The ageing hypothalamus: Impact on neuronal and glial cells that control energy homeostasis

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Abstract

The hypothalamus regulates key endocrine, homeostatic and adaptive functions, ranging from energy uptake and expenditure to circadian rhythm and reproductive functions through the coordinated activity of neuronal and glial cells. There are complex but integrated interconnections between the hypothalamic nuclei that maintain energy homeostasis through regulation of food intake and energy expenditure. Critically, the Arcuate nucleus (Arc) and the circumventricular termed the median eminence (ME) lay at the core of the energy regulation. Assumedly, age-related changes in the hypothalamic circuitry could underlie the decline in metabolism and energy homeostasis observed during ageing. Therefore, the present study is focused on elucidating the effects of ageing in the Arc-ME.

First, age-related changes in the hypothalamic neurons controlling energy balance, POMC and AgRP/NPY, were examined using immunohistochemistry and reporter mice. A reduction in the number of anorexigenic POMC neurons, but not orexigenic NPY, was associated with age. Cell death studies confirmed that programmed cell death events are not responsible for POMC decline. Second, RNA-seq analysis identified four biological processes affected in the ageing hypothalamus, including upregulation of neuroinflammation and myelin-related genes; and downregulation of genes involved in the neuronal cytoskeleton, intracellular transport and axonal growth. Third, age-related changes in the microglia (Iba1+) and astrocytes (GFAP+) suggested that reactive gliosis involving both populations develop gradually in the hypothalamus with age. Also, aged hypothalamic microglia adopt a neurotoxic/pro-inflammatory (M1) activated phenotype. Fourth, *in vivo* adult neurogenesis in the hypothalamus was assessed by cumulative BrdU labelling. The majority of proliferating cells in the ME belonged to the oligodendrocyte and microglia lineage, but a numeric decline is observed with age. Fifth, characterisation of the hypothalamic myelin pattern using immunohistochemistry revealed that myelin microstructure in the Arc-ME is impaired with age. The number and differentiation program of the oligodendrocytes was then investigated using lineage-specific markers, and an increase in number but not differences in oligodendrocyte maturation was observed. However, western blot results supported that inhibition of the intracellular trafficking of myelin components, rather than oligodendrocyte dysfunction, could underlie the defective myelin pattern observed with age.

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Abbreviations

3V	Third ventricle
AraC	Arabinofuranosyl cytosine
ACTH	Adrenocorticotrophic hormone
AD	Alzheimer's disease
AgRP	Agouti-related peptide
Arc	Arcuate nucleus
Arg1	Arginase I
AH	Anterior hypothalamus
ALS	Amyotrophic lateral sclerosis
ALDH1L1	10-formyltetrahydrofolate dehydrogenase
APS	Ammonium Persulfate
ATG5	Autophagy protein 5
ATG7	Autophagy protein 7
BBB	Brain-blood barrier
BCA	Bicinchoninic acid
BDNF	Brain-derived neurotrophic factor
βGal	β-galactosidase
Bmi1	B lymphoma moloney murine leukaemia virus integration site 1
BrdU	5-Bromo-2'-deoxyuridine
BST	Brainstem
CART	Cocaine-amphetamine-regulated transcript
CC1	Anti-adenomatous polyposis coli clone CC1
CC3	Cleaved caspase 3
CD86	Cluster of differentiation 86
Chi3l3	Chitinase-like 3
CLIP	Corticotropin-like intermediate lobe peptide
CMT	Charcot-Marie-Tooth
CNP	2′,3′-cyclic nucleotide phosphodiesterase
CNS	Central nervous system
CO_2	Carbon dioxide
CR3	Complement receptor 3
CSF	Cerebrospinal fluid

CTNF	Ciliary neurotrophic factor
CXCL5	Chemokine (C-X-C motif) ligand 5
d	day
D206	Cluster of differentiation 206
DCX	Doublecortin
DMH	Dorsomedial nucleus
DNA	Deoxyribonucleic acid
DVC	Dorsal vagal complex
DW	Drinking water
DYNC1H1	Dynein cytoplasmic 1 heavy chain 1
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EtBr	Ethidium bromide
FGF	Fibroblast growth factor
Fgf10	Fibroblast Growing factor 10
FSH	Follicle-stimulating hormone
GABA	γ-aminobutyric acid release
Gb	Gerbil
GFAP	Glial fibrillary acidic protein
GHRH	Growth hormone-releasing hormone
GLAST	Glutamate aspartate transporter
GLT-1	Glutamate transporter 1
GnRH	Gonadotropin-releasing hormone
GO	Gene ontology
GS	Glutamine synthetase
HCl	Hydrochloric acid
HD	Huntington's disease
HFD	High fat diet
hrGFP	Humanised renilla GFP
HRP	Horseradish peroxidase
Hu	Human
Iba1	Ionised calcium-binding adaptor protein-1
ICV	Intracerebroventricular
IGF-1	Insulin-like growth factor-1
IHC	Immunohistochemistry

IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit β
IL-1β	Interleukin 1β
IL-6	Interleukin 6
InsR	Insulin receptor
IP	Intraperitoneal
IRS2	Insulin receptor substrate 2
Ki67	Marker of proliferation Ki67
KIF1B	Kinesin family member 1 B
KNDy	Kisspeptin/neurokinin B/dynorphin neurons
LepR	Leptin receptor
LH	Lateral hypothalamus
LV	Lateral ventricles
m	Month
MAG	Myelin-associated glycoprotein
MAP	Microtubule-associated proteins
MB	Mammillary body
MBH	Mediobasal hypothalamus
MBP	Myelin basic protein
MC3R	Melanocortin 3 receptors
MC4R	Melanocortin 4 receptors
MCH	Melanin-concentrating hormone
ME	Median eminence
MHC	Major histocompatibility complex
MLKL	Mixed lineage kinase domain-like
MOG	Myelin oligodendrocyte glycoprotein
MOBP	Myelin-associated oligodendrocyte basic protein
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
Ms	Mouse
α-MSH	α-melanocyte-stimulating hormone
Msh1	Musashi RNA binding protein 1
mtDNA	Mitochondrial DNA
mTOR	Mammalian target of rapamycin
MW	Molecular weight
MYRF	Myelin regulatory factor

NaCl	Sodium chloride
NeuN	Neuronal nuclear protein
NF	Neurofilaments
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NG2	Nerve-glia antigen 2
NGS	Normal goat serum
nLacZ	Nuclear-targeted lacZ
NP-40	Nonyl phenoxypolyethoxylethanol
NPY	Neuropeptide Y
NSC	Neural stem cells
NSPCs	Neural stem progenitor cells
OL	Oligodendrocyte
Olig2	Oligodendrocyte transcription factor 2
OPC	Oligodendrocyte progenitor cells
OXT	Oxytocin
PAG	Periaqueductal gray
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PD	Parkinson's disease
PFA	Paraformaldehyde
PFC	Prefrontal cortex
PH	Posterior hypothalamus
PLP	Myelin proteolipid protein
POMC	Proopiomelanocortin
Pre-OL	Pre-myelinating oligodendrocyte
Prss56	Serine protease 56
PVN	Paraventricular nucleus
RIA	Radioimmunoassay
RIN	RNA integrity
RNA-seq	RNA-sequencing
ROI	Region of interest
ROS	Reactive oxygen species
Rt	Rat
RT	Room temperature

S100β	S100 calcium-binding protein β
SCN	Suprachiasmatic nucleus
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SF1	Steroidogenic factor 1
SGZ	Subgranular zone
SON	Supraoptic nucleus
Sox2	SRY-Box Transcription Factor 2
SVZ	Subventricular zone
TBST	Tris buffered saline with Tween 20
TE buffer	Tris-EDTA buffer
TEMED	Tetramethylethylenediamine
TGFβ	Transforming growth factor β
TLR	Toll-like receptors
TNFα	Tumor necrosis factor α
TRH	Thyrotropin-releasing hormone
TSH	Thyroid-stimulating hormone
TUBB3	βII-tubulin
Tuj1	Neuron-specific class III β-Tubulin
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UV	Ultraviolet
VMN	Ventromedial nucleus
vs	versus
VSP	Vasopressin
VTA	Ventral tegmental area
v/v	Volume/volume
WB	Western blot
WHO	World health organisation
w/v	Weight/volume
yr	Year

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1. General introduction

1.1. The brain and ageing

Ageing is defined as the progressive loss of physiological functions and deterioration in the maintenance of homeostatic processes over time, leading to the end of the life. Several brain functions, including cognition, circadian rhythm, regulation of energy expenditure and autonomic function, decline with age and cause significant impairment in quality of life. Ageing is also the major risk factor for most neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD) and Stroke (Hou et al. 2019; Reeve, Simcox, and Turnbull 2014). Interestingly, in mice, brain-specific genetic modifications produce a significant improvement in several physiological functions, increasing the lifespan (Kappeler et al. 2008; Satoh et al. 2013; Taguchi, Wartschow, and White 2007; Tang, Purkayastha, and Cai 2015; Zhang et al. 2013).

Different neuro-anatomical, cellular and molecular alterations in the central nervous system (CNS) are associated with ageing. Early weight studies reported an overall reduction in brain volume (Dekaban and Sadowsky 1978; Ho et al. 1980). Later studies using non-invasive imaging found that brain volume declines from about 0.1 – 0.2%/year between 30 – 50 years to 0.3 – 0.5%/year over the age of 70 years (Coffey et al. 1992; Jernigan, Press, and Hesselink 1990; Pfefferbaum et al. 1994). Moreover, the volume decline was homogeneous throughout the white matter, while the grey matter showed regional differences (Resnick et al. 2003; Scahill et al. 2003). As a consequence of brain shrinkage, the ventricular system expanded to fill the space vacated. In addition to anatomical changes, the brain also suffers changes at cellular and molecular levels that are described below.

1.1.1. Age-related neuronal changes in the CNS

At a cellular level, the main controversy has been over the presence and magnitude of the neuronal loss. Early studies in the 50s identified between 10 - 60% of neuronal loss, with some neuronal populations more affected than others (Esiri 2007). However, when the stereological-based sampling was developed in the 80s, new results concluded that age-related neuronal loss was practically undetectable (Burke and Barnes 2006; West 1993). The stereological principles allowed to get estimates from three-dimensional tissue, eliminating many of the confounding factors of the previous

studies. Findings were replicated in humans, non-human primates and rodents (Hof and Morrison 2004; Madeira et al. 1995; Merrill, Chiba, and Tuszynski 2001; West et al. 1994) concluding that cell death is not characteristic of the normal ageing process. Interestingly, an exception has been reported in non-human primates, where the area 8A of the prefrontal cortex (PFC) showed a decrease in the neuronal number of neurons, correlating with impaired performance in working memory tasks in aged monkeys (Smith et al. 2004).

Similarly to early investigations in neuronal decline with ageing, early studies identified an age-related dramatic deterioration in neuronal dendritic branching (Scheibel et al. 1975, 1976). Likewise, more recent studies using more accurate methodology reported that age-related alterations in dendritic extent are region-specific (Flood 1993). These regional differences are illustrated by studies performed in different brain areas, as an example, the dentate gyrus (a hippocampal region) showed an increased dendritic branching with age (Flood, Buell, et al. 1987). In a different subregion of the hippocampus, the areas CA1 and CA3 (Flood, Guarnaccia, and Coleman 1987; Hanks and Flood 1991) showed no changes with age. Contrarily, reduction in dendritic arborisation was observed in the PFC (Uylings 1998), suggesting the cortex is a more sensitive area to the effects of ageing.

Additionally, studies of dendritic branching in animal models confirmed human studies, and no dendritic regression was observed with age. In rats, no deterioration in dendritic extent was found in different areas across the hippocampus with age (Pyapali and Turner 1996; Turner and Deupree 1991). Conversely, the pyramidal neurons in superficial cortical layers experience an age-associated decline in dendritic arborisation (Grill and Riddle 2002). The reduction in synapse number is also characteristic of aged brains, as an example, anatomical studies in rats found a decline in synapse number in the hippocampus with age (Curcio and Hinds 1983). Electrophysiological studies and spatial memory deficits in aged rats confirmed the anatomical results (Barnes 1979; Geinisman et al. 1992).

