CHAPTER THIRTEEN

Current Proteomic Methods to Investigate the Dynamics of Histone Turnover in the Central Nervous System

L.A. Farrelly*, B.D. Dill†, H. Molina†, M.R. Birtwistle*, I. Maze*,1
*Icahn School of Medicine at Mount Sinai, New York, NY, United States
†The Rockefeller University Proteomics Resource Center, The Rockefeller University, New York, NY, United States
[1]Corresponding author: e-mail address: ian.maze@mssm.edu

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Abstract

Characterizing the dynamic behavior of nucleosomes in the central nervous system is vital to our understanding of brain-specific chromatin-templated processes and their roles in transcriptional plasticity. Histone turnover—the complete loss of old, and replacement by new, nucleosomal histones—is one such phenomenon that has recently been shown to be critical for cell-type-specific transcription in brain, synaptic plasticity, and cognition. Such revelations that histones, long believed to static proteins

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in postmitotic cells, are highly dynamic in neurons were only possible owing to significant advances in analytical chemistry-based techniques, which now provide a platform for investigations of histone dynamics in both healthy and diseased tissues. Here, we discuss both past and present proteomic methods (eg, mass spectrometry, human "bomb pulse labeling") for investigating histone turnover in brain with the hope that such information may stimulate future investigations of both adaptive and aberrant forms of "neuroepigenetic" plasticity.

1. INTRODUCTION

Eukaryotic gene transcription is a highly complex and dynamic process mediated by several critical and coordinated mechanisms that function to govern cellular diversity and plasticity. These mechanisms, and their respective timings, are critical during organismal development and are ultimately responsible for appropriate patterns of lineage specification, preservation of cellular identity, and phenotypic variation. Patterns of gene transcription, however, can be modified by a large variety of environmental exposures, thereby inducing both short- and long-term changes in gene expression, which can then act to alter the trajectory of previously "defined" cellular states.

Neuroplasticity refers to the brain's ability to adapt to changing internal and external environmental stimuli leading to changes in neuronal function, circuit formation, structural morphology, and behavior, all of which are directed, at least in part, by altered patterns of gene expression. These gene–environment interactions are multifaceted and involve several critical mediators and intrinsic mechanisms. Some of these processes are referred to as "epigenetic" and involve histone–DNA interactions that are mediated by dynamic posttranslational modifications (PTMs) (both on histones and DNA), histone variant exchange, and nucleosomal remodeling (Maze, Noh, & Allis, 2013).

Chromatin, the DNA–protein complex that functions as the defining substrate for processes regulating cellular gene expression in eukaryotes, is comprised of both genomic DNA and core basic histone proteins. The nucleosome exists as the essential repeating subunit of chromatin and consists of an octamer of highly conserved core histone proteins (H2A, H2B, H3, and H4, or variants thereof) wrapped around ~147 bp of superhelical DNA (Luger, Mader, Richmond, Sargent, & Richmond, 1997). Modulating the accessibility of genes to the transcriptional machinery via alterations

in chromatin structure has explicit implications for gene expression in brain and has been consistently linked to neuroplasticity and cognition, as well as aberrant adaptations (Maze et al., 2013). Investigating the mechanisms of epigenetic plasticity in the central nervous system is a challenging feat; however, studies of chromatin function in brain have been increasing at an exponential rate over the last decade, effectively identifying many novel "players" and mechanisms involved in the regulation of neuroepigenetic states. Such advances have largely been due to an ever-growing interest in the role of such processes in human health and neurological disease (Cramer et al., 2011).

Histone variant proteins, which vary in primary amino acid sequence from their canonical counterparts (eg, H3.3 vs H3.1 and H3.2), play a pivotal role in cellular development, lineage commitment, and transcriptional potential (Maze, Noh, Soshnev, & Allis, 2014). H3 variants are generally less diverse than those arising from the H2A and H2B families, which are more commonly linked to the direct regulation of nucleosomal stability and are accompanied by substantial variation in their amino and carboxy-terminal "tail" regions.

In recent years, it has become clear that histone variants play critical roles in the regulation of gene transcription serving to alter the PTM landscape on chromatin or to impact nucleosomal structure via (1) recruitment of distinct chromatin effector complexes or (2) sequence-based structural effects that lead to octameric instability. Interestingly, however, in postmitotic cells, canonical histones (eg, H3.1 and H3.2) are not able to incorporate into chromatin (ie, they are considered to be replication dependent) resulting in an imbalance between chromatin-associated levels of "variant" histones (eg, H3.3), which are typically incorporated into chromatin in a replication-independent manner, and canonical isoforms (Maze et al., 2014).

Recent work from our laboratory demonstrated that H3.3, but not H3.1/2, turnover in brain is extraordinarily high during early phases of neurodevelopment and remains constitutive, albeit at lower rates, for the remainder of life following rapid accumulation of H3.3 from late embryogenesis to mid-adolescence (Maze et al., 2015). We later showed, using many of the recent methods described throughout this chapter, that these histone turnover events are activity dependent, are critical for cell-type-specific transcription, are necessary and sufficient to drive both normal and aberrant patterns of synaptic connectivity/plasticity, and are essential for mammalian cognition (Fig. 1). Here, we provide a comprehensive overview of the proteomic methods, both old and new, that can be utilized to

