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Understanding the aging hypothalamus, one cell at a time

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Abstract

The hypothalamus is a brain region that integrates signals from the periphery and the environment to maintain organismal homeostasis. To do so, specialized hypothalamic neuropeptidergic neurons control a range of processes, such as sleep, feeding, the stress response, and hormone release. These processes are altered with age, which can affect longevity and contribute to disease status. Technological advances such as single cell RNA sequencing, are upending assumptions about the transcriptional identity of cell types in the hypothalamus and revealing how distinct cell types change with age. In this review, we summarize current knowledge about the contribution of hypothalamic functions to aging. We highlight recent single cell studies interrogating distinct cell types of the mouse hypothalamus and suggest ways in which single cell ‘omics technologies can be used to further understand the aging hypothalamus and its role in longevity.

Keywords

Single Cell RNA-seq; homeostasis; longevity; metabolism

Single cell technologies provide new insights into the aging hypothalamus

Aging is the most significant risk factor for a host of diseases, many of particular interest to neuroscientists, such as stroke[1], brain cancer[2], and neurodegenerative diseases[3].

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Declaration of interests

The authors declare no conflicts of interest in relation to this work.

Aging is also accompanied by alterations in basic homeostatic processes (Figure 1), many of which have been linked to disease[4–6]. The hypothalamus is a brain region that receives homeostatic information from the periphery to coordinate behaviors such as circadian rhythms, food intake, the stress response, and hormone release[7]. This brain area harbors a diverse collection of specialized neuropeptidergic neurons that regulate these distinct physiological functions. Many hypothalamic-regulated processes are altered with age, and their disruption can affect longevity and contribute to age-related disease[8–13].

Despite decades of interest in the hypothalamus and its role in aging, the transcriptional and functional complexity of the neurons in this area has remained a roadblock for the field. Recently developed technologies, such as single-cell RNA sequencing, single-cell ATAC sequencing, and spatial transcriptomics, offer new opportunities to investigate the cellular makeup and functions of the hypothalamus. In this review, we discuss findings, primarily from humans and mouse models, that implicate the hypothalamus in the aging process. We then focus on how single cell profiling technologies contribute to these discoveries, and highlight recent lines of inquiry toward the ultimate goal of promoting healthy aging and combating age-related diseases.

The hypothalamus regulates homeostatic processes that change with age

Changes in homeostatic processes such as sleep, circadian rhythms, and body composition are hallmarks of aging. The hypothalamus contains a diverse array of neuropeptidergic cell types that respond to cues from the periphery to regulate these processes and maintain homeostasis (Figure 1). Understanding how these cell types are affected during aging may be key to unlocking therapeutics to treat particular age-related conditions.

Energy homeostasis is a broad category of biological functions which include food intake, fat storage, as well as energy expenditure in the form of locomotion and body temperature maintenance. These processes are tightly regulated by a suite of neuronal subtypes across the hypothalamus (Figure 1). Aging is accompanied by alterations to body composition, including increased abdominal adiposity and decreased lean muscle mass[14]. Concomitantly, there is a decline in basal metabolic rate and total energy expenditure, and increased risk for metabolic disease such as type II diabetes[15,16]. These organismal-level changes are concurrent with changes in the hypothalamus; for example, there is reduced response to central leptin administration and reduced leptin receptor expression, increased inhibition by ARC^{AGRP} onto ARC^{POMC}, and age-related proteostasis defects. All of these changes likely contribute to age-related obesity and metabolic alterations[17–19].

Maintenance of body temperature is a homeostatic process that intersects with energy homeostasis, as food intake is affected by external body temperature, and energy is expended through heat generation in brown adipose tissue[20]. Studies in humans have shown that older individuals have a decreased average internal body temperature compared to young adults, and reduced amplitude of body temperature changes with the circadian rhythm [21,22]. Additionally, aged individuals are less able to mount a fever in response to infection [23]. Internal body temperature is maintained by the warm- and cold- sensitive thermoregulatory neurons of the preoptic area, as well as DMH^{Brs3} neurons[24,25].

Changes in circadian rhythm and sleep/wake cycles are also common in aged individuals. Middle-aged adults have advanced sleep and wake times compared to younger adults, as well as increased daytime sleeping, increased latency to fall asleep, and less deep sleep overall[8]. Circadian rhythm is controlled by neuropeptidergic cell types in the suprachiasmatic nucleus, which receive retinal inputs to entrain body rhythms to the day/night cycle. Neurons expressing hypocretin/orexin in the lateral hypothalamus ($LH^{HCRT/OX}$) are also essential for arousal[26]. With age, there is a reduction of expression of hypocretin/orexin, and reduced circadian rhythmicity of SCN neurons, which may contribute to age related changes in sleep and circadian rhythm[27,28]. Recent research has also shown that with age, $LH^{HCRT/OX}$ neurons become hyperexcitable, and pharmacological inhibition of this hyperexcitability in aged mice can restore proper sleep [29].