1.1.2. Age-related changes in glial populations in the CNS

The major types of glial cells in the CNS are astrocytes, microglia, and oligodendrocytes. Traditionally, these non-neuronal cells have been considered 'brain glue'; however, glial cells undertake multifaceted roles and are essential for the correct functioning of the brain. Interestingly, an increasing body of evidence suggests that glial function plays a critical role in brain ageing and neurodegeneration (Nagelhus et al. 2013). Main age-related changes in astrocytes, microglia and oligodendrocytes are summarised in the following sections.

1.1.2.1. Astrocytes

The astrocytes are the most abundant cell population in the CNS, outnumbering neurons (Sofroniew and Vinters 2010). Astrocytes are morphologically diverse; these cells share a star-shaped morphology with radial processes (Oberheim *et al*., 2009). In line with their morphological complexity, astrocytes undertake a plethora of different functions to maintain CNS homeostasis. Some of the multifaceted roles of astrocytes include neuronal support, synapse regulation, neurotransmitter homeostasis, glycogen storage, ion homeostasis, cholesterol synthesis and the brain-blood barrier (BBB) maintenance (Lundgaard et al. 2014; Nedergaard, Ransom, and Goldman 2003). Also, astrocytes, together with microglia, provide a defence to the CNS through a process called reactive astrogliosis (Pekny and Pekna 2014; Sofroniew 2009).

Astrocytes undertake a phenotypic switch with age: from slender and long processes in young, to thickened and short in aged animals (Castiglioni et al. 1991; Cerbai et al. 2012; Jyothi et al. 2015). Age-related morphological changes have a been reported in aged rodents (Amenta et al. 1998; Castiglioni et al. 1991) and primates (Kanaan, Kordower, and Collier 2010; Robillard et al. 2016). Also, increase in astrocytes number has been described across multiple brain areas with age, including in the hippocampus, the cortex (Amenta et al. 1998; Sabbatini et al. 1999) and the hypothalamus (Wang et al. 2006).

The glial fibrillary acidic protein (GFAP), a classic astrocytic marker, has been widely used to visualise astrocytes; however, GFAP expression is not detected in all astrocytes (Sofroniew and Vinters, 2010). Interestingly, ageing studies have reported upregulation

of GFAP expression (Clarke et al. 2018; Kohama et al. 1995; Rozovsky, Finch, and Morgan 1998; Wu, Zhang, and Yew 2005), a feature of activated/reactive astrocytes (Liddelow and Barres 2017; Sofroniew and Vinters 2010; Zamanian et al. 2012). Additional molecular markers identified include glutamine synthetase (Norenberg and Martinez-Hernandez, 1979), S100 calcium-binding protein β (S100β) (Hachem *et al*., 2005; Gonçalves, Concli Leite and Nardin, 2008), 10-formyltetrahydrofolate dehydrogenase (Aldh1L1) (Barres, 2008), glutamate aspartate transporter 1 (GLAST) (Shibata *et al*., 1997) and glutamate transporter 1 (GLT-1) (Perego et al. 2000). Although these markers are enriched in astrocytes, some of them are not specific to this glial lineage and are found to be expressed in neurons and other glial types.

Recently, transcriptomic studies provided a comprehensive database of aged astrocyte gene expression. Pathway analysis identified the upregulation of genes involved in immune pathways (Boisvert et al. 2018; Pekny and Pekna 2014). The study of individual markers in ageing astrocytes revealed upregulation of complement system genes, such as C1q, C3 and C4B; major histocompatibility complex I (MHC I) and inflammatory cytokines with chemotactic effects such as CXCL5 (Boisvert et al. 2018). The increased expression of pro-inflammatory genes pointed out that astrocytes could contribute to the low-level inflammatory state observed during ageing (Sanada et al. 2018).

In addition to changes in the reactive state of astrocytes, dysregulation genes involved in vital functions such as cholesterol synthesis, ion homeostasis, trophic factor production and BBB structure occur with age (Boisvert et al. 2018). Since the CNS cannot uptake the molecule from the bloodstream due to the BBB, astrocytes hold a significant role in the local cholesterol production. Dysregulation of the normal functioning of astrocytes may cause metabolic defects in neuronal and glial cells that depend on astrocyte cholesterol production. Moreover, aged astrocytes show a decline in the production of metabolic and trophic factors, essential for neuronal function, survival and neurogenesis (Palmer and Ousman 2018). Lastly, astrocytes are integral parts of the BBB structure, and they participate in its development, maintenance and regulation (Abbott and Friedman 2012). Disturbances in astrocytes are associated with age-related neurodegenerative disorders (Kirk et al. 2003; Zlokovic 2008). Figure 1.1 summarises the main differences observed in astrocytes function with age.

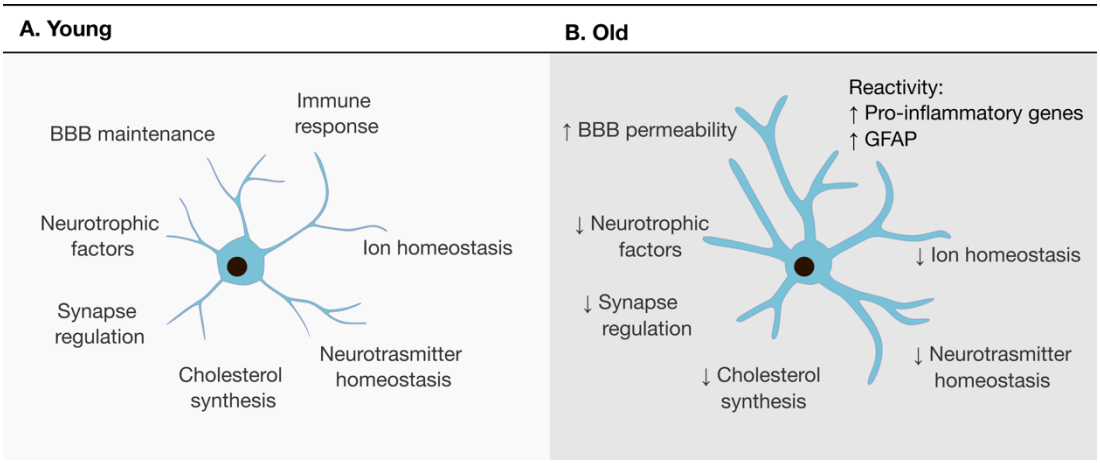


Figure 1.1. Ageing of astrocytes in the mammalian brain. (A) In the adult brain, astrocytes are part of the blood–brain barrier (BBB), maintain ion homeostasis, support and regulate neuronal transmission, are responsible of cholesterol synthesis and are involved in immune response of the brain. (B) Ageing induces molecular and functional changes in the astrocyte function. BBB permeability increases, there is a decline in the production of neurotrophic factor and cholesterol and neurotransmitter homeostasis is affected. Also, aged astrocytes display an inflammatory profile indicated by increased GFAP and complement levels.

1.1.2.2. Microglia

Microglial cells share a myeloid origin with macrophages and constitute the resident immune population of the CNS. In their immune role, microglia are responsible for the inflammatory or immune-mediated responses in the brain and can influence astrocyte activation and recruitment through their secretory profile (Liddelow *et al*., 2017). Microglia also hold multiple non-immune functions involved on the maintenance of the brain homeostasis, including synaptic organisation, control of neuronal excitability (Tay, Carrier, and Tremblay 2019), phagocytic debris removal (Janda, Boi, and Carta 2018), and contribute to myelogenesis in adult life (Grabert et al. 2016; Hagemeyer et al. 2017).

Microglial cells adopt different morphology depending on their activation state. In resting (or inactive) state, microglia exert a small cell soma and several fine, branched processes. However, during resting state, these cells are not static and continuously extend-retract their projections to explore the local parenchyma. Detection of abnormalities in the parenchyma triggers their activation, with cells adopting an amoeboid morphology with an enlarged cytoplasm and shortened processes (Davis, Foster, and Thomas 1994; Tay et al. 2019). Also, upregulation of inflammatory genes,

secretion of cytokines and expression of molecules for antigen presentation is characteristic of the activated state (Prinz and Priller, 2014).

Immunochemistry against ionised calcium-binding adaptor protein-1 (Iba1), a protein surface microglial marker, has been widely used to visualise microglia. In humans and animal models, aged microglia stained for Iba1 showed shorter and less branched microglial processes similar to activated microglia (Norden and Godbout 2013). In addition, the microglia number and density are reported to increase in several CNS regions with age, including the hippocampus (Wong 2013), cortex (Tremblay et al. 2012) and the retina (Damani et al. 2011). Aged microglia also express a plethora of pro-inflammatory markers, characteristic of the activated phenotype. These include enhanced expression of MHC II and complement receptor 3 (CR3) (Frank et al. 2006; VanGuilder et al. 2011a; Ziv et al. 2006), scavenger receptor CD68 (Godbout et al. 2005; Xie et al. 2003), and co-stimulatory molecule CD86, CD11b and CD11c integrins (Perry, Matyszak, and Fearn 1993), and Toll-like receptors (TLR) (Letiembre et al. 2007). Also, aged microglia presents an increased expression of pro-inflammatory cytokines TNFα, IL-1β and IL-6 (Campuzano et al. 2009), and anti-inflammatory IL-10 and transforming growth factor beta (TGFβ) effectors (Norden and Godbout 2013).

However, the majority of the studies used microarray or bulk RNA sequencing (RNA-seq), making it impossible to identify any regional microglial differences with age. Recently, this caveat was addressed in a transcriptomic study where microglia form different brain regions across the mouse lifespan were isolated for RNA-seq analysis. Results identified upregulation of pro-inflammatory genes in aged microglia across all regions studied. However, the magnitude and identity of inflammatory response varied according to the neuroaxis location, suggesting a differential regional response to ageing (Grabert et al. 2016).

In addition to their enhanced inflammatory response, aged microglia exert deficits in phagocytosis, involved in clearance of cellular debris and protein aggregates. As an example, myelin fragments are engulfed and degraded by microglia during myelin remodelling (Traiffort et al. 2020). However, studies suggest that an increase in myelin fragments with age can overload microglial cells, altering their homeostasis and contributing to their age-related dysfunction (Safaiyan et al. 2016).

1.1.2.3. Oligodendrocytes and Myelin

The oligodendrocytes (OLs), are glial cells responsible for myelin sheath formation within the CNS, facilitating efficient axonal conduction and providing axons with metabolic and trophic support (Y. Lee et al. 2012; Nave and Trapp 2008; Waxman 1977).

Myelin degeneration and loss of myelinated nerve fibres have been extensively reported in multiple brain regions during normal ageing, including the cortex, corpus callosum, hippocampus and the auditory nerve (Bowley et al. 2010; Peters 2002; Peters, Moss, and Sethares 2000; Sandell and Peters 2001; Wang et al. 2020; Xing et al. 2012). Defective myelination has also been observed in pathological conditions such as multiple sclerosis (MS) but also in several age-related neurodegenerative disorders (Bartzokis 2011; Braak and Del Tredici 2004; Romanelli et al. 2016). Furthermore, decreased expression of myelin-specific proteins, such as MBP and PLP, has also been reported in humans and animal models (**Table 1.1**). The age-related breakdown myelin sheaths, that provide electric insulation of axons and accelerate the transmission of electrical signals, may disrupt connectivity and reduced speed of information.

Life-long production of OLs from oligodendrocyte progenitor cells (OPCs) is required for myelination of new neuronal circuits and repair of myelin lost through natural 'wear and tear'. In rodents, production of new OLs is observed throughout the CNS, even in adulthood (Young et al. 2013). In the rodent cortex, the OL population expands between young adult and middle-aged animals, and then declines in old age (Hill, Li, and Grutzendler 2018; Hughes et al. 2018) (**Figure 1.2**). In the corpus callosum, impairment of OPC proliferation, recruitment and differentiation into myelinating oligodendrocytes has been reported with age (Psachoulia et al. 2009; Sim et al. 2002; Spitzer et al. 2019). Therefore, it has been recently proposed that the age-induced decline in OPC function underlies the myelination defects observed in healthy ageing (Neumann et al. 2019).