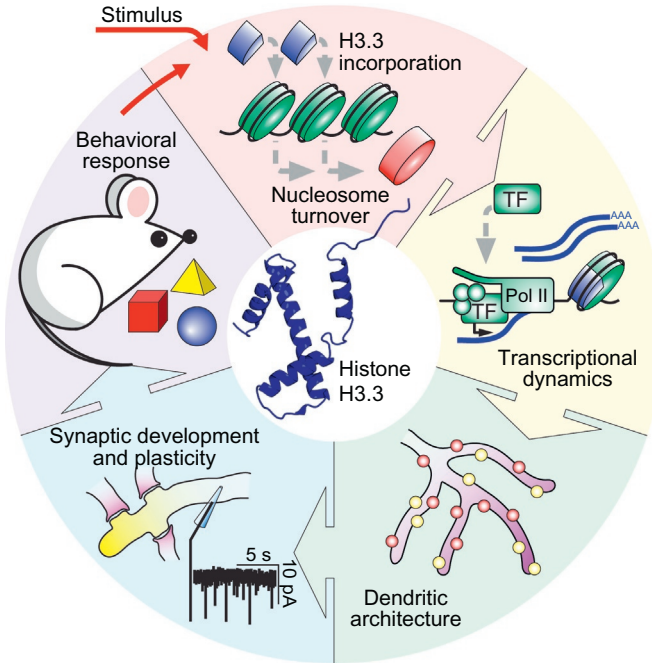

Fig. 1 Histone turnover is a critical mediator of neurological plasticity. In the CNS, during periods of heightened neuronal activity and cellular plasticity, H3.3-associated nucleosomal turnover is increased to allow for activity-dependent transcriptional plasticity that results in alterations in dendritic architecture, synaptic connectivity, and behavioral plasticity (eg, cognition).

investigate histone turnover in brain, future investigations of which will surely provide great insights into our understanding of human neurological disease and psychiatric disorders.

2. EARLY METHODS TO STUDY HISTONE TURNOVER IN BRAIN

There is a strong basis for monitoring protein synthesis and degradation in mammalian systems stemming from a basic interest in understanding cellular dynamics and their relationships to human disease. Measurements of protein turnover often require sophisticated methods that are capable of determining dynamic, and oftentimes subtle, changes resulting from the synthesis and degradation of specific polypeptides extracted from heterogeneous mixtures of both stable and dynamic molecules. Since the early 1970s, a range of methodologies have been developed. Herein, we will

briefly discuss some of the past methods used in these pioneering studies aimed at addressing the function of histone turnover.

2.1 The Introduction of Radioactive Tracers

The act of "labeling" proteins in vivo to measure protein turnover originally served to enhance the sensitivity of turnover detection and to increase quantitative measurements of protein–protein interactions. Protein labeling, in its earliest sense, was based on the incorporation of tracers into live cells. Early studies utilizing this method often took advantage of radioactivity and employed radiolabeled precursors, such as ^{35}S-labeled methionines or cysteines. Rates of protein synthesis could therefore be measured by determining the incorporation of a given label into a given cell type being investigated. In turn, protein turnover (ie, synthesis vs degradation) was then assessed through direct measurements of the incorporation and loss of the label. Radioactive labeling with ^{35}S-methionine was frequently used to label newly synthesized proteins and was therefore compatible with the study of protein turnover. An interest in investigating histone turnover commenced in the early 1970s with the overarching goal of elucidating whether histone dynamics occur independently from cellular proliferation to contribute to biological functions. Radioactive labeling, as described earlier, was used in these pioneering studies, which then "paved the way" for safer and more high-throughput methods that are utilized today. In these initial studies, Gurley and Walters radiolabeled Chinese hamster cells in tissue culture in an attempt to correlate histone turnover rates with phosphorylation events (Gurley & Walters, 1971). In doing so, they found that reductions in histone turnover correlated strongly with decreases in histone phosphorylation, suggesting that these two processes may be linked. Bondy and colleagues later measured turnover of cerebral histones using 4,5-^{3}H-leucine in the developing chick embryo and demonstrated that although all "bulk" histone fractions appeared to decay at similar rates, initially with a half-life of approximately 5 days and later with a delayed half-life of ~19 days, DNA itself was significantly more stable. They therefore concluded that histone turnover occurs independently from that of DNA (Bondy, 1971), suggesting that histone dynamics may themselves provide biological functionality in the absence of cellular proliferation.

Although informative, some of the assumptions made using these more antiquated labeling approaches were later proved to be incorrect using more sophisticated methods. For example, by labeling mammalian cells with

^{14}C-methionine, followed by hydrolysis and gas chromatography, Byvoet and colleagues originally concluded that histone methylation was irreversible following comparisons between the half-lives of histones and their methyl lysine and/or arginine components (Byvoet, Shepherd, Hardin, & Noland, 1972). Following decades of debate regarding the reversibility of histone methylation, however, Shi and colleagues identified the first histone demethylases (Shi et al., 2004), thereby refuting these earlier assumptions and changing our understanding of the stability of chromatin-templated processes.

In later studies of mouse kidney and liver cells, which are nonproliferating and terminally differentiated, specific histone variants were found to undergo continuous replacement throughout life as assessed by the incorporation of radioactive-labeled amino acids (Djondjurov, Yancheva, & Ivanova, 1983). Other classical studies used tritiated water intake in rodents to induce radiolabeling for analysis of protein turnover in rodent brain demonstrating that canonical histones (eg, H3.1 and H3.2) are remarkably stable throughout the lifetime of an animal (Commerford, Carsten, & Cronkite, 1982). Such labeling approaches work on the principle that ^{2}H atoms rapidly equilibrate across pools of water in the body following intake, whereby C—H bonds of free glycines and/or alanines, as well as several other nonessential amino acids, approach isotopic equilibrium. Protein biosynthesis then becomes the rate-limiting step for ^{2}H incorporation (De Riva, Deery, McDonald, Lund, & Busch, 2010), thus enabling extended labeling protocols for quantification of proteins exhibiting defined turnover rates (Grove & Zweidler, 1984).

2.2 Limitations of Radioactive Labeling

As discussed, protein synthesis and decay have historically been measured by incorporation and clearance of radiolabeled amino acids. Such approaches, however, are difficult to employ in animal models and are not well suited for high-throughput screening. Furthermore, many labeling procedures require the utilization of in vitro culture systems, which often do not mimic in vivo biology, especially with respect to neurons, which function as highly coordinated circuits in brain. Also, in comparison to tissue culture cells, where the amount of incorporated tracer can easily be monitored, incorporation rates in multicellular organisms are often confounded by tissue/cell-type-specific rates of protein turnover. Lastly, the use of substantial amounts of radiation carries with it obvious health and safety concerns.