Endocrine changes with aging implicate the hypothalamus

The hypothalamic-pituitary axis controls release of hormones that have profound consequences for health, including thyroid hormone, growth hormone, cortisol, and gonadal hormones[55]. While measurement of pituitary hormones, such as thyroid stimulating hormone, luteinizing hormone, and follicle stimulating hormone, can be performed in humans via blood test, studying the hypothalamic neuronal populations controlling the timing and amplitude of pituitary hormone release is difficult. Consequently, much more is known about how levels of pituitary hormones and their downstream effects change with age, than the hypothalamic neuron alterations associated with these changes. One of the changes observed with age, for example, is attenuation of growth hormone release, with decreased amplitude of pulses but not frequency of pulses[56]. Release of growth hormone from the pituitary is controlled by neurons in the arcuate nucleus that release growth hormone-releasing hormone (GHRH). However, the extent to which ARC^{GHRH} neurons are involved in age-related changes in growth hormone release not well understood. Neurons in the hypothalamus control release of other hormones known to change with age, including thyroid stimulating hormone, luteinizing hormone, follicle stimulating hormone, and adrenocorticotropic hormone. The role of hypothalamic neuronal populations in driving these age-related changes remains understudied and is an important goal for future work (see Outstanding Questions).

Age-related neurodegenerative diseases are associated with hypothalamic neuropathology

Neuropathological studies in humans implicate the hypothalamus in the development of a variety of neurodegenerative diseases. For example, neuroimaging and post-mortem analysis of human brains indicate hypothalamic atrophy may be a feature of Alzheimer's disease[57]. Interestingly, sleep disturbance and circadian changes are key features of Alzheimer's Disease and amyloid deposition is associated with poorer sleep quality[58]. Degradation of the suprachiasmatic nucleus has been reported in AD patients, and loss of orexin/hypocretin neurons occurs in advanced AD[59,60]. However, other hypothalamic regions, such as the supraoptic nucleus, seem to be spared from neurodegeneration[59].

Obesity is a major risk factor for Alzheimer's disease and alterations in body weight may precede or predict the course of neurodegenerative disease. Loss of weight late in the lifespan is associated with the development of mild cognitive impairment or preclinical Alzheimer's Disease[61,62]. In patients with Amyotrophic lateral sclerosis (ALS), hypothalamic TDP-43 inclusions were identified in one third of patient brains. In patients with inclusions in the lateral hypothalamus, body mass index (BMI) was significantly reduced[63]. Reductions in BMI are associated with accelerated disease progression in ALS, suggesting that the TDP-43 inclusions in the lateral hypothalamus may be clinically significant[64].

The involvement of the hypothalamus in neurodegenerative diseases remains critically understudied. Addressing the timing and trajectory of neuropathology in this brain region may uncover treatments for the non-cognitive symptoms of neurodegenerative disease, which would improve quality of life for the patient and caregivers (see Outstanding Questions).

The impact of energy sensing and the hypothalamus on lifespan and healthspan

A major goal in the aging field is to understand the mechanisms underlying the aging process to improve organismal healthspan, the period of an individual's life spent free from disease. The study and manipulation of organismal energy states are a mainstay of aging research, with interventions such as caloric restriction being one of the most conserved and reproducible paradigms for lifespan extension[65]. For example, in C57BL/6J mice, 20% caloric restriction (CR) increases mean lifespan by 40.6% in females and 24.4% in males compared to ad libitum fed animals[66]. Studies performed in worms[67], flies[68], and mice[69–72] implicate peptidergic neurons as key regulators of lifespan changes in response to alterations to energy sensing pathways. However, the molecular nature of the cellular changes and neuronal subtypes involved in the response to caloric restriction in mammals remains unclear, in part due to the transcriptional and functional complexity of the hypothalamus.

The mechanisms underlying lifespan extension in response to dietary restriction have been studied extensively (note that dietary restriction is an umbrella term for a variety of manipulations, and includes caloric restriction, time-restricted feeding, and specific macronutrient manipulations (e.g. carbohydrates, fats, amino acids)). These mechanisms are in part cell non-autonomous and involve nutrient sensing neurons (reviewed in [73,74]). In two invertebrate models, the effects of dietary restriction depend on neuropeptidergic energy-sensing neurons. In *C. elegans*, dietary restriction is dependent on the gene *skn-1*, which plays a role in autophagy; loss-of-function mutations in *skn-1* prevent the lifespan extending effects of dietary restriction. *Skn-1* has several isoforms, one of which is expressed uniquely in the neuropeptergic ASI neurons. Ablation of the neuron-specific *skn-1* isoform, as well as ablation of the ASI neurons themselves prevented dietary restriction from increasing lifespan[67]. Similarly, in *Drosophila*, ablation of median neurosecretory cells, which produce insulin-like peptides, extends lifespan in both dilute food conditions (dietary

restriction) and in conditions of surplus food, suggesting a cell non-autonomous effect of these neurons on mediating lifespan in response to food intake in invertebrates[68].

