representative immunohistochemistry for the microglial-enriched transcript *Il1b* demonstrating an increase in Il1b protein level (red) following LTP induction at 120' **d)** Grouped data from Il1b immunostaining (n = 2 animals, 11 slices). Scale bar = 20 µm. Data points represent the mean pixel intensities from ROIs around cell bodies and processes from single slices. p = 0.0122, error bars = SEM.

Supplemental figure legends

Figure 3-S1. Slice-induced and age-dependent differences in activation of translational signaling pathways and immediate-early gene upregulation respectively. a) Upregulation of phospho-ERK1/2 and phospho-eIF4E following cutting of hippocampal slices. Phospho levels were normalized to total levels at each time point. b) Upregulation (by TRAP) of immediate-early genes *Arc* and *Fos* in 10-12 week old mice and 10.5-12 month old mice (see methods for normalization).

Figure 3-S2. Correlation between replicates of TRAP-seq (IP) and RNA-seq (X) at each time point and condition. Pearson correlation was generated from log normalized counts comparing replicates at one time point ie 30 min compared to other 30 min samples only.

Figure 3-S3. qPCR validation of sequencing results. Every single candidate we tested is included here. a) Fold-changes of immediate early genes (IEG) by qPCR from a separate biological replicate compared with fold-changes from sequencing results. b) Fold-changes by qPCR of separate 60 and 120 minute biological replicates compared with sequencing results. c)

Fold-changes by qPCR of a separate 60 minute IP and total sample compared with sequencing results.

Figure 3-S4. RNA-seq of total RNA following LTP induction shows an increase in both number of DE transcripts and magnitude of DE over time. a) Number of DE transcripts gradually increases over time, predominantly for upregulated transcripts. b) List of the most significantly DE transcripts at 120 minutes ordered by FDR, and their respective log2 fold-changes at 30, 60, and 120 minutes. c) Heat map of significant transcripts from all time-points and their fold-changes. Color key represents log2 fold-change values. d) Large overlap of DE transcripts between early time points and 120 minutes.

Figure 3-S5. Differential enrichment of biological functions in transcripts DE only by TRAP-seq, only by RNA-seq, or by both. Gene ontology enrichment of transcripts was calculated using WebGestalt with a background transcript list derived from our RNA-seq expression levels.

Figure 3-1

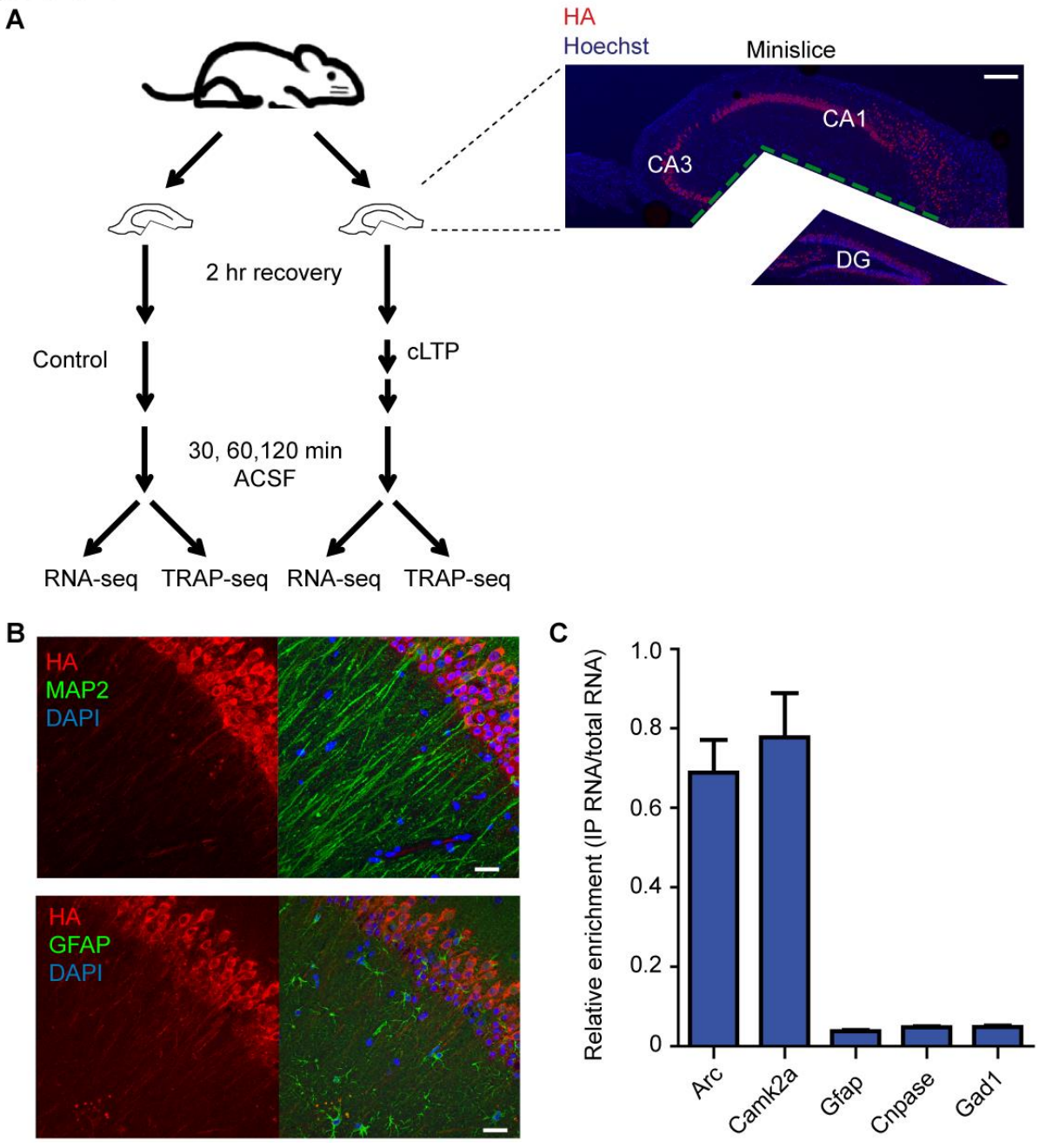
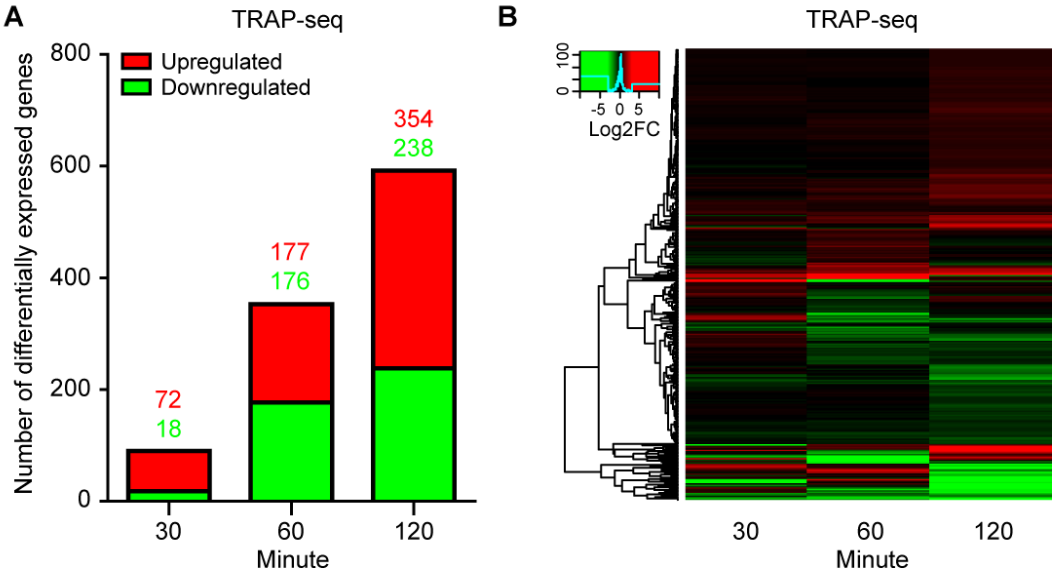

A
HA
Hoechst
Minislice
CA1
CA3
DG
2 hr recovery
Control
cLTP
30, 60,120 min
ACSF
RNA-seq
TRAP-seq
RNA-seq
TRAP-seq
B
HA
MAP2
DAPI
HA
GFAP
DAPI
C
Relative enrichment (IP RNA/total RNA)
1.0
0.8
0.6
0.4
0.2
0
Arc
Camk2a
Gfap
Cnpase
Gad1