3. CURRENT PROTEOMIC METHODS TO STUDY HISTONE TURNOVER IN BRAIN

Advances in the field of neuroproteomics have equipped modern investigators with a series of high-throughput methods that now allow for efficient investigations of histone turnover in brain. These techniques require novel methodologies of sample preparation coupled to mass spectrometry to allow for powerful analyses when combined with sophisticated bioinformatics tools.

3.1 Mass Spectrometry: A Brief Introduction

Modern high mass accuracy mass spectrometers coupled to nanoflow liquid chromatography (LC) systems have developed into a capable platform enabling sensitive, fast, and high-confidence identifications for quantifying histone isoform levels and modifications (Garcia, Shabanowitz, & Hunt, 2007). Mass spectrometry data can be acquired by a multitude of methodologies, but fundamentally consist of MS spectra with precursor ion mass-to-charge (m/z) signals and/or MS/MS spectra resulting from the gas-phase fragmentation of isolated, preferably individual, ions (Hunt, Yates, Shabanowitz, Winston, & Hauer, 1986). MS and MS/MS can either be used together or individually for peptide/protein identifications and quantification. In the most common workflows, peptides are generated by digestion of the sample protein using a sequence-specific cleavage enzyme, such as trypsin, which allows matching of proteolytic peptide fragments to predicted in silico digestion products corresponding to parent proteins in a sequence database (Aebersold & Goodlett, 2001; Dhingra, Gupta, Andacht, & Fu, 2005; Domon & Aebersold, 2006). Mass analyzers provide high resolution, high mass accuracy, and broad dynamic range. Their sensitivity and resolving power ensure that high-performance analyses of complex mixtures and their relatively low cost make them attractive instruments for a variety of laboratory settings.

Nano LC separates peptides/proteins, typically by C_{18} reversed-phase chromatography based on hydrophobicity, prior to being introduced to the mass spectrometer by nanoelectrospray (Vanhoutte et al., 1997) in a process termed "shotgun proteomics" (Wolters, Washburn, & Yates, 2001). Quantitative profiling can be further enhanced by the use of stable isotope labeling (eg, SILAC), through in situ labeling, subsequent chemical labeling, or spiked-in references, in advanced quantitation informatics pipelines.

It is without doubt that continued improvements in scan speed, dynamic range, and mass resolution will enable mass spectrometry to quantitate histone dynamics in a truly unbiased manner and become the forefront player in the field of chromatin studies. Sections 3.1.1–3.5 will review mass spectrometry-based methods to study histone turnover in brain ranging from label-free approaches to stable isotope incorporation.

3.1.1 Bottom Up vs Top Down

Proteomic analyses can be categorized into two broad categories: "top-down" analysis of intact proteins and protein complexes, or "bottom-up" analysis of proteins proteolytically digested (most commonly with trypsin) to provide smaller, more manageable peptides. While top-down approaches have definitive advantages in protein analytics including the avoidance of loss of information regarding the protein context of modifications and/or isoform identities, significantly more technological development in the areas of chromatographic separation and mass spectrometry instrumentation is required to properly resolve intact proteins given their inherently high multiple charge states during routine analyses. Bottom-up approaches, on the other hand, allow for robust characterizations, albeit via peptide-centric data.

3.2 Sample Preparation Considerations

The ultimate success of proteomic analyses begins with, and relies upon, appropriate and efficient sample processing. Histone core purifications from acid-extracted chromatin limit the dynamic range and complexity of the sample enabling higher sensitivity and detection of substoichiometric modifications without complicating downstream detection. Proteins can be digested directly in-solution or in-gel following SDS-PAGE separation, which allows for further separation from other contaminating proteins if necessary. Due to the significantly higher proportion of lysine and arginine residues in histones, posttranslational modifications are identified most successfully through propionylation blocking of lysines via propionic anhydride (Garcia, Mollah, et al., 2007); this step is often necessary to achieve reasonable sequence coverage with typical workflows. Not only does this process block trypsin digestion at modified lysines, thereby preventing the production of peptides too small to measure effectively in the optimal range for most mass spectrometers, it reduces the positive charge on peptides to reduce the dominant charge state of

most peptides toward +2 or +3, which produce the most interpretable fragmentation patterns in collision induced dissociation mass spectra (MS/MS).

3.3 Label Free vs Stable Isotope Incorporation

Numerous quantitation strategies have historically been used in proteomics and can be generally separated into label-free vs stable isotope-based approaches. In label-free quantitation, peptides are quantified and interpreted without referencing heavy-isotope versions. Spectral counting is the most straightforward label-free quantification technique, in which proteins can be quantified by the number of MS/MS events that can be attributed to them relying on the stochastic nature of MS/MS sampling. Otherwise, the ion intensity signals of precursor ions can be used to compare the relative abundance of a given analyte in subsequent LC–MS runs, either through maximal intensity or through chromatographic elution profiles. Neither method is based on absolute measurements but can be used to compare signals for a given peptide between subsequent analyses. Additionally, as ionization efficiency differs among peptide sequence and modification states, and since coeluting analytes can cause signal suppression, different peptides are not easily compared to one another directly. However, it is possible to compare relative signals of differing peptides to one another within samples, such as looking for a change in modification state or isoform stoichiometry via comparisons of the relative degree of modifications against an unmodified version.