In mice, specific manipulations to the hypothalamus are sufficient to extend lifespan. The NAD+ dependent deacetylase SIRT1 is expressed at higher levels during food restriction than during ad libitum feeding, and *Sirt1* is required for the normal upregulation of the hypocretin (orexin) receptor 2 (*Hcrtr2*) in response to DR[69]. Transgenic *Sirt1* overexpression in the lateral hypothalamus and dorsomedial hypothalamus of mice is itself sufficient to increase lifespan, with a ~16% increase in median lifespan for females and 9% for males[70]. NF-κB activity in the hypothalamus has also been shown to impact lifespan. With age, hypothalamic inflammation increases, and abrogation of inflammation by overexpressing a dominant-negative form of IκB-α which inhibits NF-κB significantly extended lifespan in male mice, although precise effect size was not reported[71]. In addition, ablation of hypothalamic progenitor cells in middle-age male mice results in a reduction in mean lifespan of 13.5%[72]. Manipulations to the hypothalamus can also improve organismal function. For example, overexpression of the nicotinamide mononucleotide transporter *Slc12a8* in the lateral hypothalamus restored skeletal muscle function of aged mice[75].

The growth hormone signaling axis has long been implicated in longevity, with mice deficient in growth hormone or lacking the growth hormone receptor living longer than their wild-type counterparts (reviewed in [76]). Growth hormone-releasing hormone (GHRH) is released by neurons in the hypothalamus to promote growth hormone release in the pituitary. In GHRH knock-out animals, lifespan is markedly increased (43% increase in median lifespan in females and 51% increase in males)[77]. Interestingly, GHRH knock-out mice display an overall increase in survival in response to caloric restriction, indicating that the mechanisms by which GHRH neurons in the hypothalamus impact lifespan are at least partially distinct from those of caloric restriction.

Together, these data implicate the hypothalamus in the central regulation of lifespan, and suggest that it may regulate multiple longevity-associated processes with additive effects on lifespan. While published studies lay a strong foundation implicating hypothalamic nuclei in healthy aging, the impact of different hypothalamic functions on longevity is only beginning to be clarified. More work is required in several areas, including reproductive aging, the stress response, social behavior, sleep and circadian rhythms, and body temperature regulation.

Longevity interventions involve multiple hypothalamic functional networks.

Distinct regions of the hypothalamus interact in order to coordinate behaviors and maintain organismal homeostasis (Figure 1). Lifespan interventions can simultaneously affect several hypothalamic functions. Thus, determining the impact of any specific hypothalamic region or cell type in longevity presents a challenge. For example, caloric restriction affects metabolic state and causes a concomitant reduction in internal body temperature[78], raising the question of the extent to which body temperature influences longevity under restricted feeding conditions. Studies have shown lifespan-extending effects of reduced internal

body temperature despite ad libitum food intake[79,80]. In contrast, mice overexpressing brain-specific *Sirt1* live longer than wild-type controls despite their higher internal body temperature and increased food intake[70], suggesting that lowered internal body temperature is one mechanism among many for lifespan extension. Further, the timing of feeding, especially in regards to circadian time of day, can affect caloric restriction paradigms. In one study, animals were fed a calorically restricted diet at the start of the active period, at the end of the active period, or throughout the day[81]. While median lifespan for all CR conditions was longer than for ad libitum animals, animals a CR diet at the start of the active period had a 35% increase in median lifespan compared to ad libitum fed animals, highlighting the intersection of circadian rhythms, feeding patterns, and longevity. Together, these data exemplify the difficulty of untangling the role of the hypothalamus and homeostatic processes in lifespan.

Finally, many studies have been hampered by the lack of specific molecular markers for cell types in hypothalamic regions or cell types of interest. It has been noted, for instance, that the lack of *Cre* driver mice to target specific cell types in the lateral hypothalamus and dorsomedial hypothalamus presents a barrier to dissecting the specific roles of these subregions in the context of studies of metabolic regulation [70]. Identifying cell type-specific molecular markers for hypothalamic cell types is a critical step toward deeper understanding of the hypothalamus in the context of aging.

Single cell ‘omics complements existing tools

Many approaches have been used to understand the role of the hypothalamus in aging and longevity, but studies have been limited by available technologies. For investigators interested in a particular cellular subtype, cell sorting (FACS, MACS) followed by bulk RNA sequencing allows for the analysis of transcriptional changes in cell types for which a reporter mouse can be used or which has known surface markers with reliable antibodies available. However, bulk sequencing approaches cannot be used to discern transcriptional and functional heterogeneity within a population. Microdissection of hypothalamic subregions for bulk transcriptomic analysis is similarly hampered by the transcriptomic diversity within the region. Low cell numbers for many neuronal subtypes, as well as difficulties in obtaining intact neurons from dissociated adult brain tissue, contribute to the challenges of these studies. The low cell number limitation can be circumvented through the use of increased animal numbers[86], but in some cases this is not a practical solution, particularly for studies involving aged mice or disease models. Techniques in which the spatial identity of a cell is preserved, such as in situ hybridization (ISH), offer an approach in which expression patterns in individual cells can be discerned[87]. However, large screens using ISH or immunohistochemistry (IHC) require probes or antibodies for known targets, and targeting multiple transcripts or proteins in a single cell is arduous. Moreover, these methods are less quantitative than available sequencing methods.