Figure 3-2



C

Gene	Description	120' log_2FC
Btg2	B-cell translocation gene 2, anti-proliferative	3.00
Nr4a1	nuclear receptor subfamily 4, group A, member 1	1.87
Junb	Jun-B oncogene	1.97
Dusp1	dual specificity phosphatase 1	1.99
Atf3	activating transcription factor 3	2.51
Klf4	Kruppel-like factor 4 (gut)	2.12
Trib1	tribbles homolog 1 (Drosophila)	2.01
Tiparp	TCDD-inducible poly(ADP-ribose) polymerase	1.71
Ptgs2	prostaglandin-endoperoxide synthase 2	1.32
Arc	activity regulated cytoskeletal-associated protein	2.09
Ppp1r15a	protein phosphatase 1, regulatory (inhibitor) subunit 15A	1.20
Hes1	hairy and enhancer of split 1 (Drosophila)	-0.82
Mylip	myosin regulatory light chain interacting protein	-0.42
Bbc3	BCL2 binding component 3	-0.67
Klf10	Kruppel-like factor 10	-0.42
Gpr146	G protein-coupled receptor 146	-0.69
Zbed4	zinc finger, BED domain containing 4	-0.81
Trp53rka	transformation related protein 53 regulating kinase A	-0.44
Pard6b	par-6 (partitioning defective 6) homolog beta (*C. elegans*)	-0.69
Ccnj	cyclin J	-0.72
Gpx3	glutathione peroxidase 3	-1.91
Lamb3	laminin, beta 3	-0.78

Figure 3-3

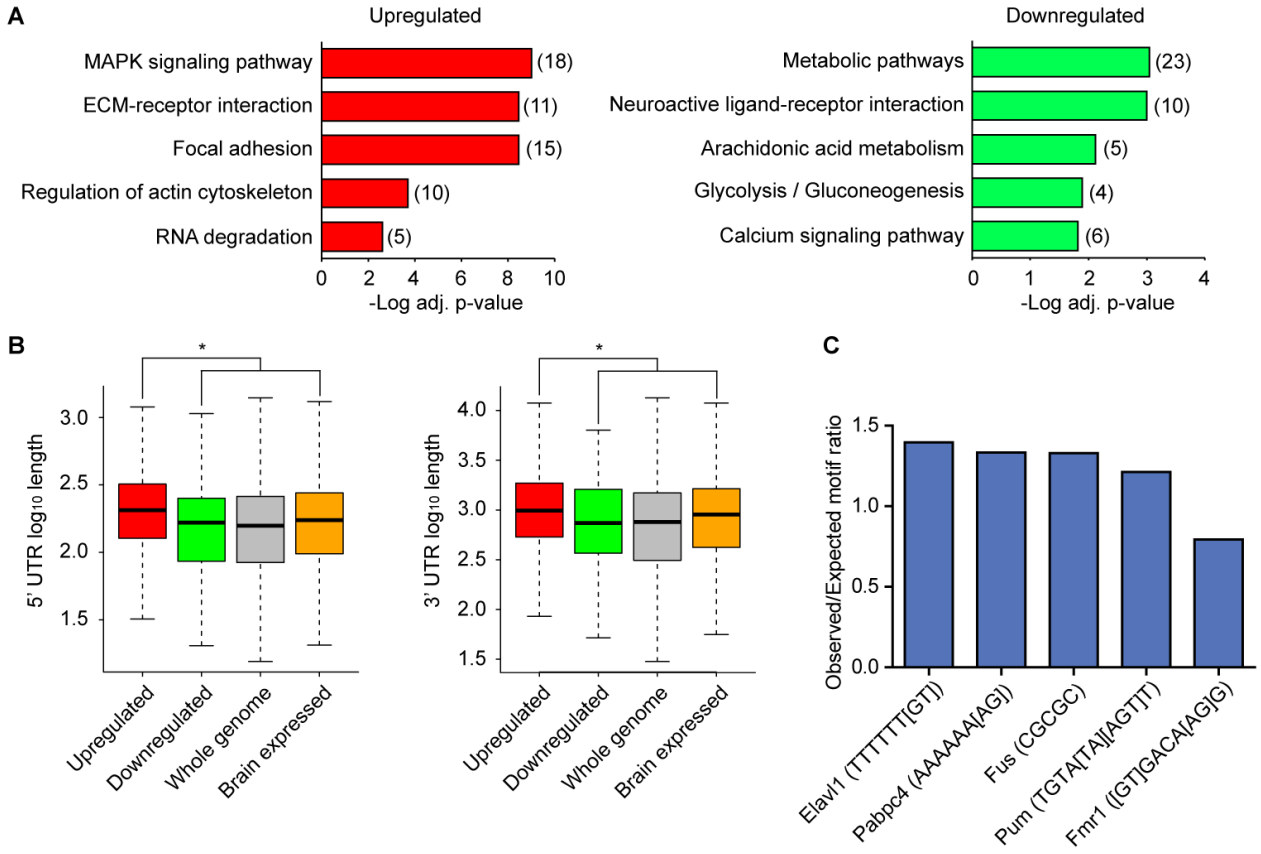
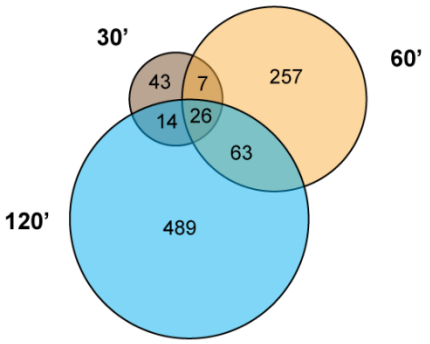
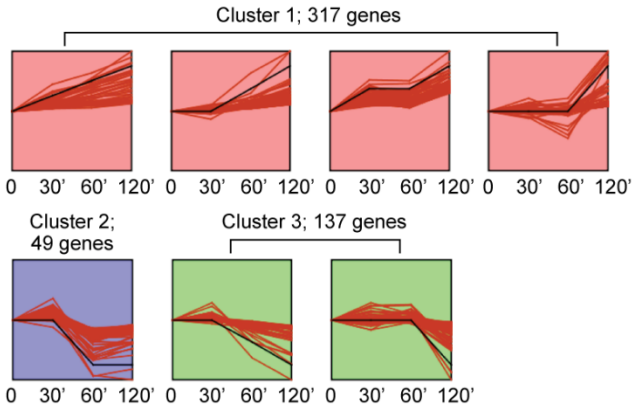

A
Upregulated
MAPK signaling pathway (18)
ECM-receptor interaction (11)
Focal adhesion (15)
Regulation of actin cytoskeleton (10)
RNA degradation (5)
0 2 4 6 8 10
-Log adj. p-value
Downregulated
Metabolic pathways (23)
Neuroactive ligand-receptor interaction (10)
Arachidonic acid metabolism (5)
Glycolysis / Gluconeogenesis (4)
Calcium signaling pathway (6)
0 1 2 3 4
-Log adj. p-value
B
5' UTR log10 length
3' UTR log10 length
Upregulated
Downregulated
Whole genome
Brain expressed
C
Observed/Expected motif ratio
Elavl1 (TTTTTT[GT])
Pabpc4 (AAAAAA[AG])
Fus (CGCGC)
Pum (TGTA[TA][AGT]T)
Fmr1 ([GT]GACA[AG]G)