Table 1.1. Age-related changes in myelin components expression across distinct CNS regions in adult mammals

Myelin components	CNS region/ Cell type	Organism	Age-related changes	Method	Age of study	Phentoype	Reference
MBP	Hippocampus	Gb	Reduction	IHC, WB	1 m, 6 m , 24 m	Decrease in myelin fiber density	Ahn *et al.*, 2017
MBP	Frontal lobe white matter	Hu	Reduction	RIA	45-60 yr and 78 yr	Not stated	Ansari *et al.*, 1985
MBP	Cortex, Cerebellum	Rt	No differences	IHC	6, 12 and 24 m	Not stated	Ciftci *et al.*, 2012
MBP, MOBP, PLP	OLs isolated from whole brain	Rt	Increase	WB	3 m, 13 m and 18 m	Not stated	de la Fuente *et al.*, 2020
MBP	Cortex, Hippocampus	Ms	Reduction	IHC	4, 13, 18 m	Decrease in myelin fiber density	Wang *et al.*, 2020
MBP, MOG	Whole brain (WB) Corpus callosum, Spinal cord, Cortex (IHC)	Rt	Reduction	IHC, WB	5 m, 18 m, 26 m	Myelin breakdown	Xie *et al.*, 2013
MBP, MOBP, MOG	Optic nerve	Rt	Reduction	Microarray	24 m	Myelin breakdown	Xie *et al.*, 2014
MBP	Auditory nerve	Ms	Reduction	IHC	1-3 m, 23-28 m	Myelin breakdown	Xing *et al.*, 2012
		Hu	Reduction	IHC	46-91 yr	Decrease in myelin fiber density	
MBP, Opalin	Cerebellum, Corpus Callosum, Hippocampus, Thalamus, Optic nerve	Ms	Opalin reduction in Cerebellum	IHC	21 d, 6 m	Not stated	Sato *et al.*, 2014

Abbreviations used: d - day. Gb - gerbil. Hu - human. IHC - immunohistochemistry. m - month. MBP - myelin basic protein. MOBP - myelin-associated oligodendrocyte basic protein. MOG - myelin oligodendrocyte glycoprotein. Ms - mouse. PLP - myelin proteolipid protein. RIA - radioimmunoassay. Rt - rat. WB - western blot. yr - year.

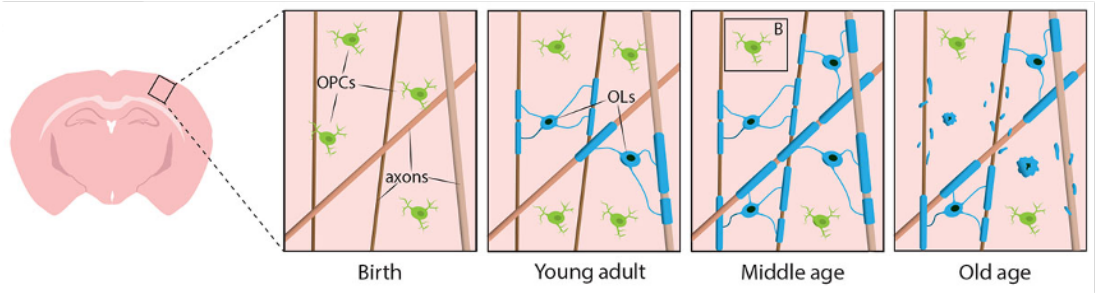


Figure 1.2. Oligodendrocyte and myelin dynamics in the mammalian cortex throughout life. Oligodendrocyte precursor cells (OPCs) continuously generate new myelinating oligodendrocytes (OLs) in the somatosensory cortex from birth up to middle age. The OL population then declines in old age, accompanied by a reduction in myelin coverage. Adapted from Williamson and Lyons 2018.

1.1.3. Possible molecular causes of brain ageing

Many hypotheses have been proposed to explain the cause of ageing and biological bases for the decline in brain functions. Summarised below are presented the main findings in the aged CNS.

1.1.3.1. Telomere shortening

Changes in telomere length have been reported in mitotic cells during ageing; however, the relationship between telomere length in post-mitotic cells and ageing is unclear. Only a few studies have examined telomere length in cells from the mammalian CNS. Telomeres shorten with every cell division and is a hallmark of replicative senescence (Harley, Futcher, and Greider 1990; Hastie et al. 1990). Telomere length is generally greater in the brain from adult and old humans compared with other systemic organs, as can be expected due to the limited cell division taking place there (Butler et al. 1998; Takubo et al. 2002). The human brain retains long telomere length in the case of a 115-year old supercentenarian (Holstege et al. 2014). In line with this, analysis of the telomere dynamics in the human pituitary gland showed that it is highly conserved throughout adult life to centenarian age (Ishikawa et al. 2012). In agreement with human findings, telomeres shorten with increasing age in the liver, lung, kidney and pancreas, but not in the brain (Cherif et al. 2003).

However, analyses of cultured rat microglia showed telomere shortening and reduced telomerase activity in ageing (Flanary and Streit 2004) and in AD (Flanary et al. 2007). Future analyses of discrete brain regions and individual neural cell types will allow the detailed characterisation of their telomere lengths during ageing.

1.1.3.2. Oxidative stress and mitochondrial dysfunction

The oxidative stress or free radical theory (Harman 1988), and its later refined version, the mitochondrial free radical theory (Alexeyev 2009), are the major theories of ageing. Both theories postulate that cellular and tissue degeneration reported during ageing is a consequence of excessive production of reactive oxygen species (ROS) and free radicals, where the mitochondrial theory positions the mitochondria at the primary source of oxidative damage.

Increasing evidence indicates that accumulation of protein, lipid, and DNA damage by free radicals, is responsible for the decline in brain functions with age. In support of this, comparison between young and old animal brains showed higher levels of ROS, mitochondrial DNA (mtDNA) damage and oxidative stress markers (Chomyn and Attardi 2003; Liu et al. 2003; Serrano and Klann 2004; Sohal et al. 1994). Also, the appearance of cognitive impairment and learning deficits strongly correlates with increasing brain oxidative damage in aged animal models (Carney and Floyd 1991; Floyd and Carney 1991). Interestingly, an inverse correlation has been shown between oxidative damage to mtDNA and maximum lifespan in the brain of mammals (Barja and Herrero 2000).

The brain is extremely vulnerable to oxidative stress due to its relatively low antioxidant defences (Ahlgren-Beckendorf et al. 1999; Baxter and Hardingham 2016; Dringen, Pawlowski, and Hirrlinger 2005). With age, there is a decline in the antioxidant activity in the brain, increasing its vulnerability dramatically to oxidative damage (Venkateshappa et al. 2012; Yehuda et al. 2002). Also, among the CNS cell types, neurons and OLs are more vulnerable to ROS mediated cellular damage due to the high demand for energy and mitochondrial activity (Fukui and Moraes 2009; McTigue and Tripathi 2008).

In summary, brain ageing is marked by reduced antioxidant defences, enhanced ROS production and oxidative stress, and mitochondrial dysfunction. Interestingly, oxidative stress is involved in maintaining the chronic inflammation observed in ageing and age-related disorders (Fuente and Miquel 2009).

1.1.3.3. Chronic inflammation and brain ageing

The term 'Inflammageing' describes the close relationship between low-grade chronic inflammation and ageing that has been linked to a broad spectrum of age-related disorders in various organs, including the brain (Franceschi et al. 2007). Major neurodegenerative diseases, such as AD and PD, present chronic activation of innate immune responses, including those mediated by microglia as a common hallmark (Glass et al. 2010).

Excessive and prolonged inflammation resulting from microglial and astrocyte activation has been reported during ageing and age-related disorders, causing neuroinflammation,

synaptic damage and, ultimately, neuronal loss (Chung et al. 2019). Also, the age-related accumulation of oxidative stress and free radical damage promotes microglia and astrocyte transition towards the activated phenotype (Norden and Godbout 2013). Moreover, recent findings showed that activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) increase in many regions of the aged mouse brain, but is most significant in the hypothalamus (Zhang et al. 2013). NF-κB is a central regulator of transcription that mediates inflammation, immune response and cell death, and has been implicated previously in the control of gene expression during ageing (Adler et al. 2007; Hayden and Ghosh 2008).

Previous studies have shown that peripheral inflammation can alter the neuroinflammation in the brain. Reportedly, pro-inflammatory mediators, such as TNFα and IL-6, are generally increased in the sera of aged humans (Krabbe, Pedersen, and Bruunsgaard 2004) and rodents (Campuzano et al. 2009; Sparkman and Johnson 2008). The circulating pro-inflammatory cytokines can reach the CNS through three routes: (1) Active transport across the BBB (Dantzer et al. 2008; Fung et al. 2012); (2) Via circumventricular organs, such as the median eminence (Roth et al. 2004); (3) Afferent nerve stimulation (e.g., the vagus nerve), which then transmit the currently heightened inflammatory status to lower brain stem regions including the hypothalamus, amygdala and bed nucleus of the stria terminals (McCusker and Kelley 2013).

1.1.3.4. Defective autophagy

Autophagy is a conserved cellular process that recycles unwanted cytoplasmic contents, including proteins and organelles, within lysosomes to maintain cellular homeostasis (He and Klionsky 2009). Few studies have covered the effects of ageing in the brain autophagy. In humans, the expression of genes related to autophagy, such as Beclin 1 (Shibata et al. 2006), Atg5 and Atg7 (Lipinski et al. 2010), is reduced in the ageing brain. Also, ageing-dependent impairment in brain autophagy has been reported in the rat (Yu et al. 2017) and the mouse models (Kaushik et al. 2011; Ott et al. 2016). Reportedly, healthy aged mice exhibit defective autophagy in the hypothalamus (Kaushik *et al*., 2012).

Interestingly, suppression of autophagy in the brain has been linked to neurodegeneration (Hara et al. 2006; Komatsu et al. 2006). In autophagy-deficient mice,

neurodegeneration is accompanied by toxic-protein aggregation of ubiquitinated proteins, similar to those observed in human neurodegenerative disorders such as Huntington's disease (HD) and AD (Bishop, Lu, and Yankner 2010). Thus, reduced autophagy and accumulation of protein aggregates may contribute to severe neuronal dysfunction with increasing age.

1.1.3.5. Evolutionarily conserved pathways involved in the determination of lifespan

Two signalling mechanisms contributing to brain ageing are the mammalian target of rapamycin (mTOR) and the insulin/insulin-like growth factor-1 (IGF-1) (IIS) pathways. mTOR and IIS pathways are very frequently altered on models of senescence and neurodegeneration, and recent studies have shown that modulating these pathways may increase lifespan.

At the molecular level, mTOR is a crucial coordinator of metabolic regulation and is involved in the regulation of numerous neurological processes, including neural development, circuit formation, and the neural control of feeding (Mori et al. 2009). Also, the mTOR pathway is recognised as the most important negative regulator of autophagy (Ganley et al. 2009; Jung et al. 2009). Interestingly, inhibition mTOR signalling by genetic or pharmacological intervention has been reported to extend lifespan in mice (Harrison *et al.*, 2009). However, the extent to which mTOR signalling modulates lifespan is not clear, but together with autophagy, has a key role in the development of age-dependent neurodegenerative diseases caused by protein aggregates (Mueed et al. 2019).

Another vital signalling mechanism contributing to brain ageing is IIS pathway. Reduced IIS pathway has been shown to extend lifespan in worms, flies and mammals (Broughton and Partridge 2009; Kappeler et al. 2008). Although reduced IIS in the CNS can extend lifespan, insulin and IGF-1 are also neurotrophic and promote neuronal survival by inhibiting apoptosis (Duarte et al. 2005). Interestingly, IRS2 (Insulin receptor substrate 2) or IGF-1 receptor knockout mice showed reduced cognitive impairment and neurodegeneration in models of AD (Cohen et al. 2009; Freude et al. 2009). Also, in AD patients, reduced expression of IGF signalling is reported (Moloney et al. 2010). The role of the IIS pathway is, therefore, debatable about its response as an effective neuroprotector as well as an indicator of the neurodegenerative process.