Methods to observe or manipulate neuronal activity rely on the existence of known marker genes. Tools such as optogenetics and chemogenetics have been essential for causally linking the activity of specific neuronal populations to changes in organismal state or behavior. In optogenetics experiments, cell type-specific activation or inhibition

of a neuronal population can be achieved for instance by combining transgenic mice expressing cell type-specific Cre drivers with Cre-dependent viral opsin expression[88]. DREADDs (designer receptors exclusively activated by designer drugs) are a class of chemogenetic tools that can be used to activate or suppress neuronal subtypes over extended timescales. Similar to optogenetic approaches, DREADDs can be expressed in a neuronal subtype-specific manner by combining viral Cre-dependent DREADD expression with cell type-specific Cre-driver mice[89]. Additionally, genetically encoded calcium sensors or voltage sensors allow researchers to record neuronal activity in awake and behaving animals (reviewed in [90]). While there are multiple strategies to target the sensors to specific cell types, including genetically encoded or viral methods, these methods require a known marker gene. Importantly, regions of the hypothalamus that impact lifespan such as the lateral hypothalamus or preoptic area are some of the least molecularly defined, and currently, specific Cre driver lines targeting these cells are lacking.

Unlike bulk RNA-seq, ISH, or IHC, single cell sequencing technologies allow for the discovery and investigation of new cell types without a priori knowledge of specific markers. In the context of the hypothalamus, single cell RNA-seq has uncovered novel neuronal subtypes and revealed previously under-reported heterogeneity among known cell types. However, how to define clusters and cell types in the hypothalamus continues to be debated. Further, experimental variables such as sample type, preparation, cell number, and sequencing depth can greatly affect downstream outcomes and complicated interpretation across datasets. To date, there is no consensus method for defining hypothalamic cell types in single cell data, and so the overall number of clusters, as well as the markers or labels used for each cluster, vary greatly from study to study. For example, in two studies examining the makeup of the whole hypothalamus using DropSeq, the number of neuronal clusters reported varied from 34 to 62[91,92]. Interestingly, the paper reporting the higher number of clusters analyzed fewer cells (3,131), while the study with the lower number of clusters analyzed over four times as may cells (14,000). Relatedly, in a recent study by our group, we sequenced nuclei rather than whole cells, and found 34 transcriptionally distinct neuronal clusters using 40,064 nuclei from the whole hypothalamus as input[93].

Somewhat counterintuitively, studies focused on particular hypothalamic subregions often report more transcriptionally distinct subclusters than are found in some studies on the whole hypothalamus (summarized in Table 1). For example, one analysis of 31,299 cells from the preoptic area found 69 neuronal clusters[94]. While computationally these clusters may represent transcriptionally distinct entities, whether these distinctions or groupings have functional relevance remains an open question (see Outstanding Questions). Moreover, since there are currently no established firm criteria for what defines and delineates a cluster, comparison between studies for validation or discovery purposes remains a challenge.

Traditionally, hypothalamic neurons have been categorized by their neuropeptide expression, or by expression of specific receptor genes. However, across the hypothalamus, single cell approaches have revealed transcriptional heterogeneity within cell populations sharing neuropeptide identity. In some cases, functional heterogeneity of the population was known prior to transcriptional profiling, as is the case with POMC neurons of the arcuate nucleus[95]. This well-studied neuronal population is known to have subpopulations

responding uniquely to serotonin or leptin[95]. In a study using SMART-Seq of sorted POMC-EGFP+ cells, four clusters of *Pomc*-positive, *Agrp*-negative cells emerged[96]. Importantly, these clusters differed based on expression of receptor genes and neuropeptide co-expression, indicating that neurons in these clusters may be sensitive to different stimuli and thus have differing functions. Single cell transcriptomic approaches present a timely opportunity to identify important subpopulations of neurons in other areas of the hypothalamus as well.

Recent single cell transcriptomic studies reveal hypothalamic neurons that may impact aging

Single cell approaches present an opportunity to identify and develop tools to target specific types of neurons that impact healthy aging, including neuronal subtypes involved in body temperature regulation, feeding and energy homeostasis, stress, and circadian rhythms. Studies using single cell transcriptomics to understand these specific functions in more depth are beginning to emerge, and here we highlight a few specific advances that uncover neuronal subtypes of interest.

Internal body temperature has been identified as a key mediator of longevity, and the search for molecular markers of hypothalamic warm-sensing neurons in the preoptic area is ongoing[79,80]. Previous research identified the genes *Bdnf* and *Adcyap1* as potential markers for this population, and these genes were co-enriched in multiple clusters in a preoptic area single cell sequencing dataset[94]. By combining transcriptomic and functional cFOS data, the authors identified a temperature-sensitive cluster that could be distinguished from other *Bdnf*+ *Adcyap1*+ clusters by its co-expression of *Sngc*. Further research is needed to fully determine the role of this *Bdnf*+ *Adcyap1*+ *Sngc*+ neuronal population in regulating internal body temperature, but this study highlights the potential of single cell transcriptomics to identify candidate neuronal populations of interest to the aging field.