Figure 3-4

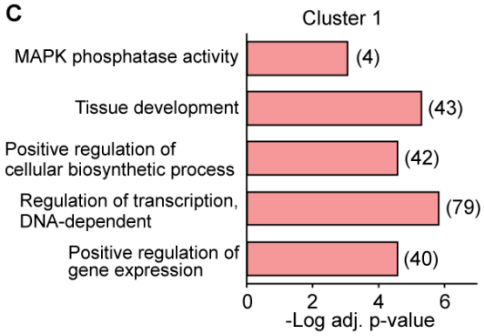
A



B



C

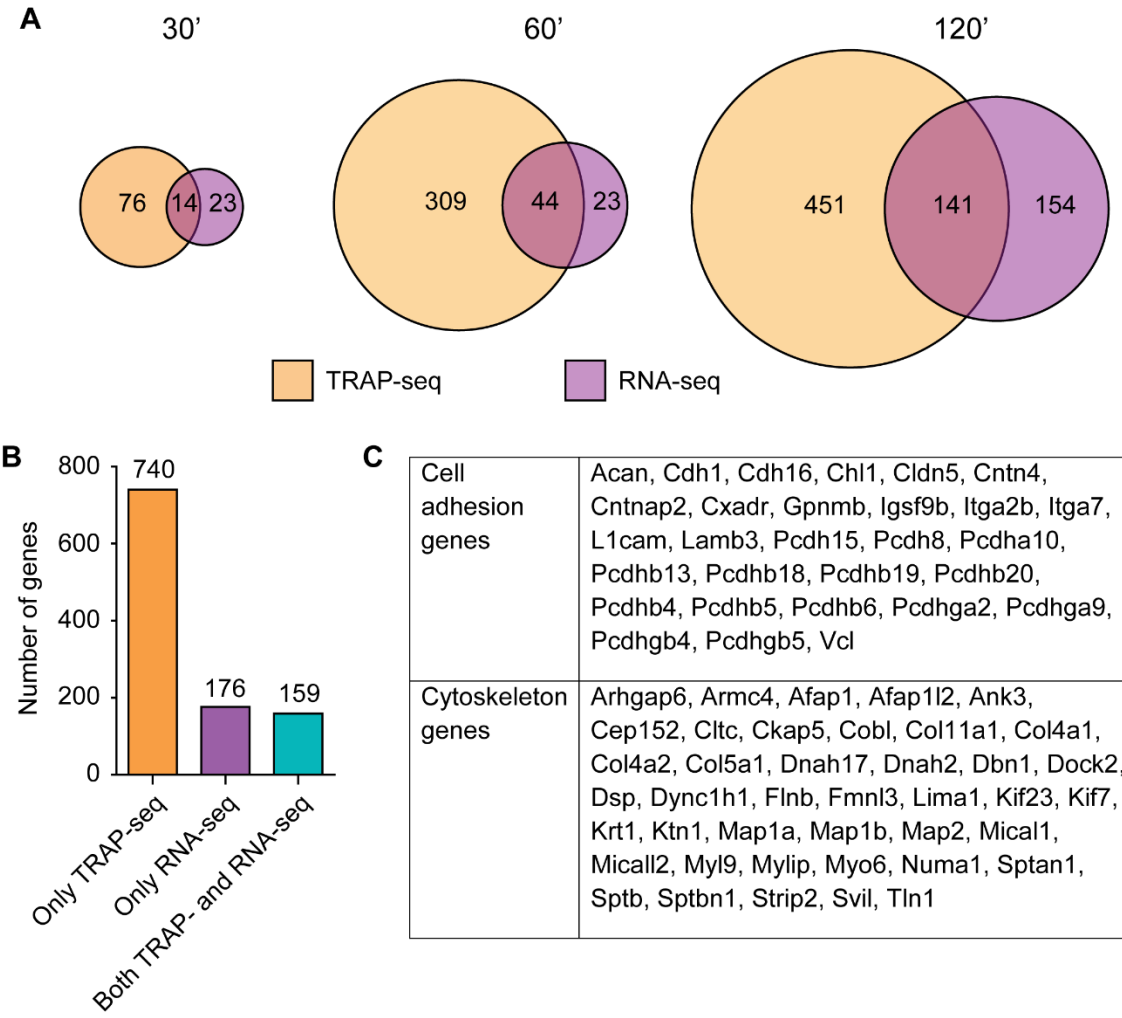


D

Regulation of transcription, DNA-dependent

Gene	30' log_2FC	60' log_2FC	120' log_2FC
Nfil3	0.27	0.59	1.24
Hey2	0.24	0.52	0.72
Satb2	0.12	0.30	0.47
Sox11	0.12	0.23	0.83
Bcl6	0.29	0.29	0.54
Tob1	-0.03	0.34	0.52
Sox9	0.27	1.05	1.49
Csrnp1	0.02	0.29	0.95

Figure 3-5



C

Cell adhesion genes	Acan, Cdh1, Cdh16, Chl1, Cldn5, Cntn4, Cntnap2, Cxadr, Gpnmb, Igsf9b, Itga2b, Itga7, L1cam, Lamb3, Pcdh15, Pcdh8, Pcdha10, Pcdhb13, Pcdhb18, Pcdhb19, Pcdhb20, Pcdhb4, Pcdhb5, Pcdhb6, Pcdhga2, Pcdhga9, Pcdhgb4, Pcdhgb5, Vcl
Cytoskeleton genes	Arhgap6, Armc4, Afap1, Afap1l2, Ank3, Cep152, Cltc, Ckap5, Cobl, Col11a1, Col4a1, Col4a2, Col5a1, Dnah17, Dnah2, Dbn1, Dock2, Dsp, Dync1h1, Flnb, Fmnl3, Lima1, Kif23, Kif7, Krt1, Ktn1, Map1a, Map1b, Map2, Mical1, Micall2, Myl9, Mylip, Myo6, Numa1, Sptan1, Sptb, Sptbn1, Strip2, Svil, Tln1

Figure 3-6

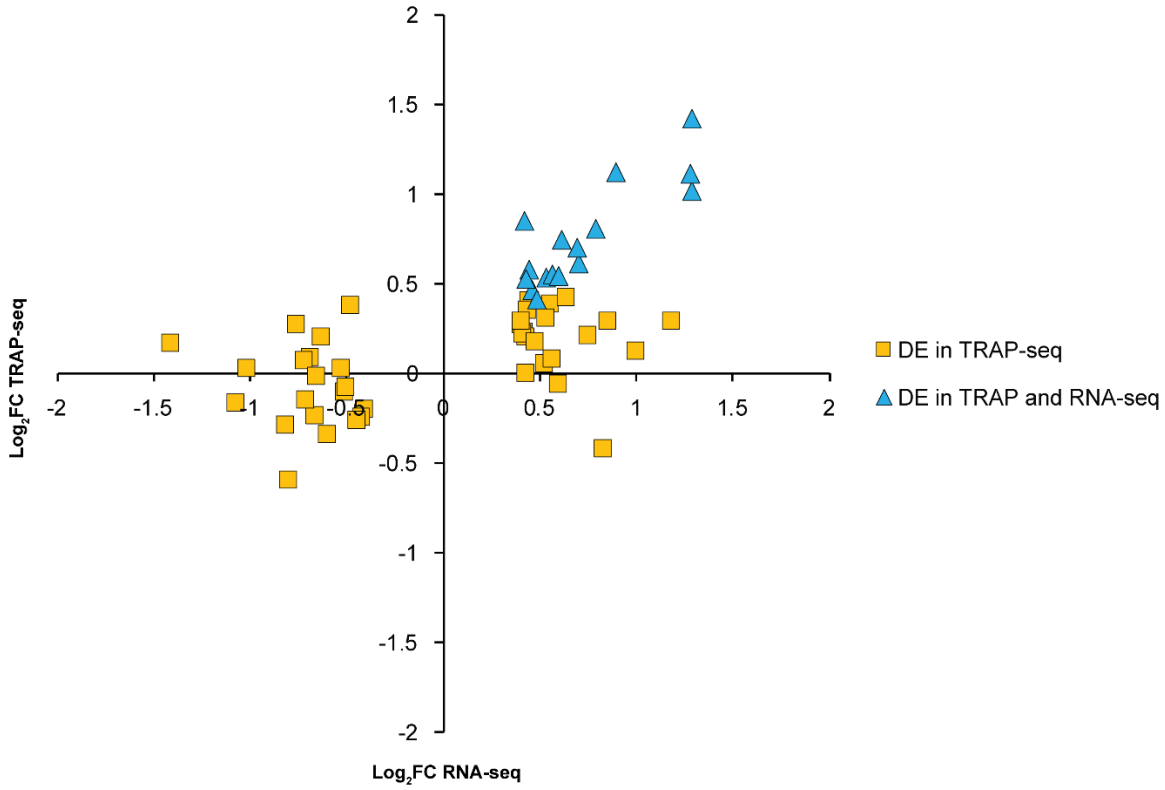

2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2
-1.5
-1
-0.5
0
0.5
1
1.5
2
Log2FC TRAP-seq
Log2FC RNA-seq
DE in TRAP-seq
DE in TRAP and RNA-seq