1.2. The hypothalamus

Over the last 20 years, there has been a growing interest in understanding the mechanisms and circuits involved in the regulation of energy and homeostasis and how these processes are cause or consequence of ageing. Energy homeostasis is defined as the balance between calorie intake and energy expenditure, which is critical for health and survival. Notably, the shift in the energy homeostasis is one of the hallmarks of the physiological changes during ageing, likely caused by the progressive impairment of mechanisms that control body homeostasis (Roberts 2000). Also, the prevalence of metabolic syndromes significantly increases with age (Hildrum et al. 2007; Saklayen 2018). Even in healthy aged people, a variation in the metabolic activity and body composition often results in sarcopenic obesity, which is characterised by an increase in the fat mass due to loss of muscles in the body composition (Lim et al. 2010). Paradoxically, Poor appetite in the elderly is quite common, resulting in an imbalance between nutrition and energy metabolism (Morley 1997; Visvanathan 2015). The consequential reduction in daily calorie and nutrient intake exacerbates the effects of ageing on a host of physiological systems – from lowered lean mass and bone density to altered gut architecture and microbiota.

The maintenance of body homeostasis is a tightly regulated and complex process facilitated by the cross-talk between the hypothalamus and peripheral organs such as the pancreas, the stomach and the gut (Brunetti et al. 2005). Peripheral signals from the bloodstream reach the brain, which interprets and integrates them to maintain the energy homeostasis. The hypothalamus, in the ventral diencephalon, is the organ responsible for integrating and responding to this variety of hormonal and metabolic signals. The hypothalamus contains highly conserved neuronal circuitries that control basic functions. thermoregulation, energy expenditure and nutrient intake, sexual and reproductive behaviour, nursing, growth and control of circadian and ultra-circadian cycles are some examples of functions under direct and indirect hypothalamic control (Chen, Maevsky, and Uchitel 2015; Melmed et al. 2016).

Furthermore, the hypothalamus contains several different types of neurons and glial cells, distributed within different nuclei that regulate homeostatic functions and play an important role in the control of the body homeostasis. During ageing, the responsiveness of these neurons to peripheral stimuli declines, causing a disruption on

the energy homeostasis control (Dilman et al. 1979). This alteration of the energy homeostasis is accompanied by the development of different age-related conditions such as reduced reproductive functions, insulin resistance, osteoporosis, loss of muscular tone and abnormalities on fat accumulation (Rehman and Masson 2001). Taking all together, the critical role of the hypothalamus in energy control and body homeostasis targets this region as a key regulator of whole-body ageing and its study mandatory to understand the causes underlying the ageing process.

1.2.1. The anatomy of the hypothalamus

The hypothalamus is a small, evolutionarily conserved brain region (Xie and Dorsky 2017) that is located below the thalamus and sits just above the pituitary and brainstem (**Figure 1.3** and **Figure 1.4**). The hypothalamus is subdivided along the rostro-caudal axis into three areas: anterior, tuberal and posterior (Schünke 2016). In the rostral part, nuclei of the *anterior region* regulate thermoregulation (Boulant 2000), sleep and circadian rhythms (Deurveilher and Semba 2003) and sexual behaviour (Paredes 2003). In the middle part, the *tuberal hypothalamus* regulates energy homeostasis (Dietrich and Horvath, 2013; Pearson and Placzek, 2013; Prevot *et al.*, 2018). In the caudal aspect, the *posterior hypothalamus* contains the mammillary bodies, involved in recollective memory, as well as areas dorsal to them like the posterior hypothalamic nuclei (Peterson and Mayes 2019; Vann 2010). At the base of the hypothalamus is located the median eminence (ME), that connects the hypothalamus with the pituitary gland through the hypophysial portal system (Yin and Gore 2010) (**Figure 1.3**).

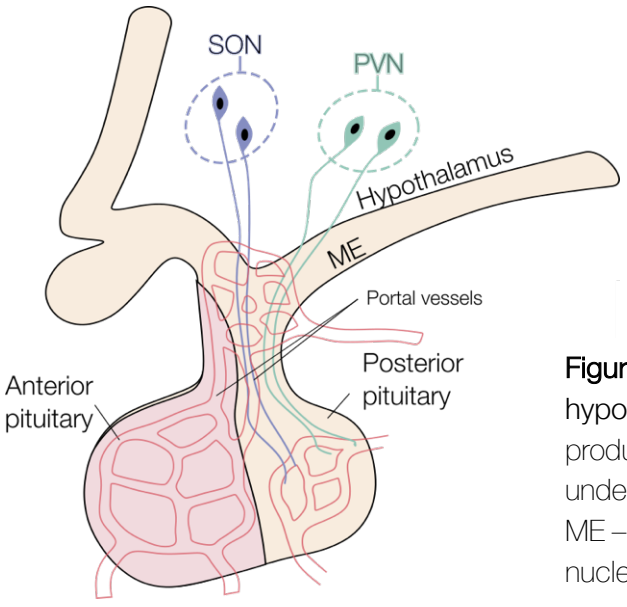


Figure 1.3. The relationship between the hypothalamus and the pituitary. The production of the pituitary hormones is under the control of hypothalamic neurons. ME – Median eminence. SON – Supraoptic nucleus. PVN – Paraventricular nucleus.

The hypothalamus links the nervous and endocrine systems via the pituitary gland. The hypothalamus controls the anterior (adenohypophysis) lobe of the pituitary through secretion of releasing hormones and inhibiting hormones that stimulate or inhibit the production of hormones in the anterior pituitary, including thyroid-stimulating hormone (TSH), adrenocorticotrophic hormone (ACTH) and follicle-stimulating hormone (FSH). Regulation of the posterior (neurohypophysis) lobe of the pituitary involves the projection of neurons from the PVN via the hypothalamohypophyseal tract to stimulate the release of oxytocin (OXT) and vasopressin (VSP) into the bloodstream (Xie and Dorsky 2017).

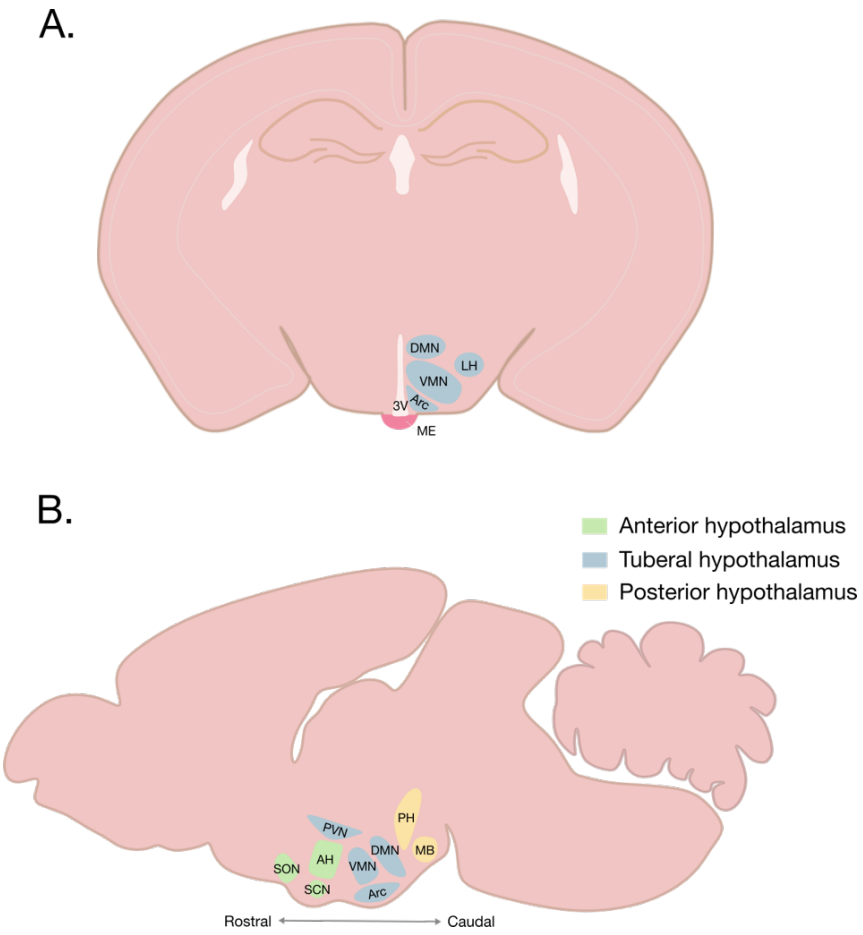


Figure 1.4. Anatomy and organisation of the hypothalamic regions in the mouse brain. (A) Schematic coronal view of the adult mouse brain depicting the location of hypothalamic nuclei present at the approximated bregma position -1.7 mm. The median eminence (ME) region is highlighted in red, below the third ventricle (3V). (B) Schematic sagittal view of the adult mouse brain depicting the location of the different hypothalamic nuclei. Tuberal nuclei (blue) depicted are the ventromedial nucleus (VMN), dorsomedial nucleus (DMH), arcuate nucleus (ARC), paraventricular nucleus (PVN) and the lateral hypothalamus (LH, coronal only). The anterior nuclei in the sagittal section are the suprachiasmatic nucleus (SCN), supraoptic nucleus (SON) and the anterior hypothalamus (AH). Posterior nuclei are the posterior nucleus (PH), and the mammillary body (MB). Generated from (Nesan and Kurrasch 2016).

1.2.2. Hypothalamic regulation of energy homeostasis

The tuberal hypothalamus contains a diverse collection of interconnected neurons and supporting glial cells organised in at least five different nuclei contained into the flanking parenchyma of the third ventricle (3V): The Arcuate (Arc), Dorsomedial (DMN), Lateral Hypothalamic (LH), Ventromedial (VMN) and Paraventricular (PVN) nucleus (**Figure 1.5**). There are complex but integrated interconnections between the hypothalamic nuclei that maintain energy homeostasis through regulation of food intake and energy expenditure. Critically, the Arc neurons lay at the core of the energy regulation (Dietrich and Horvath 2013).

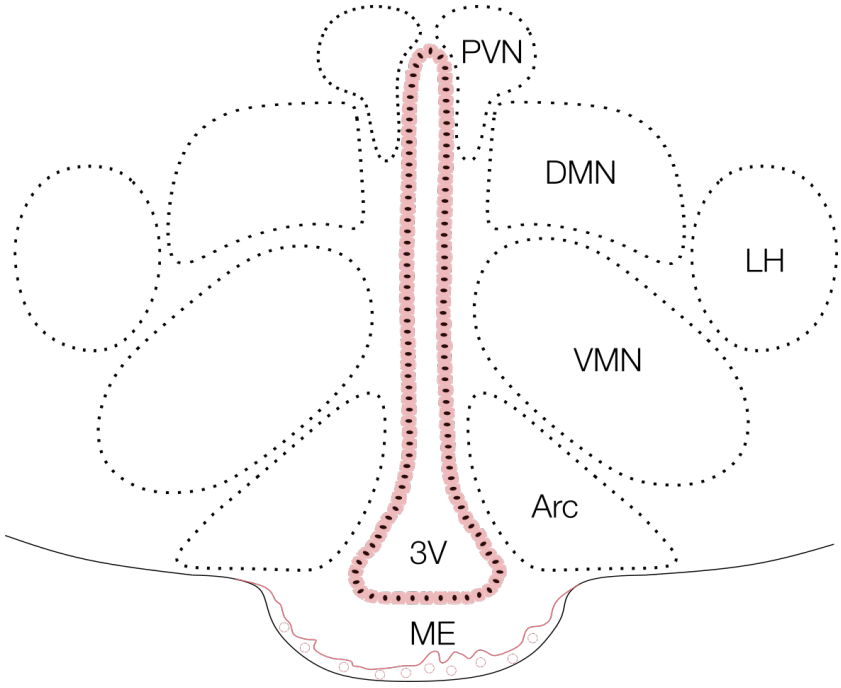


Figure 1.5. Schematic representation of the different hypothalamic nuclei related to the appetite regulation and energy homeostasis control. Pink cuboid cells represent the ependymal cells lining the third ventricle (3V). Arc – arcuate nucleus; DMN – dorsomedial nucleus; LH – lateral hypothalamus; ME – median eminence PVN - paraventricular nucleus; VMN – ventromedial nucleus.