While dietary restriction paradigms have been studied for decades, the molecular identity of cell populations involved in feeding and energy expenditure are just now being described due to advances in single cell technology. Classically, the neuron populations involved in feeding and energy homeostasis have been defined by expression of a single neuropeptide, however, single cell analysis suggests that the same neuropeptide can be expressed in functionally distinct subpopulations. For example, single cell analysis of the lateral hypothalamus confirmed two transcriptionally distinct MCH subtypes[99] as well as transcriptional heterogeneity in canonical leptin sensitive populations of POMC and AgRP/NPY neurons[96,97]. Understanding the function of these discrete neuronal populations may pave the way for pharmacological interventions targeting them to mimic the lifespan-enhancing effects of dietary restriction[116].

Changes in circadian rhythm and sleep quality are common in aging[8]. The SCN is the master regulator of the circadian rhythm, and consists of neuropeptidergic neurons which receive inputs from the retina and other areas to synchronize behavior to the light/dark cycle. Canonically, SCN VIP neurons and GRP neurons are considered to be two separate populations. However, two single cell studies have identified two distinct *Vip*+ populations,

one of which also expresses *Grp*[103,117]. Whether any (or all) of these populations are responsible for age-associated changes in circadian rhythms has not been determined, but single cell technologies have been useful to identify new neuronal populations that may be involved.

Machine learning applications in single cell analysis and aging research

Many studies have leveraged advances in machine learning (ML) to uncover processes underlying brain aging. Trajectory analysis is one method used to discover genes that underlie the transition between cell states[118]. While trajectory analysis methods were initially developed with the goal of identifying changes in cell state during development or reprogramming, two recent papers have used trajectory analysis to understand brain aging[119]. In one study on brain tissue from patients with Alzheimer's Disease, Monocle3 was used to uncover transcriptional changes across the trajectory from healthy to disease state in glial cell types[120]. In our study comparing young and aging hypothalamus, we found that a clear inflammatory signal emerged across age in microglia and macrophages[93].

One of the most well-established applications of ML in the aging field is the DNA methylation clock, in which sets of CpGs can predict chronological age with high accuracy using supervised ML methods[121,122]. Similarly, transcriptomic profiles have been used to build aging clocks to predict the age of a given cell in a single cell dataset and define the transcriptional signatures of aging[123]. For example, a study of the mouse subventricular zone neurogenic region generated transcriptomic clocks to predict a cell's chronological age. The study reported that methylation clock genes were cell type-specific but interferon and lipid metabolism signatures were shared across cell-types. Our analysis of the aging female mouse hypothalamus using single nuclei RNA-seq revealed that the X chromosome gene, *Xist*, was the most important feature to predict neuronal age using the XGBoost model[93]. Thus, ML models have been useful for identifying new markers of cellular age from single cell transcriptome data across species. In future work, integration of methylation clocks with single cell expression datasets will provide a more complete understanding of how regulatory networks impact the aging process in a cell type-specific manner.

Moving forward, with increased quantities of data, sophisticated deep learning and artificial intelligence methods can be applied to geroscience to optimize age prediction, biomarker development, and drug design[124]. However, the interpretability of deep learning models can be challenging, so in applications such as medical diagnosis, models should be carefully selected and applied.

Concluding remarks and future perspectives.

Single cell 'omics methods have ignited interest in the diverse cell types of the hypothalamus. Despite decades of interest in this brain region, many outstanding questions regarding the molecular identity of cell types controlling basic processes remain. As interest grows in defining a functional role for molecularly defined subtypes, the field will need to develop consensus definitions for specific cell types. Ideally, such consensus markers

would be stable between studies, and across sexes, strains, and age of the animals within a species. While some cell-type markers may not be conserved across taxa, markers that are expressed similarly in different species will be highly useful, particularly for investigation of the human hypothalamus.

Several methods of lifespan extension in animal models, including specific diets and manipulations to internal body temperate, involve the hypothalamus. Thus, identifying interventions that target specific hypothalamic neuronal subtypes may be a valid path toward extending healthspan without requiring arduous fasting regimens. However, as recent studies have revealed, we are only beginning to understand which cell types should be targeted, and how neurons regulating distinct processes interact. Deep sequencing of the hypothalamus across aging, under different conditions, and in disease states will provide important insight into the development of future interventions.

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Outstanding Questions.

- Many options exist to analyze single cell data and cluster cell types, however, there is high variability in number and composition of clusters across datasets generated in different laboratories. How can we improve cross-laboratory interoperability of these datasets? How well do transcriptionally defined clusters match with functionally distinct neuronal subtypes?
- In worms and flies, the proper function of neuropeptidergic energy-sensing neurons is required for the lifespan-extending effects of dietary restriction. How are hypothalamic energy-homeostasis neurons affected by dietary restriction at different ages? Which (if any) energy-sensing hypothalamic neurons mediate the effect(s) of dietary restriction on lifespan?
- The hypothalamus contains sex-specific neuronal populations and populations that function in a sex-specific manner. However, single-cell studies comparing sexes or across hormone states are rare. Do other, undiscovered, sex-specific neuronal populations or activities exist in the hypothalamus? Do these populations contribute to sex differences in lifespan or diseases susceptibility?
- There is a growing appreciation for the link between neurodegenerative diseases, such as Alzheimer's Disease, and changes in hypothalamic function. How are individual neuronal subtypes altered with age and in neurodegeneration in humans?
- Hypothalamic inflammation has been proposed as a major driver of whole-body aging, and single nuclei RNA-seq of the hypothalamus has uncovered age-related changes in female hypothalamic microglia. How does microglia-neuron crosstalk at the single cell level influence aging? How are other glial cells, such as tanycytes or astrocytes, affected by inflammation and aging?