Figure 3-7

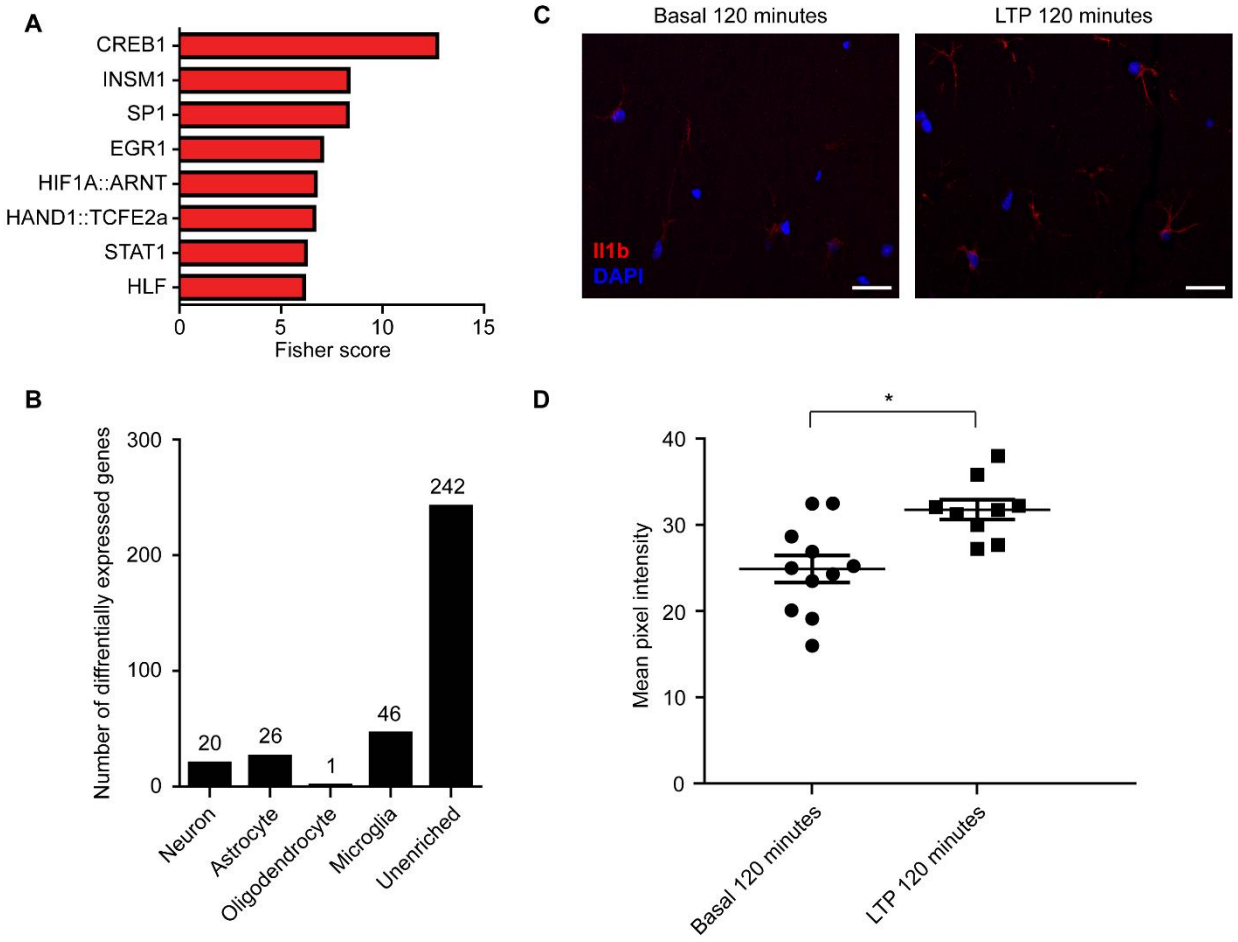

A
CREB1
INSM1
SP1
EGR1
HIF1A::ARNT
HAND1::TCFE2a
STAT1
HLF
0
5
10
15
Fisher score
C
Basal 120 minutes
LTP 120 minutes
Il1b
DAPI
B
Number of diffrentially expressed genes
300
200
100
0
20
26
1
46
242
Neuron
Astrocyte
Oligodendrocyte
Microglia
Unenriched
D
*
Mean pixel intensity
40
30
20
10
0
Basal 120 minutes
LTP 120 minutes

Figure 3-S1

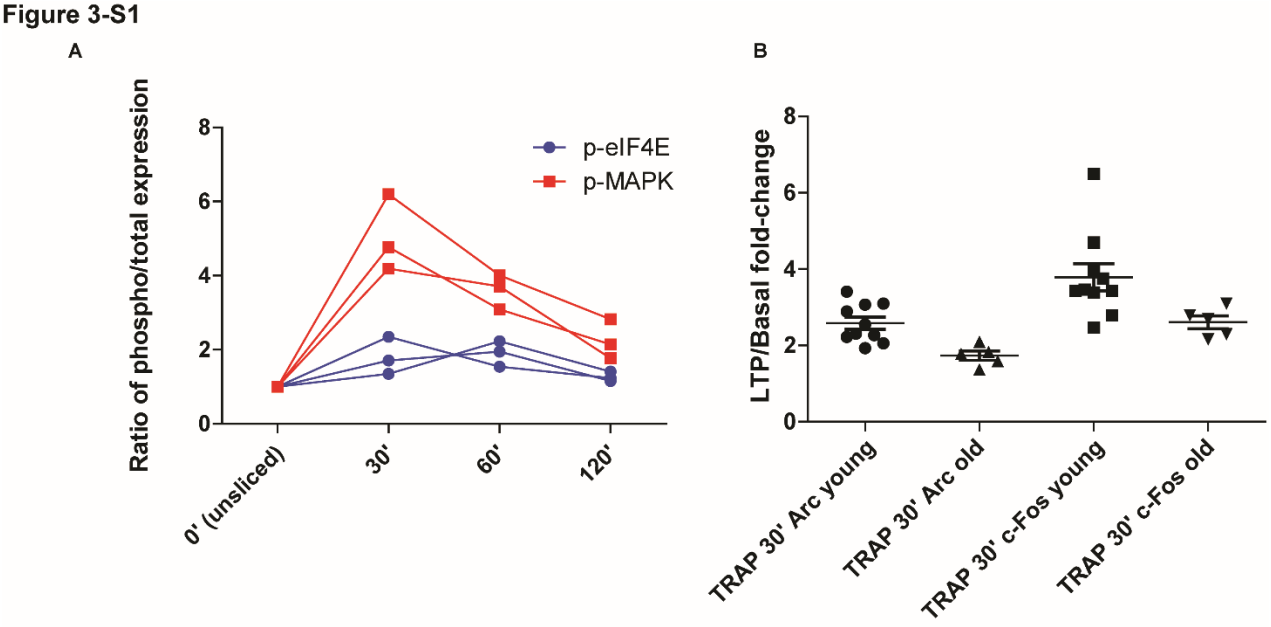
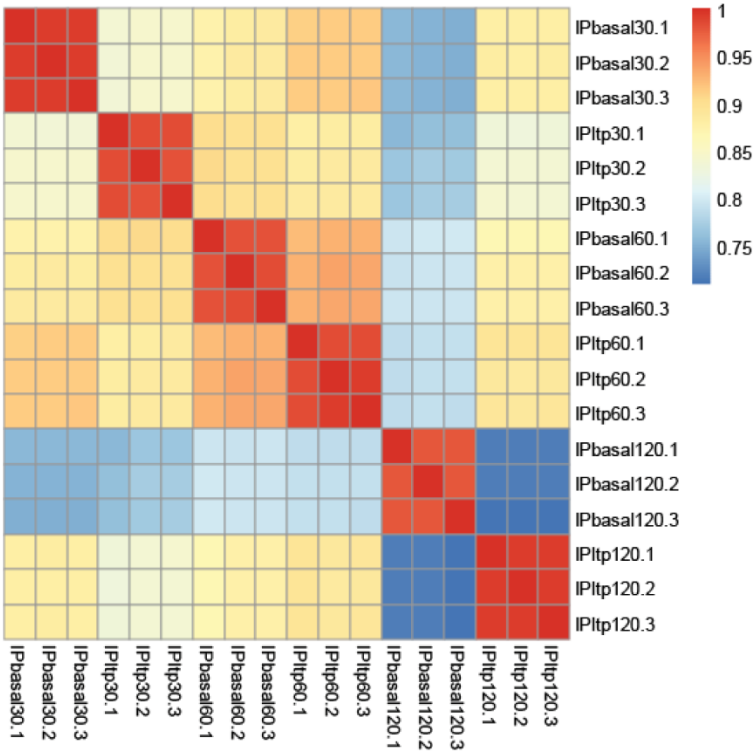

A
Ratio of phospho/total expression
8
6
4
2
0
p-eIF4E
p-MAPK
0' (unsliced)
30'
60'
120'
B
LTP/Basal fold-change
8
6
4
2
0
TRAP 30' Arc young
TRAP 30' Arc old
TRAP 30' c-Fos young
TRAP 30' c-Fos old