In isotope-based analyses, the introduction of heavy nitrogen or carbon isotopes can be used to provide highly accurate quantitation measurements between heavy and light channels, thus reducing systematic and nonsystematic variations. Isotope labeling can also remove sample preparation biases, such as differences in digestion efficiency, fractionation patterns, and sample loss during cleanup steps, such as solid-phase extraction. Isotope labeling can be accomplished through metabolic labeling with heavy isotopes of nonradioactive amino acids, such as with SILAC (Ong et al., 2002) or ^{15}N (Wu, MacCoss, Howell, Matthews, & Yates, 2004), as well as through chemical labeling approaches, such as dimethyl labeling (Arnaudo, Molden, & Garcia, 2011), isotope-coded affinity tags (eg, ICAT; Gygi et al., 1999), or isobaric tags, such as iTRAQ (Ross et al., 2004) or TMT (Thompson et al., 2003). Isobaric tags differ from aforementioned approaches due to an ability to massively multiplex samples through the

mixing of isobarically labeled peptides that can be later differentiated by specific reporter ions in MS/MS scans.

Metabolic isotope labeling has an additional application: pulse labeling to evaluate time-resolved information pertaining to net turnover: translation, degradation, and translocation. By adding heavy media for a selected period of time, "pulse" experiments can trace histone dynamics over space and time (Schwanhausser, Gossen, Dittmar, & Selbach, 2009).

In some experimental scenarios, it is desirable to measure the absolute, rather than relative, abundance of proteins. While absolute quantitative data cannot be directly determined from quantitative values, since peptides have different physical properties that dictate ionization efficiency, absolute quantitation can be derived by comparisons to spiked-in internal standards of defined abundance (Gerber, Rush, Stemman, Kirschner, & Gygi, 2003).

3.4 Acquisition Types

Aside from quantitative schemes for measuring abundance of peptides/proteins, another important consideration is the methodology of data acquisition. In classical LC–MS/MS experiments, peptides were selected for fragmentation to derive peptide identity based on selection of the most abundant ions at a given time during the LC timescale, termed data-dependent acquisition. Particularly in analyzing complex samples, a primary limitation to peptide sampling is the duty cycle of the mass spectrometer measurements, as this occurs on a chromatographic timescale. While faster mass spectrometers have continually been developed, low-abundance proteins and modifications are still prone to under sampling.

Targeted acquisition resolves issues of undersampling and improves sensitivity limitations through *a priori* determination of masses corresponding to analytes of interest. This can be accomplished through the use of triple quadrupole mass spectrometers by selected reaction monitoring (Lange, Picotti, Domon, & Aebersold, 2008), which measure individual fragment ions after fragmenting selected precursor masses, or by mass spectrometers more routinely utilized for data-dependent acquisition, such as an Orbitrap (ThermoScientific, San Jose, USA) or Q-TOF (Sciex, Toronto, Canada) by high mass accuracy monitoring of full fragmentation patterns of selected ions, termed parallel reaction monitoring (Peterson, Russell, Bailey, Westphall, & Coon, 2012). By selecting peptides of interest, in particular peptides that discern histone isotypes (eg, H3.1 vs H3.2 vs H3.3) or represent modification sites, reproducibility and sensitivity of detection can be

maximized, thereby limiting noise and irrelevant signals, as well as facilitating the most accurate possible quantifications. The primary drawback to targeted analysis is that subsequent reanalysis of a dataset for previously unknown analytes is hampered, since data for these would not necessarily be collected, as would be the case for data-dependent approaches. An additional acquisition approach, termed data-independent analysis, is possible for "archival" data collection through an intentionally wide isolation protocol prior to fragmentation; however, this comes at the greatest expense of sensitivity and discernment of related analytes (Gillet et al., 2012).

3.5 Mass Spectrometry-Based Methods to Study Histone Turnover in Brain

The introduction of high-throughput MS-based proteomic techniques in the investigation of chromatin dynamics has provided us with a new understanding and appreciation of the nucleosome. The earliest insights arose in the early 2000s and introduced a game-changing dimension to what we know about histone proteins and their respective functions. This was, with the aid of MS, the realization of new PTMs including O-linked β-*N*-acetyl glucosamine (O-GlycNac) and lysine modifications such as propionylation, crotonylation, succinylation, and malonylation (Sakabe, Wang, & Hart, 2010; Tan et al., 2011; Xie et al., 2012). And most recently, MS was a key player in the groundbreaking finding of histone turnover as a previously undocumented regulator of synaptic formation and memory (Maze et al., 2015). Mass spectrometry can be used to study the dynamics of PTMs in the total histone pool by briefly labeling newly synthesized histones with a heavy isotope, such as ^{15}N or ^{13}C. Histone modifications can then be determined by MS for both the old and new histones based on mass differences between these two pools. Incorporation of heavy amino acids into a peptide leads to a known mass shift compared to the peptide that contains a natural (ie, "light") version of the amino acid. In contrast to early labeling studies, the isotopes of SILAC amino acids are, as the name indicates, stable, thereby differing dramatically from radioactive approaches (Mann, 2006). SILAC studies of histone turnover by Zee and colleagues have revealed a similar turnover rate for core histones, except for those of the H2A variant family (Zee, Levin, Dimaggio, & Garcia, 2010). Moreover, acetylated histones were recently shown to display faster turnover rates in comparison to unacetylated or methylated histone proteins. Additionally, histone modifications relating to active gene transcription report higher turnover rates in comparison to their silent counterparts.

Our latest pioneering study using SILAC in the investigation of histone turnover in postreplicative neurons (Fig. 2) found that in mice, approximately one-third of the total H3.3 pool was replaced over a 4-week period, even during times in which the overall amount of H3.3 remained unaltered. This demonstrated, contrary to dogma, that H3.3 undergoes constitutive turnover in neurons, while H3.1 and H3.2 remain static (Zovkic & Sweatt, 2015). In sum, mass spectrometry-based methods have allowed for groundbreaking strides in our understanding of neuroepigenetics, methods that can now be applied to both basic and translational investigations.