HIGHLIGHTS

- Homeostatic processes such as food intake/energy balance, sleep and circadian rhythms, and stress responses are controlled in part by specific neurons of the hypothalamus. These processes are altered with age, and can contribute to increased age-associated disease.
- Despite decades of interest in the hypothalamus, molecular markers for many of the specific neuronal subtypes in the hypothalamus remain elusive.
- Recently, single cell RNA-sequencing technologies have uncovered new and functionally distinct neuronal subtypes in the hypothalamus. Studies have also revealed significant transcriptional and functional heterogeneity among cell types previously thought to be homogeneous.
- Comprehensive transcriptomic mapping across hypothalamic cell types will provide important insights into the aging process and may pave the way for new healthspan-enhancing interventions.

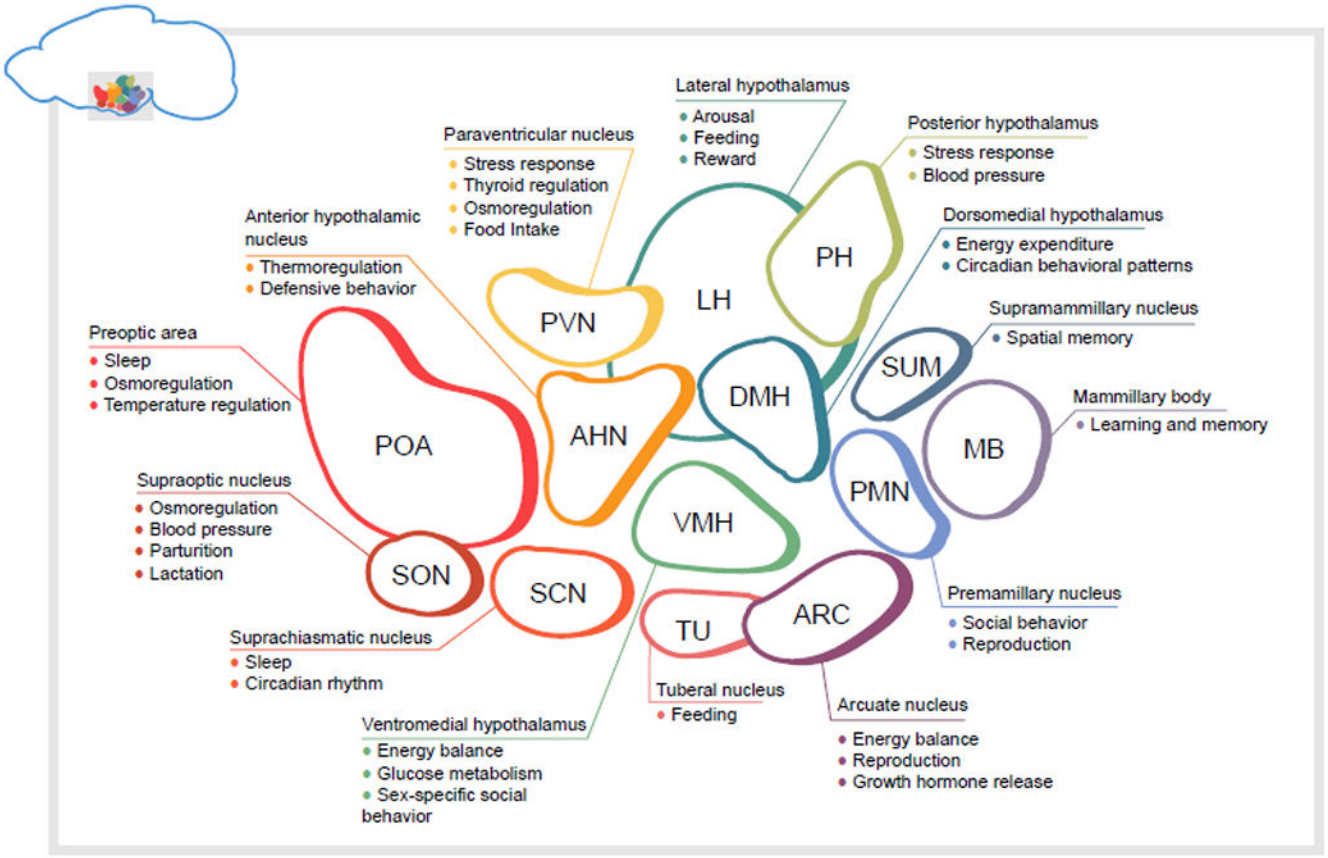

Figure 1. Schematic of hypothalamic nuclei and their functions.