Figure 3-S2


IPbasal30.1
IPbasal30.2
IPbasal30.3
IPltp30.1
IPltp30.2
IPltp30.3
IPbasal60.1
IPbasal60.2
IPbasal60.3
IPltp60.1
IPltp60.2
IPltp60.3
IPbasal120.1
IPbasal120.2
IPbasal120.3
IPltp120.1
IPltp120.2
IPltp120.3
1
0.95
0.9
0.85
0.8
0.75

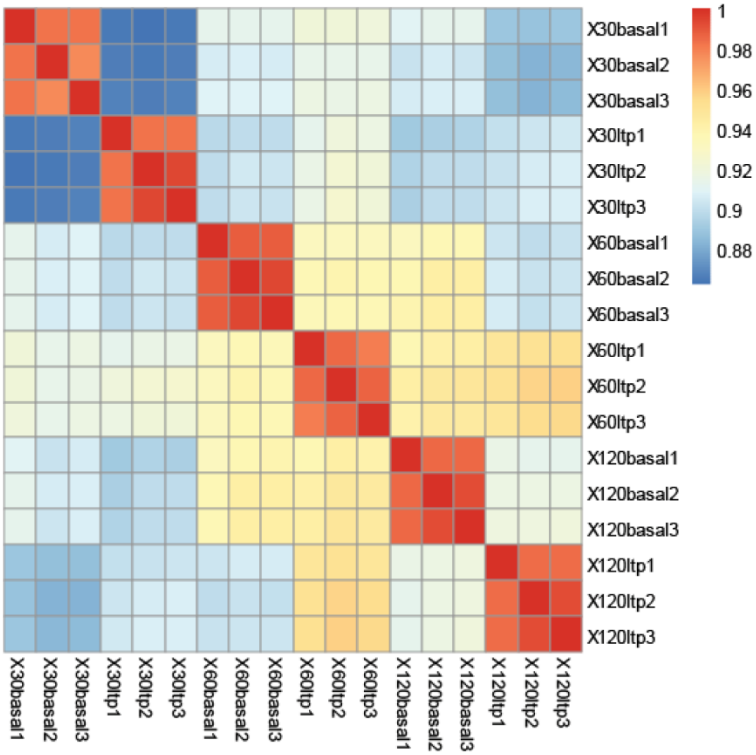

X30basal1
X30basal2
X30basal3
X30ltp1
X30ltp2
X30ltp3
X60basal1
X60basal2
X60basal3
X60ltp1
X60ltp2
X60ltp3
X120basal1
X120basal2
X120basal3
X120ltp1
X120ltp2
X120ltp3
1
0.98
0.96
0.94
0.92
0.9
0.88

Figure 3-S3

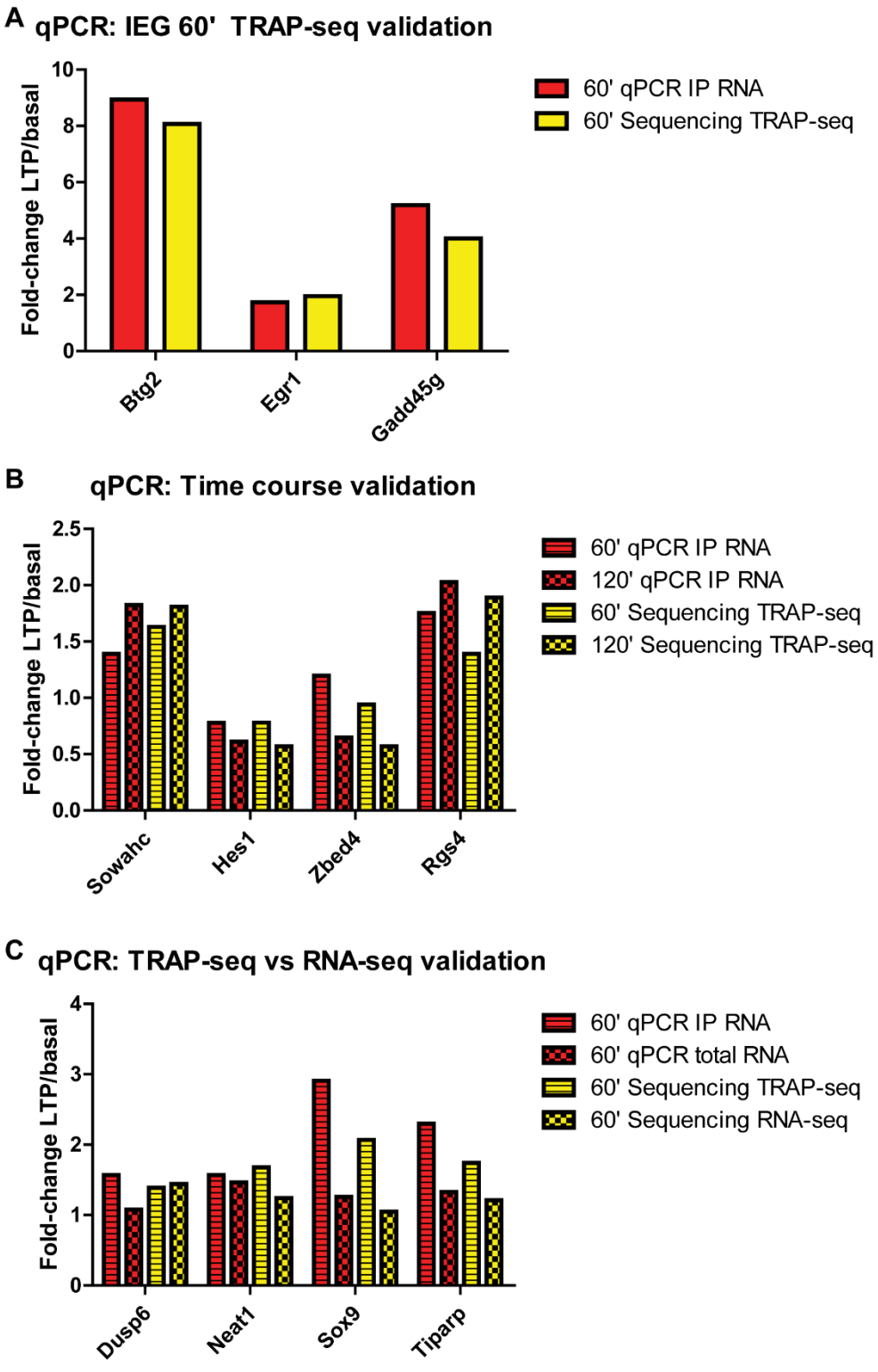
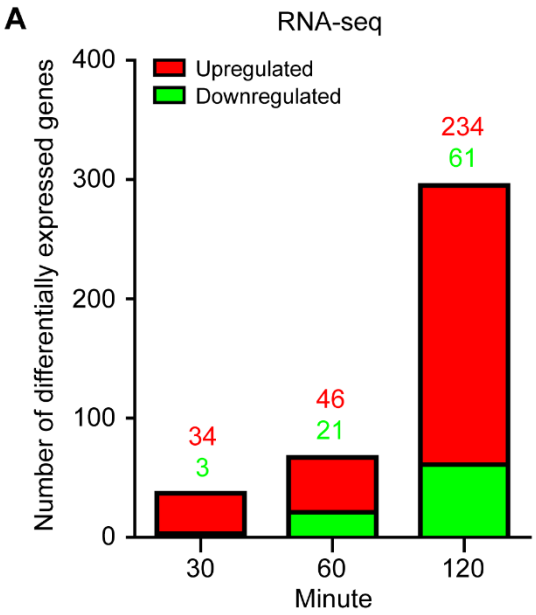
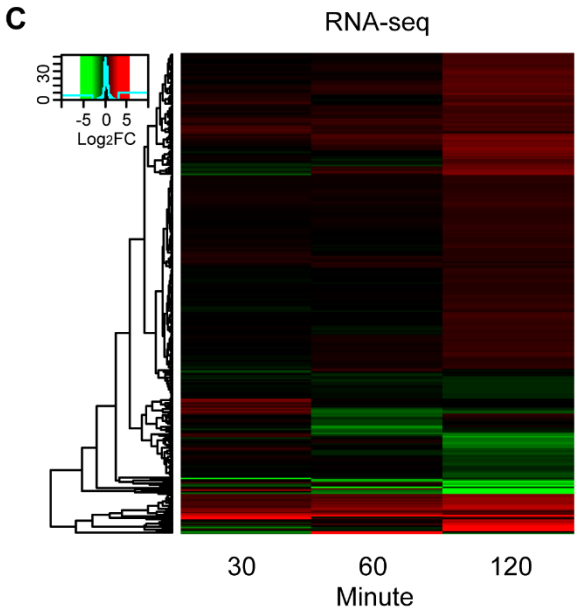