4. RETROSPECTIVE BIRTH DATING OF HISTONES IN HUMAN POSTMORTEM BRAIN

4.1 "Bomb Pulse Labeling" Coupled to Accelerator Mass Spectrometry

Until approximately 10 years ago, our understanding of cell turnover in human brain was greatly restricted. This was due to a general lack of methods available to study organic synthesis and decay in human tissues, where pulse-chase-labeling techniques were generally not feasible. The technique referred to as "bomb pulse labeling," however, revolutionized our ability to resolve these complicated phenomena and will serve as the focus of Sections 4.1 and 4.2.

High levels of open-air nuclear weapons testing from the mid 1950s to the early 1960s resulted in a dramatic increase in global levels of radioactive ^{14}C in the atmosphere. Elevated levels of ^{14}C resulting from this so-called bomb pulse effectively provided us with a novel means for monitoring organic synthesis and decay in human tissues, as ^{14}C levels have remained in exponential decay since the cessation of open-air bomb testing in the early 1960s. Thus, the principle of "bomb pulse labeling" assumes that most molecules within a given cell, with the exception of genomic DNA, are in a constant stage of dynamic regulation. In line with this assumption, molecules of ^{14}C that become integrated into genomic DNA should therefore reflect the level of ^{14}C in the atmosphere at the time of incorporation (Spalding, Bhardwaj, Buchholz, Druid, & Frisen, 2005). In other words, if a new neuron is born at time "X" and ^{14}C levels in the atmosphere at time "X" = "Y," then one can assume that if that cell persists until a later time of "Z" (as would be presumed to be the case for many nondividing cells), then levels of ^{14}C in the DNA of this cell should remain at Y over the course of time

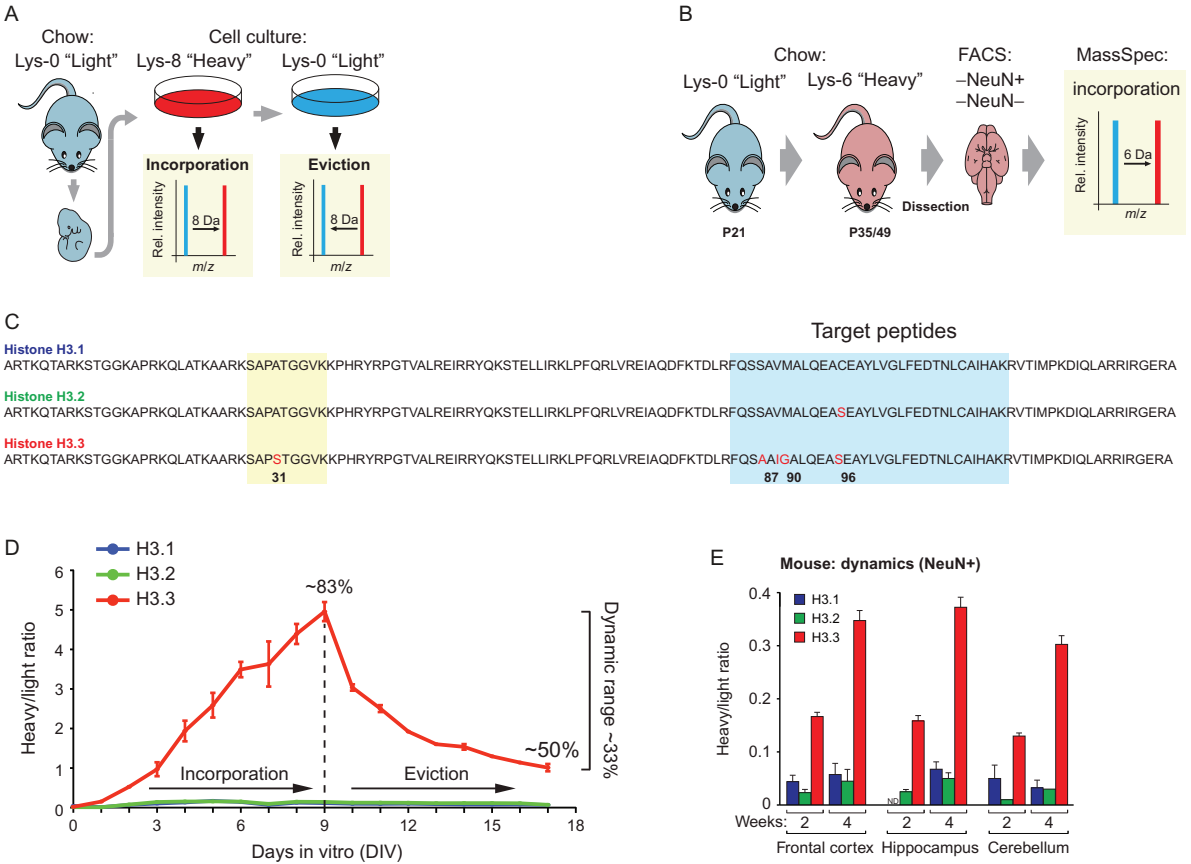

Fig. 2 See legend on next page.

approaching that of "*Z*". In line with such assumptions, pioneering studies by Frisen and colleagues were the first to demonstrate that levels of ^{14}C in human neuronal genomic DNA (isolated from postmortem tissues) closely correspond with atmospheric levels and can consequently be exploited to establish the timeline in which DNA is synthesized and new neurons are born (Spalding et al., 2013, 2005). By determining the age of DNA, which is reflected in the number of radioactive carbon atoms incorporated vs atmospheric ^{14}C levels, one can determine exactly when a cell is created utilizing accelerator mass spectrometry (AMS) to examine carbon turnover.

Until recently, dogma in the neuroscience field had long postulated that the adult human brain did not create new neurons, although several groups utilizing histological and/or autoradiography-based techniques had firmly established that adult neurogenesis, within specific regions of the brain (eg, hippocampal dentate gyrus), represents a phenomenon that persists throughout life (Altman & Das, 1965; Eriksson et al., 1998), similar to findings in rodents (Kuhn, Dickinson-Anson, & Gage, 1996). Using AMS, Spalding et al. were the first to conclusively determine the age of neurons in adult human hippocampus from postmortem neuronal DNA and concretely established the existence of adult human neurogenesis.