In the following, the figure's nuclei are described counterclockwise, starting with the preoptic area. Many of the specific behaviors described relate to studies in rodents, although some of the functions are relevant to other species as well. **POA**. Preoptic area neurons are involved in sleep and temperature regulation[25]. **SON**. The supraoptic nucleus contains two major populations: vasopressin (encoded by *Avp*) expressing neurons control osmoregulation, while oxytocin neurons (encoded by *Oxt*) play an essential role in parturition, lactation, and other social behaviors[30,31]. **ANH.** The anterior hypothalamic nucleus is understudied, but has a known role in defensive attack behaviors[32]. **SCN**. The suprachiasmatic nucleus is the core of the internal circadian clock, and entrains body processes and behaviors to the light cycle. **TU.** TU^{Sst+} (encoding somatostatin) neurons are involved in feeding[33]. **ARC.** The arcuate nucleus of the hypothalamus is a critical region for energy homeostasis and reproduction[34,35]. ARC^{Kiss1} (kisspeptin neurons) are essential for proper pulsatile release of reproductive hormones [34]. **DMH.** Leptin sensitive neurons in the dorsomedial hypothalamus are essential for energy expenditure via brown adipose tissue thermogenesis, as well as the circadian timing of feeding and activity[36]. DMH^{Brs3} neurons are involved in heart rate and maintenance of body temperature[24]. **VMH.** Leptin sensitive neurons on the ventromedial hypothalamus protect against diet-induced obesity, and regulate the interaction between estrogen and body composition[37,38]. Glucose-excited and glucose-inhibited neurons in the VMH control whole-body glucose homeostasis[39]. VMH^{Esr1} neurons coordinate social behaviors such as attack and

mounting[40]. **PMH.** The ventral premamillary nucleus links conspecific odorant cues and energy balance signals to reproductive function[41,42]. The dorsal premammillary nucleus controls escape behavior in response to threat[43]. **MB.** The mammillary bodies encode information about head direction, and projections from the mammillary bodies to anterior thalamic nuclei are necessary for spatial memory[44]. **SUM.** The supramamillary nucleus projects to the dentate gyrus and is involved in spatial memory tasks and can promote hippocampal neurogenesis[45,46]. **PH.** The posterior hypothalamus is activated under chronic unpredictable stress[47,48]. Together with the supramamillary nucleus, stimulation of the posterior hypothalamus can induce theta oscillations in the hippocampus[49]. **LH.** Lateral hypothalamus neurons are implicated in arousal and feeding, especially motivation to eat[26,50]. **PVN.** The paraventricular nucleus relays information from the hypothalamus back to the body. PVN^{Trh} neurons release thyrotropin-releasing hormone to the pituitary to control the hypothalamic-pituitary-thyroid axis. PVN^{Crh} expressing neurons are the central regulators of the hypothalamic-pituitary-adrenal (HPA) axis [51,52]. PVN^{MC4R} neurons receive inputs from hypothalamic nuclei such as ARC and promote feeding[53,54].