A
qPCR: IEG 60' TRAP-seq validation
Fold-change LTP/basal
10
8
6
4
2
0
Btg2
Egr1
Gadd45g
60' qPCR IP RNA
60' Sequencing TRAP-seq
B
qPCR: Time course validation
Fold-change LTP/basal
2.5
2.0
1.5
1.0
0.5
0.0
Sowahc
Hes1
Zbed4
Rgs4
60' qPCR IP RNA
120' qPCR IP RNA
60' Sequencing TRAP-seq
120' Sequencing TRAP-seq
C
qPCR: TRAP-seq vs RNA-seq validation
Fold-change LTP/basal
4
3
2
1
0
Dusp6
Neat1
Sox9
Tiparp
60' qPCR IP RNA
60' qPCR total RNA
60' Sequencing TRAP-seq
60' Sequencing RNA-seq

Figure 3-S4





B

Gene	30' log_2FC	60' log_2FC	120' log_2FC
Arc	0.94	1.32	1.76
Btg2	1.25	1.84	2.29
Egr4	0.86	1.36	2.05
Junb	0.85	1.14	1.72
Gm13889	0.76	1.06	1.52
Nr4a1	0.33	0.83	1.55
Fos	0.61	0.93	1.71
Ptgs2	0.29	0.60	1.20
Dusp1	0.33	0.86	1.28
Rgs4	0.19	0.49	0.91
Rasl11a	0.02	0.42	1.49
Fosb	0.19	0.65	1.21
Errfi1	0.12	0.35	0.95
Klf4	0.15	0.58	1.31
Tiparp	0.10	0.27	0.93
Thbs1	-0.06	0.42	1.48
Dusp6	0.29	0.53	1.09
Sik1	0.12	0.44	1.25
Cyr61	0.25	0.51	1.07
Egr2	0.65	1.33	2.24

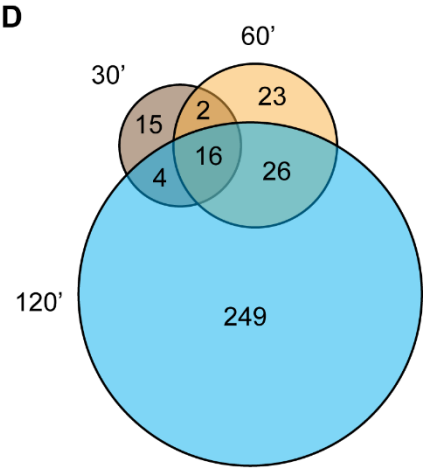
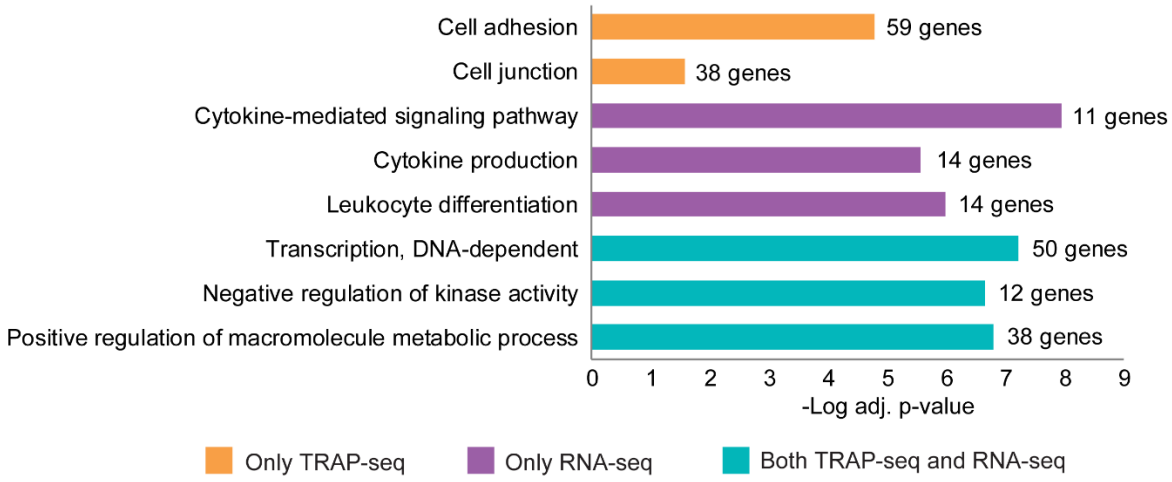


Figure 3-S5


Cell adhesion
59 genes
Cell junction
38 genes
Cytokine-mediated signaling pathway
11 genes
Cytokine production
14 genes
Leukocyte differentiation
14 genes
Transcription, DNA-dependent
50 genes
Negative regulation of kinase activity
12 genes
Positive regulation of macromolecule metabolic process
38 genes
0 1 2 3 4 5 6 7 8 9
-Log adj. p-value
Only TRAP-seq
Only RNA-seq
Both TRAP-seq and RNA-seq

Chapter 4

Whole genome techniques in neuroscience: considerations for comparisons of transcriptome results across different studies

Introduction

The complexity of the brain has required an increasing specialization of neuroscientific domains or fields of study. From this stratification, great progress has been made in elucidating the functions of the nervous system at genetic, molecular, cellular, circuit and behavioral levels. However, successful attempts to unify findings between the fields have been largely lacking. One issue is that techniques and approaches used to study neuroscience at one particular level generate data that is incomprehensible to other levels. The demand for different tools to drive progress within a specific level of analysis of neuroscience has resulted in a polarization of understanding; researchers studying the same level may appreciate developments in their level, but outsiders are unable to understand the experimental results and are unable to critically consider the claims made of the research. The utility of this data is thus negatively impacted, as studies in other fields that are designed around the results may be based on blind faith in the presented interpretation rather than rigorous scientific rationale. Therefore, discussions about critical factors that would allow for a cross-discipline understanding of data will be important for the continued progress of neuroscience research.

This issue is best demonstrated by the growing popularity of whole-genome approaches in studies of the molecular and genetic processes underlying neuronal physiology and organismal behavior. Usually, these approaches aim to quantify expression of genes from cell-types, development profiles, or regions of the brain [54,55,67], or to quantify differences in expression of genes between two states (stimulated against unstimulated neurons, different cell-types, etc.)[90,104]. For example, in the field of hippocampal-dependent learning and synaptic plasticity, transcriptomic approaches are increasingly used to characterize the differential expression of

genes following induction of different long-term potentiation (LTP) protocols or memory training paradigms (see Table 4-1). The persistence of LTP and memory requires new gene expression[44,45], making whole-genome methods (such as microarrays and RNA sequencing, or RNA-seq) an attractive strategy to identify critical genes underlying plasticity. An additional benefit of these approaches is that these methodologies identify not only individual differentially-expressed genes, but also elucidate broad molecular processes and networks that may be involved in synaptic plasticity. However, the variability of the reported results from whole-genome studies in the context of synaptic plasticity and physiology, and the difficulty in accessing the raw data, significantly limits the potential of genome-wide studies in understanding brain function. Two important and interrelated standards need to be implemented by the scientific community to overcome these limitations: explicit, appropriate and standardized criteria for determining significance of data, and standardized, easily-accessible formats for presentation of primary, raw sequencing data.

Standards for significance

Significance is widely associated with a 0.05 probability that the result is due to chance (p-value < 0.05), as this is the standard accepted by the scientific community to indicate that a finding is unlikely to be due to chance. However, the failure in understanding the importance of appropriate statistical tests, and the limitations of these tests, has affected the reproducibility of results across all fields of neuroscience[105,106]. For example, when testing for significant differences between two conditions for multiple variables at once (such as in electrophysiology when measuring conductance, post-synaptic potentials, mini frequencies, decay constants, etc.), a statistical threshold of $p < 0.05$ for each variable does not fit the widely-accepted criteria of a

finding above chance. As the number of variables tested increases, the probability of finding a false positive also increases. This is simply because when more measurements are made, there is a higher probability that one positive result is just due to chance. For example, when testing 100 variables at once, one would expect that just by chance there would be 5 significant hits with a $p < 0.05$ cutoff. In other words, with an established $p < 0.05$ cutoff, while testing of one variable would have a 0.05 probability of a significant result being due to chance, two tests would have a 0.1 probability, three tests a 0.15 probability, and so on. This is above the generally accepted probability of a chance result that would normally be accepted. Thus, a multiple comparisons adjustment needs to be applied[107,108]. This is particularly important for whole-genome studies, since these experiments effectively carry out statistical testing for differential expression of up to 24,000 different genes.