Although much of the recent work utilizing AMS had focused exclusively on cellular turnover in human brain (ie, DNA measurements), we recently set out to investigate whether protein dynamics, specifically within the context of histone turnover, could similarly be assessed using this method. To do so, we performed high-performance liquid chromatography (HPLC) to purify

Fig. 2 Mass spectrometry-based assessments of histone turnover in neurons using SILAC. (A) Schematic of SILAC to assess chromatin-associated H3.x incorporation and eviction from cultured neurons. (B) Schematic describing the SILAC mouse model to assess chromatin-associated H3.x incorporation in adult neurons. (C) Amino acid sequences for H3.x proteins highlighting differences between H3.1 vs H3.2 and H3.3 (H3.3, *red*). Putative target peptides for mass spectrometric analysis in the N-terminal tail (*yellow*) or histone core (*blue*) are indicated. Target peptides in *blue* are used for all subsequent analyses. (D) SILAC time course of H3.x chromatin incorporation and eviction in mouse embryonic neurons over the course of 17 d in vitro. Percentages reflect H3.3 peptide labeling by SILAC. (E) SILAC LC–MS/MS analysis of H3.1/2 vs H3.3 in NeuN+ mouse chromatin from multiple brain structures after 2 or 4 weeks of feeding on a heavy lysine (6 Da) diet. *Panels (D) and (E) displayed with permission from Maze, I., Wenderski, W., Noh, K. M., Bagot, R. C., Tzavaras, N., Purushothaman, I., et al. (2015). Critical role of histone turnover in neuronal transcription and plasticity.* Neuron, 87*(1), 77–94.*

chromatin-associated H3.3 from postmortem human brain, followed by AMS to examine H3.3 synthesis and decay rates throughout development and into adulthood. In doing so, we demonstrated that not only does H3.3 remain dynamic in neuronal chromatin throughout the lifetime of an individual, but also that rates of turnover differ dramatically during different stages of development, in line with a newly established role for these turnover events in synaptic connectivity and plasticity (Maze et al., 2015).

4.2 Bomb Pulse Labeling: Analytical Considerations

Open-air bomb testing resulted in essentially a global ^{14}C pulse-chase experiment from which one might infer turnover properties of various biological quantities. For example, depressed ^{14}C ratios in individuals born after the pulse initiation, or increased ^{14}C ratios in individuals born before the pulse initiation, suggest turnover. However, such conclusions still leave many important questions. For example, how quick is the turnover? Is turnover restricted to a subset of cells or a particular pool of molecules within a cell? Do turnover rates change with age? How does accumulation or depletion of the analyte (eg, due to cell growth/death, protein level increases/decreases, or isoform switching) affect these ratios and conclusions?

Drawing more precise inferences from ^{14}C ratios to gain insight into such questions is not straightforward for several reasons, many of which we detail later. These reasons to be outlined arguably apply not only to histone proteins, but most analytes, although here we focus on how to resolve them in the context of histone turnover. This analysis task is facilitated by interpreting the ^{14}C ratios in the context of a mathematical model that formalizes particular assumptions related to these (and other) questions posed earlier. The general approach is to write systems of differential equations that account for rates of ^{14}C uptake and removal due to the biological mechanisms deemed important for the questions at hand. These equations can be solved to predict how ^{14}C levels change over time in an individual with certain characteristics, as defined by the translation of biological assumptions into equations. Coherence of such equation-based predictions with observed ^{14}C levels from an experimental cohort suggests that the assumptions are consistent with reality (although such coherence never conclusively proves mechanism). Alternatively, incoherence strongly suggests that the assumptions do not reflect reality. Inherent in these equations are rate constants (and other parameters) whose estimated values will allow for quantitative and precise insight about turnover.

One of the potentially largest confounds in such analyses is that histone turnover can arise from cell birth/death or from subcellular histone turnover (ie, from transcriptional regulation). Simply having a ^{14}C ratio indicative of turnover does not on its own tell us which of these mechanisms may be responsible, and moreover, to what extent. If one has measurements of ^{14}C turnover in DNA, however, then this can be used to constrain the contribution of cell birth/death independently. Inherent in this analysis is the reasonably fixed ratio of histones/DNA carbons (Maze et al., 2015). This analysis requires the assumptions that (i) subcellular DNA turnover (ie, due to damage and repair) is negligible compared to that of cell turnover—which fortunately is typically the case (but may not always be), and also (ii) the overall cell number within the tissue of interest is essentially constant over a timescale of years. This is reasonably the case in many brain regions, but may not be in others or in different tissues (or disease contexts), so caution must be taken to evaluate this assumption critically. If the cell number in the tissue of interest is not constant over an individual's time span, the situation is not unworkable, but rather requires more information to constrain and formalize how cell birth and death rates change over time. For example, knowing the dynamics of cell number over time, and how the balance of cell proliferation and cell death dictate those dynamics, would be sufficient.

Another significant issue is accumulation (or depletion) of the histone of interest over time. In our work on H3.3, we found that its levels increase approximately threefold from birth to adolescence. Thus, one would expect changes in ^{14}C levels simply due to such accumulation, regardless of turnover. Like above with cell turnover, though, knowledge of such accumulation/depletion dynamics allows one to account for its contribution on observed ^{14}C levels (Fig. 3).