The central melanocortin system is the primary regulator of feeding behaviour and energy homeostasis via the opposing action of two main neuronal types in the Arc nucleus – the orexigenic (appetite-increasing) neurons expressing agouti-related peptide (AgRP) and neuropeptide Y (NPY) (Hahn et al. 1998); and the anorexigenic (appetite-suppressing) neurons expressing proopiomelanocortin (POMC) (Cone et al. 2001). The

anorexigenic and orexigenic neurons (first-order neurons) exert their functions on downstream targets (second-order neurons) that express the of melanocortin 3 and 4 receptors (MC3R and MC4R) and NPY receptors (Y1-Y5) located in the PVN, the DMN and the LH (Betley et al. 2013; Cone et al. 2001; Hahn et al. 1998).

The energy homeostasis is primarily defined by the balance between the firing rate of POMC and NPY/AgRP neurons, and the firing rate of the neurons regulates feeding behaviour and body weight (Cone et al. 2001). Due to its proximity to the fenestrated capillaries present in the ME (Ciofi 2011), the Arc neurons may receive hormonal and metabolic cues from the bloodstream. Among these are the satiety signal leptin, which activates POMC and inhibits NPY/AgRP neurons (Cowley, Smart, and Diano 2001); and ghrelin, a gastric hormone which is produced during hunger and has the opposite effect (Cowley 2003). Also, Arc neurons are known to be responsive to insulin, glucose, gonadal steroids and interleukin-1β (Blum, Roberts, and Wardlaw 1989; Cone et al. 2001; Kohno and Yada 2012; Varela and Horvath 2012). Intracerebroventricular (ICV) injection of insulin into the 3V has been reported to inhibit Npy expression and decreasing energy intake (Obici *et al*., 2002). In turn, glucose modulates both POMC and NPY neurons, promoting POMC neuronal activation but inhibiting NPY/AgRP neuronal activity (Parton *et al*., 2007). Finally, interleukin-1β activates a subpopulation of POMC neurons in the Arc and promotes their release of the anorexigenic α-melanocyte-stimulating hormone (α-MSH) (Scarlett *et al*., 2007).

Together with first-order neurons in the Arc, neurons of the VMN, DMN, PVN and LH also regulate energy homeostasis. In the PVN, where MC3R and MC4R are densely expressed, the orexigenic and orexigenic peptides produced by Arc neurons regulate the release of thyrotropin-releasing hormone (TRH) (Fekete, Légrádi, Mihály, Huang, et al. 2000; Fekete and Lechan 2007; Lechan and Fekete 2006) and corticotropin-releasing hormone (CRH) (Fekete, Légrádi, Mihály, Tatro, et al. 2000; Lu et al. 2003) from PVN neurons. The DMN hosts orexigenic neurons expressing NPY that regulate energy homeostasis but are not directly responsive to leptin (Bi, Robinson, and Moran 2003; Chao et al. 2011). In the VMN, neurons express steroidogenic factor 1 (SF1) and the brain-derived neurotrophic factor (BDNF), which have anorexigenic effects (Dhillon et al. 2006; Rios 2013). In turn, neurons in the LH express the orexigenic neuropeptides orexin a and b that promote food intake; and the melanin-concentrating hormone (MCH), which enhances energy conservation and promotes food intake (Barson,

Morganstern, and Leibowitz 2013). The hypothalamic neurons in these nuclei form complex networks within and outside of the hypothalamus that regulates energy homeostasis (Timper and Brüning 2017).

1.2.2.1. The Arcuate nucleus: the orexigenic and anorexigenic neurons

The Arc nucleus contains two key neuronal populations involved in the control of food intake and energy balance, POMC and NPY. In addition to POMC and NPY, other neuronal types have been described in the Arc nucleus, such as dopaminergic (Zhang and Van Den Pol 2016), γ-aminobutyric acid release (GABA) (Kong et al. 2012), somatostatin (SST) (Campbell et al. 2017) and kisspeptin/neurokinin B/dynorphin (KNDy) neurons (Kumar et al. 2015; Sanz et al. 2015).

POMC and NPY/AgRP neurons are present in the hypothalamus during embryonic development, with the expression of POMC earlier (E10.5) than NPY expression (E14.5). Padilla *et al*., showed that nearly one-quarter of the mature NPY neurons share a common progenitor with POMC. Even though the origin of these neurons is embryonic, it has been demonstrated that their production continues after birth (Chaker et al. 2016; Gouazé et al. 2013a; Niels Haan et al. 2013; Li, Tang, and Cai 2012; Pierce and Xu 2010).

The orexigenic neurons exert their effect through the release of the neurotransmitters AgRP and NPY (Hahn et al. 1998). AgRP is an endogenous inverse agonist of the melanocortin receptor, specifically MC3R and MC4R, expressed by neurons located in the PVN and LH (Betley et al. 2013; Hahn et al. 1998); while NPY is an agonist of the NPY receptors (Y1-Y5) (Fetissov, Kopp, and Hökfelt 2004). In addition to their direct effect to the PVN, the NPY/AgRP neurons innervate and inhibit the activity of POMC neurons via direct neuropeptide action through the Y2 receptor and γ-aminobutyric acid (GABA) (Garcia de Yebenes et al. 1995). During the fasting state, there is an increase in the expression of both of these mRNA, thus, suggesting that hypothalamic NPY/AgRP neurons are activated by fasting to stimulate food intake (Hahn et al. 1998).

Germline knockout for AgRP, NPY, or double knockout NPY/AgRP mice are viable and exhibit normal feeding behaviour and energy homeostasis (Qian et al. 2002). Different reports that NPY Knock-out showed a reduced food intake and anxiogenic-like

phenotype (Bannon et al. 2000). In contrast, AgRP neurons ablation in adult mice produces extreme anorexia and ultimately starvation (Gropp et al. 2005; Luquet 2005). It has been suggested that these different results are the consequence of an adaptive mechanism that occurs only during the development or the neonatal period when the circuit is not fully formed and is more plastic, but not in adulthood (Bewick 2005; Luquet 2005).

The anorexigenic neurons express POMC precursor which undergoes post-translational processing giving rise to different active peptides: The melanocyte-stimulating hormones (MSHs: alpha, beta and gamma), adrenocorticotrophic hormone (ACTH) and β-endorphin (Millington 2007) (**Figure 1.6**). The α-MSH released by POMC neurons acts upon MC4R and MC3R in the hypothalamus as a melanocortin agonist, suppressing feeding, and PVN neurons are one of the main targets (Millington 2007). In addition, POMC neurons express other peptide and non-peptide transcripts such as cocaine-amphetamine-regulated transcript (CART) (N. Vrang et al. 1999), nociceptin, acetylcholine (Meister et al. 2006), GABA and/or glutamate (Dennison et al. 2016; Jarvie and Hentges 2012; King and Hentges 2011). Recent studies described different subtypes within the POMC population, implying that POMC neurons are not a homogeneous population and have functional heterogeneity (Campbell et al. 2017).

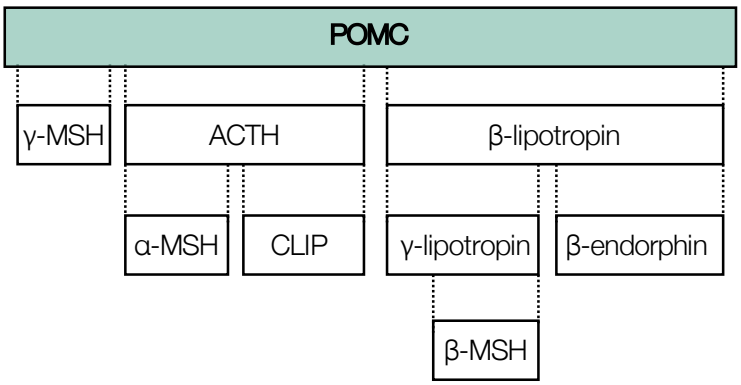


Figure 1.6. Melanocortin peptides ACTH and MSHs (alpha, beta and gamma) derive from post-translational processing of POMC precursor. POMC is also the precursor for opioid peptides β-endorphin and CLIP (corticotropin-like intermediate lobe peptide) Adapted from (Millington, 2007).

Transcriptomic studies have revealed that caloric restriction reduces hypothalamic Pomc mRNA expression (Mizuno et al. 1998), whereas expression is increased in overfed rats (Hagan et al. 1999). In a situation of negative energy balance, as in fasting, the expression of Pomc is decreased. However, during a state of energy surplus, AgRP levels are diminished, and Pomc levels are elevated. Interestingly, Pomc knockout mice exhibit hyperphagia and obesity (Yaswen et al. 1999) similarly to the phenotype reported in humans carrying mutations in the POMC gene (Krude et al. 1998).

1.2.3. The Arc-ME system

The Arc has a unique anatomical relationship with the BBB due to its proximity to the ME (**Figure 1.7**). The ME is one out of eight circumventricular organs in the brain (porous BBB) and plays an important role in the neuroendocrine regulation, facilitating the communication between the hypothalamus and the peripheral endocrine system (Fry and Ferguson 2009; Oldfield and McKinley 2015). In the ME, the signal interchange occurs in both directions. The axons of Arc neurons release hypothalamic hormones into the fenestrated capillaries that carry blood to the pituitary. Moreover, blood-borne signals can diffuse from the ME to the Arc, thereby providing the Arc access to fluctuations of different peripheral signals on the bloodstream (Ciofi 2011).

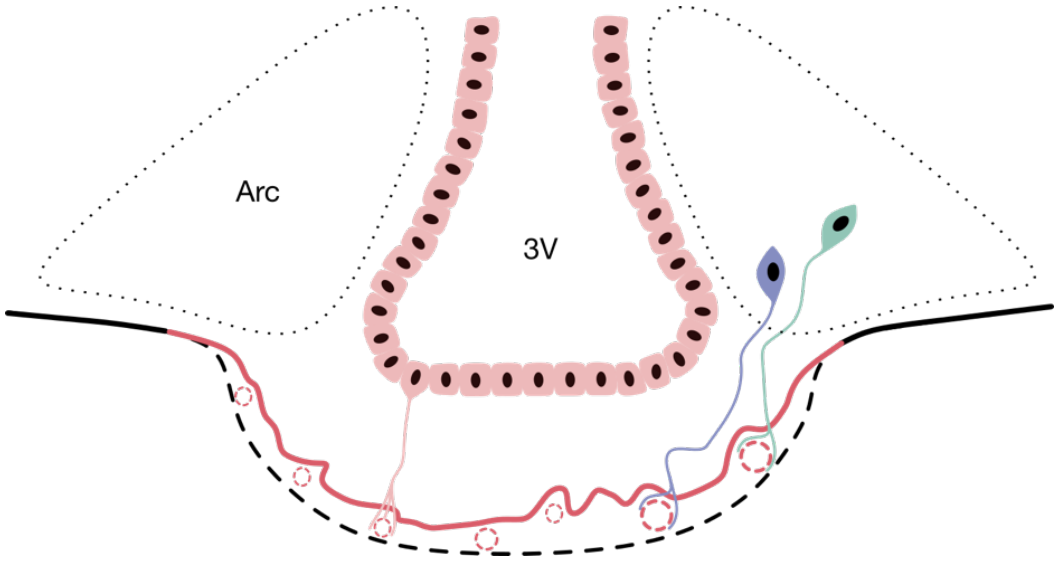


Figure 1.7. Schematic coronal representation of the Arc-ME region. The position of the Arc is depicted. 3V – third ventricle. Arc – arcuate nucleus.

The cell bodies, dendrites and a long segment of the axons of Arc neurons are protected by the BBB. In contrast, the terminal part of these axons can contact the BBB-free regions of the ME (Rodríguez, Blázquez, and Guerra 2010). Interestingly, NPY/AgRP neurons are unique among hypothalamic neurons as their cell bodies are located outside the BBB, which makes them more sensitive to small changes in circulating leptin (Olofsson et al. 2013) and toxins (Yulyaningsih et al. 2017).