Of the 25 articles included here[56–60,85,109–127] that report differential expression following memory training or LTP induction paradigms, no two research groups determined differential expression of transcripts using the same metrics (Table 4-1). In both microarray and RNA-seq studies, basic considerations such as whether to include statistical testing, whether to correct for multiple comparisons in the statistical testing, and whether to incorporate any other cutoffs differed by research group. Both the lack of statistical testing and the lack of multiple comparisons corrections for these tests result in increased numbers of false positives when carrying out thousands of statistical tests. While a good portion of the studies in Table 4-1 have justified their criteria (or lack thereof) by validations through other means, it is unclear whether these validations are simply the most likely candidates to be differentially expressed (ie testing only the most differentially expressed genes) or whether they represent a true sampling of the detected differentially-expressed population. Many different analysis packages are now available

and widely used for the analysis of high-throughput data[63,128] that correct for multiple comparisons, which should be heavily favored when selecting analysis pipelines for high-throughput data. A nearly identical problem was experienced and addressed by the neuroimaging field[129], which routinely deals with over 100,000 voxels per experiment and has learned the importance of multiple testing corrections.

Even after correcting for multiple comparisons, statistically significant noise is still common when comparing differences between conditions. One major source of noise derives from the severely underpowered nature of whole-genome experiments. Studies routinely utilize two or three replicates per condition and test for differential expression; for any other methodology this number of replicates would be considered insufficient. As a result, the expression levels of samples in one condition may be slightly greater than another just due to chance, and this difference may be considered statistically significant. Thus, although statistical corrections may alleviate some issues of false positives, it is insufficient for such low replicate numbers to account for them all. In support of this, a recent study tested 48 biological replicates of a two-condition experiment to compare results from low replicate numbers and high replicate numbers[130]. When randomly sampling three replicates from the 48, the study found a true positive rate of only 20-40% for three replicates relative to analysis done on all 48, even *after* multiple comparison corrections (of note, the authors found that a robust fold-change cutoff increased the fraction of true positive results to ~80%). Thus, even with proper statistical approaches, the severely underpowered nature of whole-genome studies makes them prone to false positive results. Replicates for the studies included in Table 4-1 were between 2 to 6 per study, falling well within the range of replicates that are highly susceptible to inaccurate interpretation of results.

Another source of statistical noise is directly tied to the expression levels of genes, with lowly-expressed genes sometimes showing highly significantly differential expression. While the rough considerations for defining significance in current analysis pipelines include fold-change between conditions, variance between replicates, and expression level, the modeling of the data distribution fails at the extreme ends of any one of these criteria. When the observed distribution of the data falls well outside of the assumptions critical to the statistical modeling, results from significance testing are not interpretable. Lowly-expressed genes tend to have a much higher fold-change difference and higher proportional variance between replicates, resulting in increased numbers of false positives at this expression level. This effect is compounded by small replicate sizes. No studies included in Table 4-1 utilize an expression level cutoff, despite the inability of pure statistical analyses to properly account for these factors.

From this emerges a clearer picture of the considerations required to more rigorously analyze whole-genome data. I propose that all experiments utilizing whole-genome tools should take into consideration three factors when deciding on significance cutoffs, as a single cutoff is not sufficient to mitigate the number of false positives within datasets. An adjusted p-value cutoff, a fold-change cutoff, and an expression level cutoff should all be used to increase the true positive rate of experimental results. The exact cutoffs do not necessarily have to be the same between studies (as this will depend on the variance between replicates, number of replicates, etc. of each individual study), but some cutoff with these criteria should at least be included. One important note is that a fold-change cutoff for statistical significance is not indicative of a biological threshold for an effect, as smaller changes in important genes could still have a measurable biological impact. However, due to the small replicate numbers for most studies, difficulty in distinguishing between a false and true positive necessitates a fold-change cutoff to

minimize spurious results that arise purely from statistical noise. With these considerations, we may gain a more accurate characterization of the full molecular systems underlying complex neuroscientific processes, and move beyond studying only the most highly differentially-expressed genes that have served as lynchpins for different fields.

Accessibility of data

One of the attractive qualities of utilizing whole-genome approaches is the view that complex genetic and molecular pathways may help explain higher-level functions of the nervous system. Thus, making whole-genome data accessible (and interpretable) to researchers in all fields of neuroscience should be a priority. Currently, there are no standards for publishing large datasets that are generated from whole-genome studies. Individual studies parse their starting data into lists of genes they wish to discuss, and consequently only publish the parsed list of genes. Most of the gene tables end up in supplemental data, meaning that there is minimal or nonexistent editorial oversight by journals that publish the data. As a result, each list of genes published in a study has a variable list of qualifiers attached to it. Some lists include a combination of expression levels, fold-changes, and p-values, while others include nothing but the gene name and the qualifier referenced in the paper (see supplementary tables of Lee et al., 2016 and Cajigas et al., 2012). All of these values would have a profound impact on decisions regarding future experimentation, yet they are omitted in most studies.

A recent example of the highly limited nature of published data comes from a study that examined differences in gene expression between dorsal and ventral hippocampal neurons[132]. The published list of differentially-expressed transcripts between dorsal and ventral hippocampal gene expression only includes those genes with a fold-change greater than three. This high fold-

change cutoff is quite rigorous, and serves the authors' intent to only consider genes that are heavily enriched in one or the other hippocampal region for their studies. However, researchers interested in a specific gene or families of genes might find that a 3-fold cutoff may be too great, as an entire family of genes enriched by 2-fold would still be interesting and possibly functionally relevant. Researchers in other areas, such as physiologists or behavioralists, may instead want to look at specific genes and decide for themselves whether the degree of enrichment or non-enrichment is relevant to their own fields. This is not possible with a published gene list that only includes the authors' criteria, but would be possible if the pre-filtered data were accessible somewhere. Compounding the problem is the fact that every study has a different standard for significance, which makes a direct comparison between published study results impossible without acquisition and manipulation of the original data files. For labs that regularly analyze this kind of data, obtaining and using the raw data is still a hassle; for labs with no experience handling this data, this is nearly impossible. These issues limit the accessibility of whole-genome data for the researchers who may benefit the most from it, and limit their ability to critically consider the data for themselves.

One important point to emphasize is that statistical significance is not created equal. This becomes especially apparent when all replicate data is made available. While variability is taken into consideration when testing for significance, no statistical test can fully account for the variability if it is too large. Higher variability leads to an increase in the likelihood of a significant result being a false positive. But, aggregated or collapsed significance results hide this variance and may mislead the reader into believing a finding is more robust than it actually is. Journals are pushing to enforce scatterplots instead of plunger graphs for this very reason; whole-genome data should be held to the same standard. No published data in Table 4-1 includes

individual replicate information, despite this being a critical determinant of significance. By publishing the expression levels of each replicate for each condition, others who use the data would be able to see the degree of reproducibility and decide for themselves whether it is appropriate for their own interests.

In theory, all of this information is contained within uploaded data to the GEO database run by NCBI. All of the raw data files—both raw sequencing files and unparsed output files from analysis pipelines (usually a table)—are uploaded from data that is published in journals. However, these files are not standardized in a meaningful way and are not interpretable for researchers with minimal bioinformatics background. In uploaded tables to the GEO database (and even in many published tables), there are ambiguous labels for the columns that supposedly contain the data that led to the authors' conclusions. Each study's spreadsheets are formatted differently and often do not include necessary descriptors for table contents. Many experiments also do not include individual replicate data in the raw data. Having an easily-interpretable table with all of the important values will be critical if others are to use this data.

One important consideration that is not fully addressed here is the effect of using different analysis pipelines to analyze data. The choice of analysis pipeline has a significant effect on the numbers of significant genes that are identified[133,134]. This difference between results likely arises from the differences between the assumptions made about the data by each analysis tool and how closely the assumptions match the experimental data. Thus, the appropriate tool must be considered on an experiment-to-experiment basis. Regardless, including the important information outlined above from even a single analysis package would be a large improvement over existing data accessibility.

From this, a number of suggestions can be implemented to facilitate the access to and interpretation of whole-genome data. Published data should be carefully annotated and at a minimum should include a statistical metric, expression levels, and fold-changes for all genes listed in the supplementary tables. Raw data and data tables uploaded to GEO should be annotated appropriately, regardless of analysis pipeline used, so that others with minimal expertise can easily understand the information contained within the different columns produced from the analysis programs used. The raw data table should also include separated data from individual replicates for all genes in the genome. Implementing these suggestions will allow scientists from all areas of research to use the generated data and draw their conclusions based off their own critical examination.