Given data on only a single subject, limited information can be inferred from ^{14}C data. In our experience, data from multiple subjects born both prebomb pulse and postbomb pulse, with a variety of birth and death years, significantly enhance one's ability to draw precise conclusions. In the case of H3.3, we found that data from individuals born prebomb pulse helped us to constrain the turnover rates later in life, whereas data from individuals born postbomb pulse helped us to constrain turnover rates early in life. Thus, identifying potential changes in turnover rates as a function of aging depends on picking individuals with a variety of birth and death ages pre and postbomb pulse. Although not yet performed, we postulate that such analyses

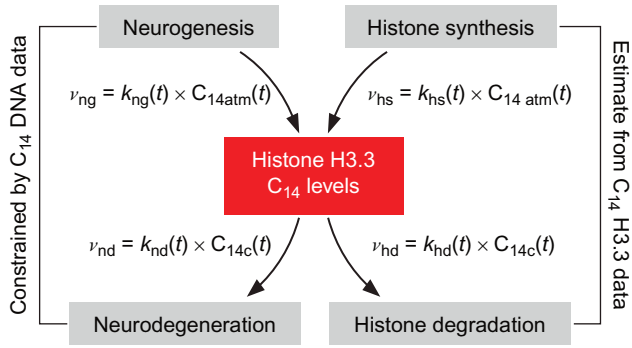

Fig. 3 Modeling nucleosomal turnover in human brain with AMS. The rate of fluctuation of ^{14}C levels in histone H3.3 pools from human brain tissue depends on the rates of (1) cellular (neuronal and glial) proliferation and death (eg, neurogenesis vs neurodegeneration), (2) histone H3.3 synthesis and accumulation in brain chromatin, and (3) degradation, which can be directly monitored using "bomb pulse labeling." With this information, one can effectively model H3.3 turnover rates as proportional to the current atmospheric $^{14}C/^{12}C$ levels and degradation rates as proportional to current cellular $^{14}C/^{12}C$ levels. *Displayed with permission from Maze, I., Wenderski, W., Noh, K. M., Bagot, R. C., Tzavaras, N., Purushothaman, I., et al. (2015). Critical role of histone turnover in neuronal transcription and plasticity.* Neuron, 87*(1), 77–94.*

can be applied to future investigations aimed at uncovering the role of histone dynamics in neurological disease states and may open the door to a new understanding of the role of histone regulation in brain.

5. CONCLUSION

Here, we have attempted to provide researchers with an updated "toolkit" of current proteomic methods for use in the dissection of histone turnover events in brain with the hopes that such information will stimulate deeper investigations into the underlying mechanisms of neuroplasticity and neurological disease. It is without doubt that the field of neuroepigenetics is progressing rapidly toward significantly enhanced and higher throughput methods to monitor the rates and functions of histone turnover in brain. It is our hope that by introducing these principles and protocols that researchers across diverse disciplines may now apply these tools in their specific research programs to address critical mechanistic questions relating to the role of histone turnover in brain development and human health.

6. METHODOLOGY: PREPARING CHROMATIN FROM NEURONS FOR MASS SPECTROMETRY ANALYSIS OF HISTONE VARIANTS AND TURNOVER

See Fig. 4 for schematic of analysis pipeline.

Note: The nucleus and the cytoplasm are highly distinct with respect to their macromolecular composition, and separation of these fractions allows for efficient and sensitive downstream proteomic analyses. To extract chromatin from various cell types in brain (or from primary cultures), the following stepwise protocol is ideal and is compatible with SILAC-based experiments.

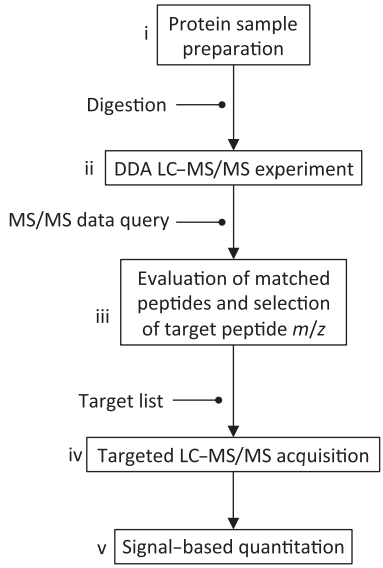

Fig. 4 Typical workflow for LC–MS-based quantitation of histone proteins. (i) High-quality histone-enriched protein samples, including nuclear fractions and chromatin, can be generated so that downstream fractionations are unnecessary (eg, for typical histone enrichment protocols, such as histone acid extraction (Shechter, Dormann, Allis, & Hake, 2007), histone signals are approximately 10-fold higher than the most abundant "nonhistone" protein in the sample). (ii) Digested samples are analyzed in data-dependent acquisition (DDA) experiments, (iii) which allows assessment of samples, including digestion efficiency and modification types, to select appropriate mass-to-charge ratios for peptides to be (iv) targeted in quantitative analyses. Preferably, samples are analyzed in biological and technical replicates to enable statistical analyses. (v) Target peptide signals can further be extracted and processed using appropriate software.

If one aims to isolate chromatin from intact brain, additional steps aimed at isolating neurons from nonneuronal cells are necessary due to the heterogeneity of brain tissues. To do so, fluorescence-activated cell sorting, otherwise referred to as "FACS," can be used. In short, this involves preextraction of nuclei in hypotonic lysis buffer, followed by ultracentrifugation and immunotagging with the neuronal specific marker NeuN using antibody-based methodologies that have been previously described (Matevossian & Akbarian, 2008).

Step 1: Chromatin isolation from neurons

Primary neurons

- First, cells are washed 2× in phosphate-buffered saline, collected using a sterile cell scraper, and then spun in a table top centrifuge at 1500 × *g* for 5 min.
- Pelleted cells are then resuspended in 200 μL of buffer A [10 m*M* HEPES, pH 7.9, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.34 *M* sucrose, 10% glycerol, 0.5 m*M* phenylmethylsulfonyl fluoride (PMSF), 0.1% Triton-X, 10 m*M* sodium butyrate, plus EDTA-free protease inhibitors (Roche, Basel, Switzerland), and PhosSTOP phosphatase inhibitors (Roche)].
- Resuspension of the cell pellet can be achieved in several ways. We recommend utilizing a Dounce homogenizer, or alternatively, they can be broken up and resuspended by drawing the cell suspension/buffer solution through a 22-gauge needle and 1-mL syringe.
- Homogenized samples are then incubated on ice for approximately 30 min, briefly vortexing the samples every 5 min.
- At this point, the sample is then ready to undergo initial crude fractionations. Samples are centrifuged at 1300 × *g* for 5 min to separate the supernatants (crude cytosol*) from the pellets (nuclei).