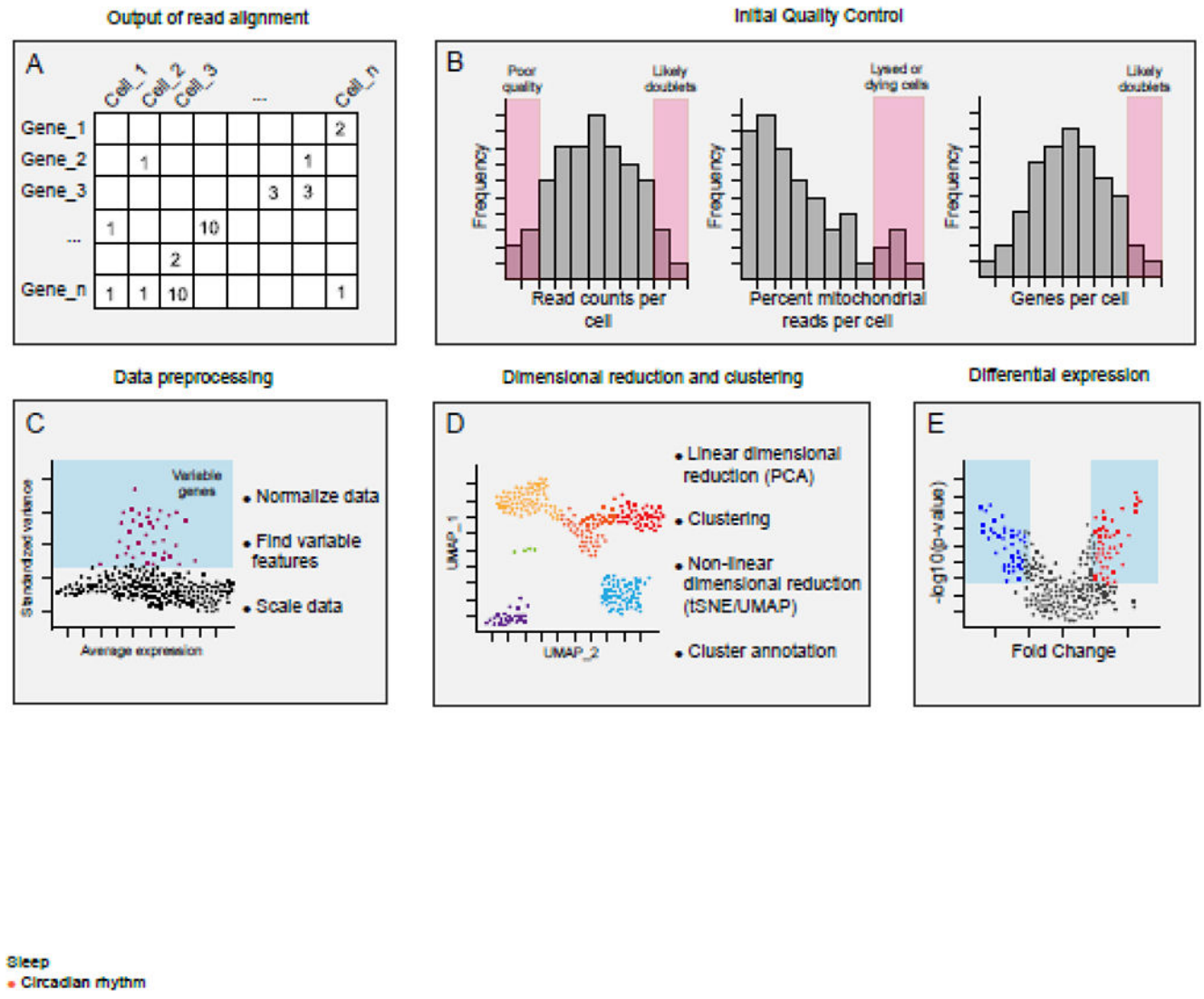

Figure 2. Analysis pipeline for single cell RNA-seq experiments.

A) The output of most alignment software is a barcode by gene matrix. Due to variability among cells, and the technical limitations of single cell RNA-seq sampling, datasets are sparse[82]. B) Quality control and filtering steps are essential and impact downstream analysis. Threshold values should be carefully considered and can vary depending on the tissue analyzed and the sample preparation[83]. The number of counts and features per cell can indicate whether a cell is low quality (few counts or features), or a doublet (very high features compared to other cells). However, some cell types may have more features than others, so strict cut-offs may remove sources of legitimate biological variability. Similarly, the percentage of mitochondrial reads can indicate whether a cell is dead or dying, but some cells do have naturally occurring higher mitochondrial counts. C) Data preprocessing steps include data normalization, the identification of highly variable genes, and data scaling. The subset of genes which are highly variable can be used for downstream analysis. D) Single-cell datasets have high dimensionality, therefore dimensional reduction is used for clustering and data visualization. The results of clustering can vary based

on upstream quality control[83]. E) Testing for differential gene expression between groups is a major goal of many studies. Because of the sparse nature of single cell data distributions, single cell RNA-seq can require different statistical approaches from traditional bulk approaches. Pseudoreplication, in which cells from the same animal are treated as statistically independent replicates, can be avoided through the use of mixed models[84,85].

Table 1.

Single cell studies of adult mouse hypothalamus

Region/cell type	Approach	Total cells	Number of neuronal clusters	Ref.
Whole hypothalamus	Drop-Seq	898	62	[92]
Arcuate nucleus and median eminence	Drop-Seq	20,921	34	[97]
Whole hypothalamus	Drop-Seq	14,000	34	[91]
Sorted POMC-EGFP neurons	SMART-seq	163	5	[96]
Whole CNS, including hypothalamus	10x	509,876	15 peptidergic	[98]
Preoptic region	Drop-Seq	31,299	69	[94]
Lateral hypothalamic area	10x	7,218	30	[99]
Lateral hypothalamic area	Drop-Seq	20194	4	[100]
Ventrolateral ventromedial hypothalamus	SMART-seq and 10x	4574 (SMART-seq), 41,385 (10x)	29	[101]
Sorted *Gipr*^{EYFP} cells	10x	2420	1	[102]
Suprachiasmatic nucleus	Drop-Seq	62,083	16	[103]
Sorted SF1-tdTomato neurons	SMART-seq	530	6	[104]
Whole hypothalamus	10x	51,199	45	[105]
Arcuate nucleus	10x	21,017	14	[106]
Whole hypothalamus	10x	125,224	50	[107]
Whole hypothalamus	10x	15,291	N/A	[108]
Ventral posterior hypothalamus	10x	16,991	20	[109]
Median eminence and mediobasal hypothalamus	10x	54470	1	[110]
Sorted CRH-tdTomato neurons	SMART-seq	254	5	[111]
Arcuate nucleus	10x	7136	4	[112]
Sorted *Hcrt*-dsRed neuronal nuclei	10x	N/A	4	[29]
Median eminence	10x	28,292	1	[113]
Anterior ventral preoptic area	10x	16,430	23	[114]
Whole hypothalamus	10x	40,064	35	[93]
Arcuate nucleus	10x	19,995	1	[115]
Ventral posterior hypothalamus	10x	16,991	20	[109]