The Arc-ME system is in close proximity to the 3V where a population of stem cells lining the floor of the 3V has been described – the tanycytes. Tanycytes contact Arc neurons and the ME trough their processes and it has been postulated that they function as neuromodulating cells since they can regulate the availability of hormones and metabolites from peripheral tissues to Arc neurons (Balland et al. 2014; Collden et al. 2015) and the cerebrospinal fluid (CSF) contained in the 3V (Orellana et al. 2012).

The ME has four distinctive regions (from dorsal to ventral): 3V zone, myelinated axon zone, neural profile zone, and capillary zone. The ME contains nerve terminals and glial cells but is mostly devoid of synapses, neuronal perikarya, and dendrites (Bitsch and Schiebler 1979; Fekete and Lechan 2007). Tanycytes processes can be observed across the 4 regions, with the end feet contacting the basal lamina (Collden et al. 2015; D. A. Lee et al. 2012; Rizzoti and Lovell-Badge 2017). The neuronal profile zone (unmyelinated neuron processes targeting portal vasculature) contains hypothalamic releasing and inhibiting hormone neural processes, such as CRH, somatostatin, TRH, growth hormone-releasing hormone (GHRH), gonadotropin-releasing hormone (GnRH) neurons (Ojeda, Lomniczi, and Sandau 2008; Yin and Gore 2010). The myelinated axon zone contains nerve terminals from VSP, OXT (Holmes et al. 1986; Yin and Gore 2010) and LepR-expressing neurons (Djogo *et al*., 2016).

Different types of glial cells within the ME compartment have been identified: astrocytes, microglia, tanycytes (Yin and Gore 2010) and OPCs (Djogo et al. 2016; Kokoeva, Yin, and Flier 2007). The glial cells in the ME interact with neurons and the portal capillary system, being important for the correct function of the portal. Interestingly, during the ageing process, tanycytes suffer morphological changes in ME, the projections become thicker and disorganized and their cytoplasmatic content in lipid droplets (a unique feature of tanycytes) increase (Brawer and Walsh 1982; Zoli et al. 1995).

Interestingly, the communication within glial cells-nerve terminals has been implicated in the control of different neuronal functions, such as the hormone release and neuronal processes maintenance. The best-documented case is the GnRH hormone release, which is controlled by tanycytes and astrocytes interaction with the GnRH terminals in the ME (Parkash et al. 2015; Yin et al. 2009). In the case of LepR-expressing neurons, NG2-glia in the ME plays an important role in the maintenance of their processes (Djogo et al. 2016).

1.2.4. The hypothalamic stem cell niche

In the adult mammalian brain, it is now known that new neurons and glial cells continue to be generated after the embryogenesis period (Eriksson et al. 1998). Adult neurogenesis involves the proliferation of neural stem/progenitor cells (NSPCs), cell survival, cell death, migration, differentiation and, finally, their functional integration. Neural stem cells (NSC) are multipotent cells able to differentiate through different neural lineages with self-renewal ability; while progenitors are proliferating cells with committed differentiation potential (Pino et al. 2017).

Adult neurogenesis does not occur throughout the whole brain and is limited to anatomically circumscribed zones, termed neurogenic niches. The universally accepted neural stem cell niches are located in the subventricular zone (SVZ), lining the walls of the lateral ventricles, and the subgranular zone (SGZ) of the hippocampus. Besides the canonical neurogenic niches, the hypothalamus has emerged as a novel region for postnatal and adult neurogenesis. Within the hypothalamus, two main potential neurogenic sites have been identified: the tanycytes lining the 3V and the parenchymal region.

Hypothalamic tanycytes emerge during late embryonic development (E17 in mice) and occupy the floor and ventro-lateral walls of the 3V (Altman and Bayer 1978). Their apical poles contact the CSF, and their basal extensions extend to portal vessels or contact different hypothalamic nuclei, predominantly the Arc. Due to their anatomical position, it has been suggested that tanycytes may link the CSF to neuroendocrine events (Rodríguez et al. 2005). Tanycytes are subdivided into alpha (α) and beta (β) subtypes depending on their location along with the 3V and gene expression profile (**Figure 1.8**)

(Goodman and Hajihosseini 2015). The β-tanycytes (β1 and β2) domain occupies the most ventral floor of the 3V, extending their projections into the ME, while simultaneously forming a barrier between the CSF and parenchymal nuclei – the CSF-ME barrier. The α-tanycytes (α1 and α2) are situated more dorsally along the ventro-lateral walls of the 3V, adjacent to the parenchymal DMN and VMN. Tanycytes express multipotent NSC markers such as Sox2 (SRY-Box Transcription Factor 2), Nestin, Bmi1 (B lymphoma Moloney murine leukaemia virus integration site 1 homologue) and Msh1 (Musashi RNA Binding Protein 1) (Goodman and Hajihosseini 2015; Niels Haan et al. 2013; Lee et al. 2016; Li et al. 2012; Robins, Stewart, et al. 2013; Zhang et al. 2017). In addition to the tanycytes, another ependymal cell type, the ependymocytes, with a cuboidal morphology line the 3V. The Ependymocytes are situated more dorsal and form transition zones interdigitating with tanycytes (Hajihosseini et al. 2008).

Even though both tanycytes subtypes share common features, such as the presence of long radial process projected into the parenchyma and the lack of beating cilia, they also display multiple differences. In addition to their different location along with the 3V, α-tanycytes extend only a single villus into the 3V, whereas β-tanycytes extend multiple villi into the 3V space and show blood vessel contact. Furthermore, the majority of β-tanycytes carry primary cilia (Miranda-Angulo et al. 2014), possibly related to cilia–mediated signalling. The expression profile of the alpha-tanycytes is more similar to the ependymocytes, expressing markers such as S100β, GFAP (Niels Haan et al. 2013) and GLAST, and their progeny is mainly parenchymal astrocytes (Chaker et al. 2016; Robins, Trudel, et al. 2013). In contrast, β-tanycytes express several growth factor genes such as Fgf10 (Fibroblast Growing factor 10), FGF-receptors 1 and 2 and CNTF (Ciliary neurotrophic factor) (Goodman and Hajihosseini 2015). In regards to cell progeny, β-tanycytes generate mainly neuronal types that integrate into different hypothalamic nuclei involved in energy balance and appetite such as Arc and VMN (Lee *et al.*, 2012; Haan *et al.*, 2013; Chaker *et al.*, 2016).

Tanycytes occupy a privileged position in the hypothalamus by possessing contacts with both the CSF of the 3V and peripheral signals such as hormones and metabolites through fenestrated capillaries. Furthermore, β2-tanycytes contact the fenestrated capillaries of the ME and sense factors from the bloodstream. Subsequently, β2-tanycytes can transport various factors or signals derived from the bloodstream to

specific regions within the hypothalamus (Balland et al. 2014; Rodríguez et al. 2005) CSF of the 3V.

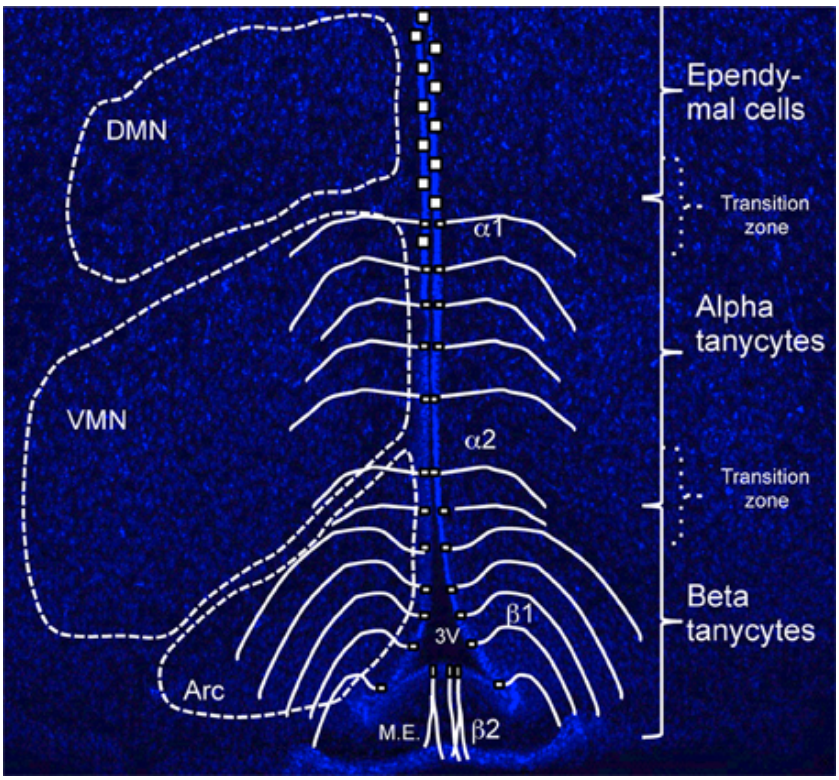


Figure 1.8. Schematic representation of the cell types of the hypothalamic neurogenic niche lining the 3V. Abbreviations: DMN – dorsomedial nucleus. VMN –ventromedial nucleus. Arc – arcuate nucleus. 3V – third ventricle. ME – median eminence (Goodman and Hajihosseini 2015).

In addition to tanycytes, NG2 (Nerve-glia antigen 2) cells have been identified in the parenchymal region of the hypothalamus. NG2 cells have been demonstrated to proliferate in the adult CNS and are well known for their role as OL progenitors (Dimou and Gallo 2015). The hypothalamic NG2 cells are proliferative and express the stem cell marker Sox2 (Robins, Trudel, et al. 2013).

Genetic fate mapping based on inducible Cre recombinase expression under the promoter of NG2 has demonstrated that a high proportion of hypothalamic NG2-glia differentiate into OLs whereas only a small proportion (8.6%) give rise to neuronal fate cells. The neuronal daughter cells derived from NG2-glia show an immature neuronal phenotype and seem to receive input, indicative of their electrical integration in local hypothalamic circuits. Thus, NG2-glia can take neuronal fates and mature into functional

neurons, indicating that NG2 glia contributes to the neurogenic capacity of the adult hypothalamus (Robins, Trudel, et al. 2013). Also, recent studies have shown that NG2 cells function is connected with leptin signalling in the Arc neurons, suggesting that not only tanycytes but also NG2-glia could act as neuromodulating cells in the Arc-ME system.

In summary, previous evidence suggests that tanycytes lining the 3V and NG2-glia constitute different stem cells populations in the adult hypothalamus. However, the fate of their progeny and their capacity to generate new-born cells across the lifespan is still under debate.

1.3. Hypothesis and objectives

The ultimate control of the appetite and energy balance is located in the hypothalamus, with the Arc nucleus at the core of this regulation. In the Arc nucleus, different neuronal and glial cell populations coexist, and the balance between the activity of these populations is of major importance in regulating energy balance and metabolism. Notably, dysregulated energy metabolism is one of the hallmarks of ageing, likely caused by the progressive impairment of mechanisms that control body homeostasis (Roberts 2000).

Therefore, one may hypothesise that ageing is associated with a reduction in the number, relative ratios and/or connectivity of appetite-regulating neuronal subtypes and glial cells in the hypothalamus accompanied by alterations in hypothalamic transcriptome and a decreased neurogenesis in the hypothalamus. To address this hypothesis, the following objectives were set:

1. To determine age-related neuronal numbers and distribution of the two main populations of neurons (anorexigenic and orexigenic) present in the Arc nucleus of the murine hypothalamus.

2. To identify age-related transcriptional changes in the mouse hypothalamus through RNA-seq analysis. The genes with the highest age-related changes will be confirmed by imaging and/or molecular biology techniques and scrutinized against published literature.

3. To determine age-related changes in astrocyte and microglial cells present in the Arc nucleus of the mouse hypothalamus using immunohistochemistry and western blot.

4. Determine the level and magnitude of age-related decline in the hypothalamic neurogenesis using proliferation labelling methods and immunohistochemistry.

5. To identify age-related changes in the oligodendrocyte population and the myelination pattern in the mouse hypothalamus using immunohistochemistry and western blot techniques.