  *Isolation of pure cytosol involves an additional centrifugation step at 20,000 × *g* for 5 min to remove insoluble materials.

Both primary and FACS (see note earlier) purified neuronal nuclei

- Nuclear pellets are then washed once more with 200 μL of buffer A (without Triton-X) and then resuspend in 100 μL of buffer B [3 m*M* EDTA, 0.2 m*M* EGTA, 0.2 m*M* PMSF, 10 m*M* sodium butyrate, plus EDTA-free protease inhibitors, and PhosSTOP phosphatase inhibitors] (note: pelleted nuclei should appear white and opaque).
- Nuclear samples in buffer B are then incubated on ice again for 30 min, briefly vortexing the tube every 5 min.

- Following incubation, nuclear samples are centrifuged at 1700 × *g* for 5 min at 4°C to further fractionate the nuclear sample into a supernatant (soluble nuclear fraction) and final pellet (chromatin).
- Chromatin pellets can then be washed in 200 μL of buffer B prior to further processing into purified histones for mass spectrometry (note: chromatin pellets should appear glassy and translucent).

Step 2: Acid extraction of histones from chromatin

- Deviating slightly from published protocols (Shechter et al., 2007), chromatin pellets are then resuspended in 400 μL of 0.4 N sulfuric acid. This addition acts to solubilize histone proteins. Samples are then drawn through a 22-gauge needle and 1-mL syringe to remove any clumps that may be present.
- Samples are then incubated on a rotator for 30 min at 4°C. This step can be extended to overnight if desired, but we find 30 min to be sufficient to recover high yields of histone for subsequent processing.
- Following incubation, samples are centrifuged at 16,000 × *g* for 10 min at 4°C. This step acts to remove nuclear debris and nonacid soluble proteins.
- Histone containing supernatant is the carefully transferred into fresh 1.5-mL tubes.
- Trichloroacetic acid is then added to the supernatants to a final concentration of 33%, followed by gentle inversions to mix. Samples are then incubated overnight at 4°C. This is done to precipitate histone proteins from the acid solution.
- The next day, histones are pelleted by centrifugation at 16,000 × *g* for 10 min at 4°C, followed by subsequent removal of the supernatant. Take caution not to disrupt the histone pellet, which will likely appear as a smear along the side of the tube.
- Ice-cold acetone is then used to wash (2 ×) the histone pellets, typically using 1 mL/wash, followed by and centrifugation at 16,000 × *g* for 5 min at 4°C.
- Following the final wash, all supernatants are removed and histone pellets are allowed to air-dry for 15–20 min at room temperature.
- Pellets are then solubilized in up to 100 μL of water, taking note to wash the sides of the inner tube (note: if sufficient amounts of histones are present, resuspension in water should cause sample bubbling during pipetting. Once bubbles are present, the samples are now ready for subsequent processing).

Step 3: In solution digestion for mass spectrometry analyses

- Resuspended samples are then prepared with 8 *M* urea in 0.1 *M* ammonium bicarbonate (ABC). This serves to solubilize and denature proteins, making them more susceptible to subsequent enzymatic cleavage.
- *Reduction*: Dithiothreitol is added to a final concentration of 5 m*M* for 30 min at room temperature (note: it is very important to never heat with urea present, in particular with histone samples containing high contents of lysines that can be carbamylated) to disrupt disulfide bonds within and between proteins.
- *Alkylation*: Add iodoacetamide (IAA) to a final concentration of 15 m*M* and incubate for 30 min at room temperature in the dark. Alkylation with IAA increases the mass of a peptide by 57.021464 Da for each cysteine present.
- Samples are then diluted with 0.1 *M* ABC to reduce urea concentration to <4 *M*.
- *First digestion*: LysC is added at 1:50. This endoproteinase specifically hydrolyzes proteins at the carboxyl side of lysine, and efficient protein digestion can be completed in 6 h at room temperature.
- Dilute sample to <2 *M* urea with 0.1 *M* ABC.
- *Second digestion*: Trypsin (1:50) is added to the sample to further digest the proteins, also hydrolyzing following lysine residues but also arginine, and is incubated overnight at room temperature shaking.
- Trypsin digestion is stopped by adding trifluoroacetic acid (TFA) (0.5%, v/v). By lowering the pH of the solution, trypsin can no longer actively digest proteins.
- Samples are next desalted and concentrated using a C_{18} Stop And Go Extraction (StAGE) tip (Rappsilber, Ishihama, & Mann, 2003).
- Samples are then dried down in a speed vacuum centrifuge before resuspension in a mass spectrometry compatible buffer for analysis, such as 0.4% acetic acid (v/v) and 5% acetonitrile (v/v) in HPLC grade H_2O.

Step 4: Mass spectrometry analysis for identification of neuronal histone peptides

- Peptides of interest can be analyzed by a high-performance LC–MS/MS setup such as a Q-Exactive coupled to a Dionex NCP3200RS HPLC setup (ThermoScientific, San Jose, USA).

- *Data acquisition*: Depending on LC conditions including gradient, up to the 20 precursor ions are subjected to MS/MS per MS survey scan.
- *Analysis of data*: Software, such as "ProteomeDiscoverer/MASCOT," can be used to interrogate raw data which included querying MS/MS spectra against Uniprot's complete human or mouse proteome databases concatenated with common known contaminants (Bunkenborg, Garcia, Paz, Andersen, & Molina, 2010).
- For label-free quantitation experiments, the area of the target peptides is used, whereas for SILAC experiments, ratios can be calculated as heavy over light as previously described (Maze et al., 2015).

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