Concluding remarks

The explosion of high-throughput data has been and will continue to be invaluable in providing insight into the different functions of the brain. However, to prevent the needless redundancy of experiments and to expedite the discovery of new findings, the community must work to improve the standards by which it assesses the data, reports the data, and publishes the data, to help others use the data as well. Science is a collaborative endeavor by nature, and is increasingly requiring the efforts of multiple groups to rigorously tackle complex biological questions. It would be in the scientific community's best interest to help each other in unraveling the mysteries of the brain by facilitating the communication of information between the neuroscientific disciplines.

Chapter 5

Conclusions

The results from the RNA-seq and TRAP-seq (Chapter 3) raise a number of important questions about gene expression during LTP and provide a framework for future experimental designs. The most obvious experiments to complete following this work would be to select candidates that have not been previously implicated in LTP (such as examining transcription factors that have not been implicated), or candidates belonging to functional categories that have been underexplored in the context of LTP (such as genes encoding metabolic functions), and determine if and how they act to influence LTP persistence. Beyond just examining gene expression changes in neurons, experiments should also use a combination of cell-type specific unbiased whole-genome approaches to identify gene expression changes in non-neuronal cell-types alongside candidate gene studies to determine the function of these genes in non-neuronal cells. This would add to the ever-growing list of genes and cellular functions that play an important role during LTP induction. Perhaps with careful selection of interesting candidates, previously-unidentified important molecular processes will be revealed following LTP induction. However, given the numbers and temporal dependency of differentially-expressed genes, interpreting the results from these experiments in the context of LTP may prove challenging.

Another critical avenue that has remained untouched is determining the effect of different stimulation paradigms on gene expression. Given that LTP is only one form of gene expression-dependent synaptic plasticity, other forms of plasticity may require distinct genetic programs for their persistence[135,136]. For example, the natural extension from this study is to understand the effect of neuromodulators on gene expression during LTP, or to understand the effect of long-term depression (LTD) inducing-stimulation on changes in gene expression. One longstanding question about LTP and LTD is: how do molecules engaged in both LTP and LTD-inducing stimulation act to affect synaptic strength in opposite ways[137,138]? The background expression

and activity of other genes may provide a completely different context between LTP and LTD and consequently may change the molecular/cellular functions of the molecules activated during both LTP and LTD. This in turn would lead to a differential effect on long-lasting physiological changes.

Experiments analyzing gene expression following a combination of different stimulations over some period of time would provide insight into a stimulation paradigm that more closely represents the stimulation patterns in an awake, behaving animal. Indeed, even the criticality of gene expression following LTP induction is not limited by time but by stimulation intensity[18], demonstrating that stimulation following the previously-defined early, critical period of gene expression for LTP persistence can and does affect gene expression. With LTP or LTD induction experimental designs, there is generally a single period of time when the stimulation is given which is then followed by hours of recording the changes in synaptic strength using a test pulse of some low frequency. However, as the nervous system is constantly receiving inputs and the circuits involved in a given memory are constantly being activated to some degree, the subsequent inputs that a circuit receives after a memory has been formed (ie when LTP is induced) may alter gene expression differently than in a "naïve" circuit. For example, if LTD is first induced in the Schaffer collateral pathway of the hippocampus, and then followed by LTP induction, how would gene expression following LTP induction differ between this scenario and a scenario where LTD was not first induced? Using another example, LTP induction initially precludes further potentiation of synapses for a period of time (~3 hours), but later, synaptic strength can be potentiated even further with another round of LTP induction[139]. How would gene expression changes differ between the first round of LTP induction and the second round? To my knowledge, this question has remained wholly unaddressed.

One intriguing observation is that even non-LTP-inducing stimulation leads to upregulation of immediate early gene expression critical for LTP[140]. Thus, what differentiates gene expression when comparing a strong stimulation that induces LTP against a weak one that does not? Two possibilities are: 1) the difference lies in the magnitude of differentially expression, or 2) the identities of genes differentially expressed are distinct; these possibilities are not mutually exclusive. Whole-genome approaches could be used to more accurately determine the differences in gene expression induced by two strengths of stimulation. Taking this concept to the extreme: is there *any* magnitude of stimulation that does not result in some form of new gene expression synthesis? With the observation that local protein synthesis can be influenced by spontaneous release of vesicles[141], and the demonstrated importance of local translation in plasticity[142], this would suggest that even the smallest quantal unit of neurotransmission can influence gene expression in a biologically meaningful way. Yet, this is complicated by the finding that LTP-inducing stimulation does not induce LTP unless given during the positive phase of hippocampal theta oscillations[143]. From this perspective, not even a strong stimulation can induce the necessary gene expression changes to induce LTP unless coordinated with the background theta oscillation. To truly begin to understand the finer scale gene expression that may be occurring in different forms of neuronal stimulation, more sensitive and temporally-controllable tools for identifying and profiling local translation events are required. This highlights the complexity and challenges in understanding the function of gene expression in both LTP and the nervous system as a whole.

A parallel question of reducibility exists for gene expression changes that are discovered from whole-genome experiments: how many of the identified gene expression changes truly *matter* for LTP? On one extreme, it is possible that all identified genetic changes are just

consequences of a single molecule acting as a supreme master regulator. On the other extreme, it is possible that every single genetic change is required for the expression of LTP, and missing any single one abolishes LTP. Both possibilities are equally impossible—the truth probably lies somewhere in between these two extremes. However, currently there is no easy way to reconcile the hundreds to thousands of genes that are identified in any differential expression experiment and functional testing on a candidate basis. The combinatorial effects of multiple genes on this scale would be impossible to predict, and careful manipulations of even single genes are challenging in the nervous system. Although something can be learned from single gene experiments, the following example illustrates some of the challenges and considerations that must be accounted for when interpreting single gene studies.

In the context of gene expression and LTP, there are two scenarios that could happen if a given gene is knocked out and LTP persistence is tested. One is that knocking out the gene removes the persistence of LTP. From this observation, one would conclude that the gene is critical and a "significant" molecule for LTP. But if the gene that was knocked out was a transcription factor[144] or a phosphorylation site on a translation factor was removed[145] (or theoretically even any gene), there would almost certainly be widespread changes in the entire molecular milieu with these manipulations. For example, the effects on LTP of knocking out transcription factor *x* might in fact be due to the actions of a completely separate transcription factor *y* that only binds in the absence of *x*; this aberrant binding is the true cause of the effect, yet without further experimentation one would conclude that *x* is a "memory molecule". Conversely, knocking out a gene may have no effect on LTP persistence. From this observation, one would then conclude that this gene is not critical for LTP persistence. In reality, the

knockout may perturb the entire system in a way where functional compensation occurs[146]. Thus, the way a system *can* work does not mean it is the way the system *does* work normally.

Although demanding, the designing of experiments to challenge a scientist's personal hypothesis and rule out likely alternative explanations is the cornerstone of science. Failure to do so is an example of the congruence bias[147] in action, and is most likely one contributor to the issue of irreproducibility in science. Statistical problems in science are a major concern for reproducibility[105,106] (see Chapter 4) alongside a highly political, often incomprehensible peer-review system, but this is theoretically correctable with increased funding for replicate numbers and a more rigorous and transparent peer-review system. However, the risk of cognitive biases in scientific research is a far more difficult problem to root out[148]. In parallel with the congruence bias, scientists are affected by the Dunning-Kruger effect[149], otherwise termed "the double burden of ignorance": one is wrong about something and is unaware of his/her own wrongness. (An important note is that the Dunning-Kruger effect should not be thought of as occurring on the level of an individual, but instead should be thought of as occurring on the level of subject matter—we are all ignorant about something and unaware of this fact). As scientists we are trained to believe we are scholars of objectivity, but in reality we are just as susceptible to certain cognitive biases as anyone else. We are not above the influence of cognitive biases and we are also unable to recognize this fact—the Dunning-Kruger effect. Thus, it takes a significant amount of self-awareness and self-doubt to recognize when one may be wrong, and being aware of one's limits of knowledge is critical for not overcommitting to wrong ideas. This is a major hurdle in educating all practitioners of science in being self-critical and objective.

In conclusion, science is a difficult, lifelong endeavor that can be as wearisome as it is wondrous. What little incremental knowledge we may gain from this pursuit is, hopefully, "worth it" in the grand scheme of things. In some cases it is. In some case it is not. But, despite all the flaws in the practice and practitioners of science, science remains the best and only system by which to pry into the inner workings of the world.

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