2. Material and methods

2.1. Transgenic animals

Mice maintained on a mixed C57BL6/129Ola genetic background and were housed under standard conditions (12-hour light/dark cycle, temperature between 20 and 24 ºC, chow and water ad libitum) unless stated otherwise. All animals were maintained, bred, treated and culled in compliance with terms of a Home Office Project Licence (P1980162E). Analyses were performed in males and female brain tissue. Experimental groups of each genotype were defined as young adult (3 – 7 months old), middle-aged (10 – 14 months old), and old animals (18 – 24 months old; **Figure 2.1**). Only healthy, disease free mice were used in this study. **Figure S1** included weight data for animals used in this study with no weight loss observed with increasing age.

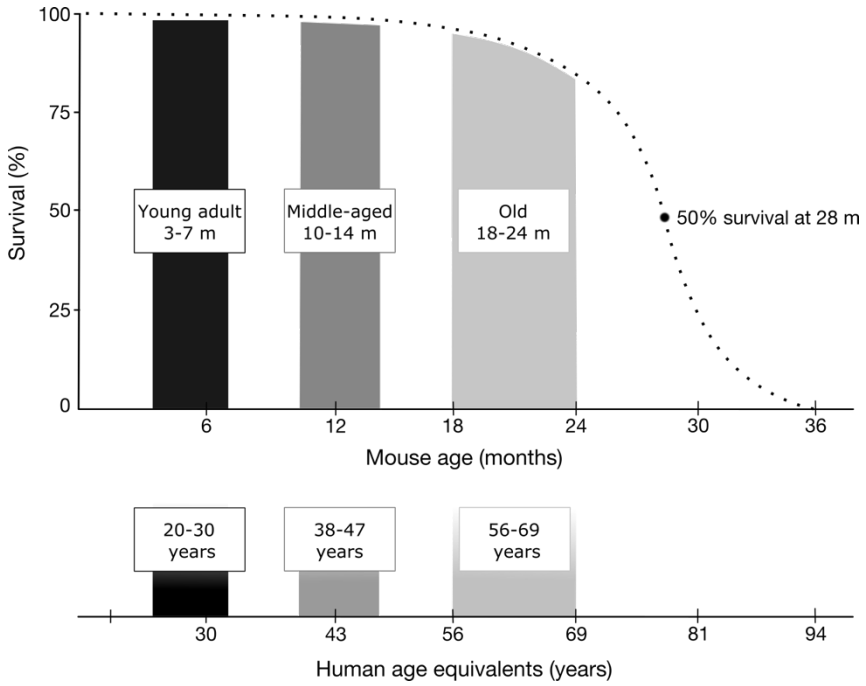


Figure 2.1. Life stages in mice in comparison to humans. Experimental mouse age groups are indicated, together with their human age equivalences. Figure prepared with data from "The mouse in biomedical research" (Flurkey, Currer, and Harrison 2007).

2.1.1. Npy-hrGFP

Npy-hrGFP (also named Npy-GFP) transgenic reporter mice were obtained from the Jackson Laboratory (#006417), which exhibits humanised Renilla GFP (hrGFP) expression under the control of the Npy promoter (van den Pol *et al.*, 2009) (**Figure 2.2,**

A). Expression of the reporter gene is observed in neurons from the Arc nucleus of the hypothalamus, the dentate gyrus of the hippocampus and the cerebral cortex.

2.1.2. Pomc-EGFP

Pomc-EGFP (also named Pomc-GFP) transgenic reporter mice were obtained from the Jackson Laboratory (#009593), which exhibits enhanced green fluorescent protein (EGFP) expression under the control of the Pomc promoter region (Newell-Price, 2003) (Figure 2.2, B). EGFP expression is observed in the Arc nucleus of the hypothalamus, melanotrophs/corticotrophs of the pituitary gland, and in a subpopulation of newly born granule neurons of the dentate gyrus of the hippocampus. EGFP expression is also observed in the nucleus of the solitary tract of the medulla.

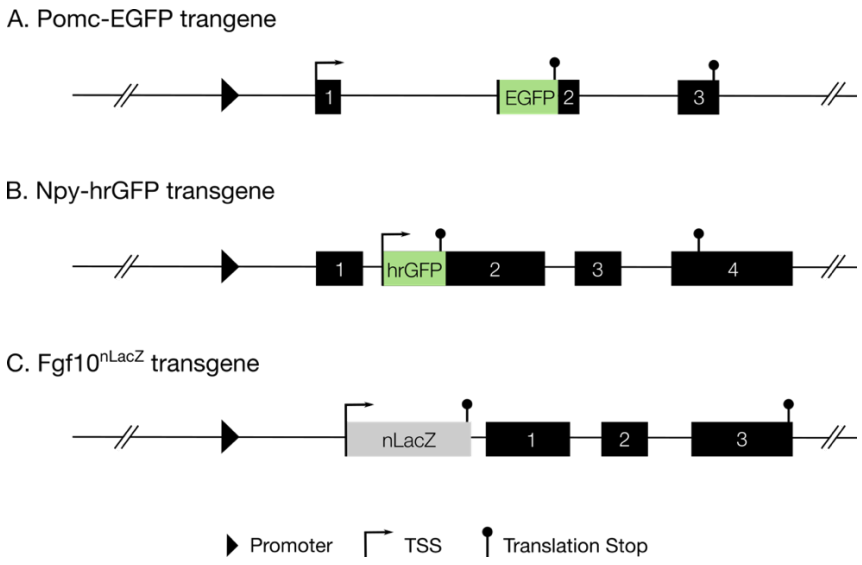


Figure 2.2. Schematic representations of transgenic mouse alleles utilised. (A) The Pomc-EGFP and (B) the Npy-hrGFP reporter line. Black boxes – exons. green boxes – reporter gene. The transcriptional start site (TSS) and the translation stop are indicated.

2.1.3. Fgf10-LacZ

The Fgf10^{LacZ} transgenic mouse line has the nuclear-targeted lacZ (nLacZ) transgene inserted into Fgf10 regulatory regions 114 Kb upstream of the Fgf10 coding sequence (Fig 2.2 C; (Hajihosseini et al. 2008; Kelly, Brown, and Buckingham 2001). In

these mice, expression of LacZ closely matches expression patterns of Fgf10 and can be used for lineage tracing of Fgf10 expressing cells due to the bacterial origin of the protein product β-galactosidase (β-Gal).

2.1.4. Breeding and genotyping

Genomic DNA for genotyping was extracted from 2 mm ear biopsies and digested overnight at 55 °C in 50 µl of digest buffer (1 M Tris, 0.5 M EDTA, 5 M NaCl, 2 mg/mL SDS) supplemented with 7 µg/mL Proteinase K (Sigma, Japan). Following extraction, genomic DNA was precipitated using 50 µl of isopropanol, spooled out using sterile pipette tips and resuspended in 30 µl of 30% TE buffer (3mM Tris, 0.3 mM EDTA) overnight at 37 °C in the water bath.

The DNA was subjected to Polymerase Chain Reaction (PCR) using the Roche Expand-PCR kit (Roche, Switzerland). Primers and PCR cycles and are described in Table 2.1 and 2.2, respectively. The PCR product was run on ethidium bromide (EtBr)-stained 1% (w/v) agarose gel, and the amplified product was visualised using a UV transilluminator.

Table 2.1. Primers for genotyping

Allele	Primers (5'- 3')	Product size (bp)
Pomc-EGFP	AAGTTCATCTGCACCACCG	WT: 324 Pomc-GFP: 173
	TCCTTGAAGAAGATGGTGCG	
	CTAGGCCACAGAATTGAAAGATCT	
	GTAGGTGGAAATTCTAGCATCATCC	
Npy-hrGFP	TATGTGGACGGGGCAGAAGATCCA	WT: 400 Npy-GFP: 300
	CCCAGCTCACATATTTATCTAGAG	
	GGTGCGGTTGCCGTACTGGA	
$Fgf10^{nLacZ}$	GCATCGAGCTGGGTAATAAGCGTTGGCAAT	WT: 600 LacZ: 800
	GACACCGACACAACTGGTAATGGTAGCGAC	
	CGAGTGGAGCATGTACTTCCGTGTCCTGAA	
	TCCCTACCCAGTCACAGTCACAGCTGCATA	

Table 2.2. PCR programmes for genotyping

Programme name	Temperature (ºC)	Time length (s)	Cycle repeats
Pomc-GFP	94	120	1
	94	30	15
	58	30	
	68	70	
	94	30	17
	58	30	
	68	70 (+ 20s each cycle)	
	68	420	1
	6	600	1
Npy-GFP	94	120	1
	94	30	10
	61	30	
	68	160	
	94	30	17
	61	30	
	68	160 (+ 40s each cycle)	
	68	420	1
	6	600	1
$Fgf10^{nLacZ}$	94	120	1
	94	30	15
	62	45	
	68	60	
	94	30	17
	62	30	
	68	60 (+ 40s each cycle)	
	68	420	1
	6	600	1

2.2. Animal experiments *in vivo* – BrdU labelling

To study proliferation, animals were treated for 15 days with 5-Bromo-2′-deoxyuridine (BrdU, Sigma) in drinking water *ad libitum* (as 1 mg/ml solution containing 0.25 mg/ml glucose) before sacrifice. BrdU is a synthetic thymidine analogue that is incorporated in DNA of cells entering in S-phase of the cell cycle and, is therefore often used to assess cell proliferation (Wojtowicz and Kee, 2006). Fresh BrdU-solution was supplied every 48 hours, and the drinking bottles were protected from light. Stability of BrdU in this paradigm was validated previously (N. Haan et al. 2013).

2.3. Tissue Processing

For vibratome sectioning, mice were sacrificed by CO_2 asphyxiation, followed by transcardial perfusion with 4% (w/v) paraformaldehyde (PFA, pH 7.4, diluted in PBS 1X). Then, brains were dissected out and fixed by immersion in 4% PFA overnight at 4 °C. After overnight fixation, brains were dehydrated in a series of ethanol dilutions for 1 hour each: 30%, 50%, 70%, 90% up to absolute ethanol and stored at 4 °C until required.

Alternatively, for protein or RNA extraction, animals were sacrificed by cervical dislocation. Them, the hypothalami were dissected out, flash-frozen in centrifuge tubes on liquid nitrogen and stored at -80 °C until use.

2.4. Vibratome tissue sectioning

Prior sectioning, brains were rehydrated through a series of ethanol dilutions consisting of 1 hour per dilution: 90%, 70%, 50%, 30%, and PBS. Then, brains were embedded in 3% (w/v) agar, incubated for 30 minutes at 80 °C and left to set in coronal orientation. Vibratome sections were generated on a Leika VT1200 (Leika, Germany) at 60 µm thickness and stored serially in a 48-well plate containing PBS 1X until use.

2.5. Immunolabelling

2.5.1. Antigen retrieval

2.5.1.1. Citrate pre-treatment

Where necessary and before the blocking step of the immunohistochemistry, sections were placed into 10 mM citrate buffer (10 mM tri-sodium citrate, 0.05% Tween-20; pH 6.0); for 10 minutes at room temperature (RT). Subsequently, sections were placed in pre-warmed 10 mM citrate buffer and left for 15 minutes at 70 °C. The sections were left to cool down to RT and washed twice in PBS for 10 minutes each.

2.5.1.2. HCl pre-treatment

To enable the nuclear penetration of the BrdU antibody, before the blocking step, sections were incubated in a solution of HCl 2 M for 1 hour at 37 °C in the water bath and subsequently washed twice for 10 minutes in PBS 1X at RT.

2.5.2. TUNEL assay

To assess apoptosis via DNA fragmentation, the Apoptag TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay was performed (kit S7165; Merck Millipore, Massachusetts, USA), according to the manufacturer recommendations. The different reagents were applied directly on slide-mounted sections in a humidified chamber. Alongside hypothalamic sections and as a positive control, sections containing the SGZ of the LV were also assessed for TUNEL staining. To remove false positives, the nuclear morphology and chromatin condensation in TUNEL+ cells were also examined. When double-labelling with immunohistochemistry was required, the TUNEL protocol was performed prior to the blocking step in (1.5.3).

2.5.3. Immunolabelling of vibratome sections

Vibratome sections containing the whole extension of the Arc nucleus (from bregma -0.94 mm to -2.5 mm, approximated) and control regions (SGZ of the LV) were

selected for immunolabelling by comparison to the Allen Brain Atlas (Franklin and Paxinos 2007). To increase antibody binding and penetration in the tissue, the immunolabelling protocol was performed on free-floating sections contained in glass vials. Unless stated otherwise, the different steps were performed at RT and on a rocking platform.

To block non-specific binding sites and permeabilise sections simultaneously, sections were incubated for 2 hours in a solution containing 20% (v/v) NGS, 1% (v/v) Triton X-100 prepared in PBS 1X. Subsequently, sections were incubated with relevant primary antibodies (Table 2.3) made in 0.2% (v/v) NGS, 0.1% (v/v) Triton X-100 diluted in PBS 1X overnight at 4 ºC. The following day, sections were washed five times, allowing 1 hour per wash, in a solution containing 0.2% (v/v) NGS, 0.1% (v/v) Triton X-100 diluted in PBS 1X to remove the non-bound antibody and incubated overnight at 4 ºC with the appropriate secondary antibodies (Table 2.4) made diluted in 0.2% (v/v) NGS, 0.5% (v/v) NP-40 in PBS 1X. Where required, for biotin-conjugated secondary antibodies, sections were washed five times, allowing 1 hour per wash, in 0.2% (v/v) NGS, 0.1% (v/v) Triton X-100 prepared in PBS 1X, and incubated in streptavidin-conjugated antibodies made in 0.2% (v/v) NGS, 0.1% (v/v) Triton X-100 diluted in PBS 1X overnight at 4 ºC.

The final day, sections were washed six times with PBS 1X, 30 min per wash, and stained with 5 µg/mL Hoechst 333452 in PBS 1X for 30 minutes. After counterstaining, sections were mounted in the slides with Vectashield (Vector Laboratories, California, USA) mounting medium, cover-slipped and sealed by nail varnish.

2.6. Western Blotting and Protein Analysis

2.6.1. Protein extraction from hypothalamic tissue

Hypothalamic tissue was homogenized with ice-cold RIPA lysis buffer (10 µl/mg of tissue; 150mM NaCl, 1% [v/v] NP-40, 0.5% [w/v] sodium deoxycholate, 0.1% [w/v] SDS, 50mM Tris pH 8.0), containing Halt protease and phosphatase inhibitors (1:100; ThermoFisher, Massachusetts, USA), using the bead-beating system TissueLyser LT (Qiagen, France) in series of 1-2-1 minutes. Homogenates were then incubated on an orbital shaker (Cole-Parmer, United Kingdom) for two hours at 4 °C, and cleared to remove debris by centrifugation at 12,000 rpm for 20 minutes at 4 °C. The resulting supernatant was collected, and the total protein concentration of each sample was determined using the colorimetric Pierce BCA protein assay (ThermoFisher), according to manufacturer's instructions.

Samples were prepared by mixing 100 µg of protein with 4X loading dye (62.5 mM Tris base pH 6.8, 2% [w/v] SDS, 10% [v/v] glycerol, 0.02% bromophenol blue and 12.5% [v/v] β-mercaptoethanol) in a final volume of 25 µl, boiled for 5 minutes at 95 °C, and kept at -20 °C until required. As an exemption, for PLP detection, samples were not boiled during the preparation.

2.6.2. SDS polyacrylamide gel electrophoresis and transference

The SDS polyacrylamide gel was composed of a 5% stacking gel (126 mM Tris pH 6.8, 5% [v/v] Acrylamide; 0.2% [w/v] SDS; 0.1% [v/v] Ammonium Persulphate; 0.3% [v/v] TEMED); and a resolving gel of 6, 7, 10 or 12% (375 mM Tris pH 8.8, 6-12 % [v/v] Acrylamide 29:1; 0.2% [v/v] SDS; 0.1% [v/v] Ammonium Persulphate; 0.25% [v/v] TEMED), depending on the target protein (Table 2.5). Samples were loaded alongside the PageRuler Plus protein ladder (ThermoFisher) and run in electrophoresis buffer (25 mM Tris base, 192 mM Glycine, 1% [w/v] SDS) at 70 V until the dye front surpassed the bottom of the stacking gel, and then at 100 V until the dye front crossed the lower end of the gel.

After electrophoresis is completed, proteins were transferred to nitrocellulose membranes (0.45 µm pore size, Thermofisher) using the mini trans blot cell system (Bio-Rad, California, USA) containing ice-cold transfer buffer (20 mM Tris base, 154 mM Glycine, 0.01% [w/v] SDS, 10% [v/v] methanol) for 90 minutes at 50 V at 4 °C. Alternatively, to transfer high molecular weight proteins (KIF1B and DYNC1H1), the transference was done at 20 V overnight at 4 °C.

2.6.3. Membrane blocking, protein detection and visualisation

Once the transference was complete, membranes were incubated with the blocking solution containing TBST (TBS 1X, 0.1% [v/v] Tween-20) and 5% (w/v) semi-skimmed milk, for 2 hours at RT on a rocking platform. After blocking, membranes were directly incubated with the relevant primary antibodies prepared in TBST and 1% semi-skimmed milk (w/v), overnight at 4 °C on a tube roller (Cole-Parmer).

After that, membranes were washed with TBST four times, 10 minutes each wash, and incubated with relevant secondary antibodies conjugated with horseradish peroxidase (HRP) prepared in TBST and 1% semi-skimmed milk (w/v), for 2 hours at RT. After washing again with TBST twice for 10 minutes each wash, membranes were incubated in Pierce ECL plus western blotting solution (Thermofisher) for 2 minutes and developed using the ChemiDocTM Imaging System (Bio-Rad). The full list of primary and secondary antibodies can be found in Table 2.5 and 2.6, respectively.

In order to re-probe the membranes for different proteins, membranes were incubated in stripping buffer (62.5 mM Tris base pH 6.7, 2% [w/v] SDS, 0.78% [v/v] β-mercaptoethanol) for 30 min at 55 °C with gentle rocking, washed four times in TBST for 10 minutes each wash, and blocked again before applying the primary antibodies.

2.7. Microscopy

All fluorescent images were acquired with the Zeiss Apotome Imager M2 microscope (Axiovert 200M) equipped with a Zeiss AxioCam HRm, using the Axiovision 4.8 software. Pictures were captured in serial z-stacks (1 µm of thickness) under X10 (air), X20 (air), X40 (oil) and X63 (oil) objectives. The approximated bregma positions of

each brain section where determined by consultation of the Allen Mouse Brain Atlas (Franklin and Paxinos 2007).

2.8. Image analysis and quantifications

2.8.1. Pomc and Npy quantifications

The Volocity 6.3 software (Perkin Elmer, Massachusets, United States) was employed to generate three-dimensionally reconstructed images, create composites and to modify brightness and contrast to make visible low stained cells. Using the three-dimensional images, the total number of hypothalamic GFP+ cells in each brain section was counted in approximated 24 serial sections per brain ranging from bregma -0.94 mm to -2.5 mm. The Volocity automated cell counter was used for Npy-GFP+ cells, while manual counting was preferred for Pomc-GFP+ cells. Both hemispheres were used for the counting, and results are represented as the average cell number of both hemispheres. To avoid double-counting, only the middle 40 µM of each 60 µM-thick section was analysed. The Npy: Pomc ratio was calculated using the average number of Npy-GFP+ and Pomc-GFP+ per hemisphere obtained in *Chapter 3.*

2.8.2. GFAP and Iba1 quantifications

The GFAP+ and Iba1+ cell and size number were quantified within a specified ROI (region of interest; a rectangle of area 0.134 mm^2) using the ImageJ software (https://imagej.nih.gov/ij/). The counting method consisted of positioning the ROI covering the whole Arc nucleus region and counting manually the positive cells contained within the ROI (represented as GFAP+ or Iba1+ cells/mm^2). To quantify the cell size, the same ROI was used, and the total area covered by GFAP+ or Iba1+ signal was divided by the number of GFAP+ or Iba1+ cells contained within the same area (represented as GFAP+ or Iba1+ cell size).

2.8.3. BrdU quantifications

The total number of BrdU+ cells was counted manually within the mediobasal hypothalamus using the ImageJ software, in approximated 24 serial sections per brain ranging from bregma -0.94 mm to -2.5 mm. To obtain the percentage of BrdU+/Olig2+ and BrdU+/Iba1+ cells in the Arc-ME, double-labelled (BrdU+/Olig2+ or BrdU+/Iba1+) and single-labelled cells (BrdU+) were counted manually within a specified ROI (a square of area 0.0016 mm^2). To improve accuracy, the ROI was positioned in, at least, three different regions of the Arc-ME within the same section.

2.8.4. Olig2 and CC1 quantifications

The number of Olig2+ and CC1+ cells were counted manually within a specified ROI (a square of area 0.0016 mm^2) using the ImageJ software, in approximated 12 sections ranging from bregma -1.58 mm to -2.3 mm. The counting method consisted of positioning the ROI within the Arc-ME nucleus and counting manually the positive cells contained within the ROI (represented as Olig2+ or CC1+ cells/mm^2). To improve accuracy, the ROI was positioned at least in three different regions of the Arc-ME within the same section.

2.9. RNA-sequencing

Hypothalami form young and old mice (n = 5 for each age group) were isolated and stored in RNA later at -80 °C. Then, the RNA from hypothalamic tissue was extracted by Dr Tarang Mehta, using the RNeasy Plus Mini Kit (Qiagen), achieving RNA integrity (RIN) in the range of 9 – 10 (Agilent Bioanalyzer Total RNA Pico Assay).

The samples were sent to Novogen (Cambridge, UK), where the RNA-Seq experiment was performed. Libraries were constructed and sequences using Illumina HiSeq2000. Each sample had: a sequencing depth of at least 20 million reads per sample. Differential gene expression analysis was carried out by Dr Simon Moxon who pseudoaligned reads to the Ensembl *M. musculus* cDNA release (version 97) (Yates et al. 2020) using Kallisto (Bray et al. 2016). Sleuth (Pimentel et al. 2017) was used to calculate gene-based differential expression from Kallisto count data.

2.10. Statistics

Statistical analysis for multiple comparisons was performed with one-way or two-way ANOVA followed by Tukey's post hoc test using GraphPad Prism (v. 6). Significance was only considered for p-values lower than 0.05 (*p<0.05, **p<0.01, ***p<0.001). Data is represented as mean ±SEM.

For immunohistochemistry, western blot and RNA-seq studies, *n* numbers relate to the number of mouse brain/hypothalamus analysed within the experimental paradigm. Quantification of cell numbers was measured from approximately 12 – 24 sections per brain, depending on the experiment (see *section 2.8*). The specific number of mice analysed for each experiment is described in *Chapters 3, 4, 5, 6* and *7*, with a minimum of 3 mice brains per age group analysed in each experiment.

Table 2.3. Primary antibodies used for immunohistochemistry

Name	Host	Manufacturer	Comercial reference	Dilution	Antigen retrieval
α-APC (CC-1)	Ms	Millipore	MABC200	1/500	No
α-ASCL1	Ms	Santa Cruz	sc-374104	1/100	No
α-BrdU	Rt	Abcam	ab6326	1/500	HCl
α-Cl Caspase 3 (CC3)	Rb	Cell signaling	9661	1/1000	No
α-DCX	Ms	Santa Cruz	sc-271390	1/100	No
α-GFAP	Ms	Millipore	MAB360	1/1000	No
α-GFP	Rb	Abcam	ab290	1/1000	No
α-IBA1	Rb	Wako	019-19741	1/1000	No
α-MBP	Ms	Abcam	ab62631	1/500	No
α-MLKL	Rt	Millipore	MABC604	1/100	No
α-NeuN	Ms	Millipore	MAB377	1/500	Citrate
α-NF	Ms	Biolegend	SMI 312	1/500	No
α-OLIG2	Rb	Millipore	AB9610	1/500	No
α-PLP	Ms	Bio-Rad	MCA839G	1/250	No
α-S100β	Ms	Abcam	ab4066	1/200	Citrate

Rb (rabbit), Ms (mouse), Rt (rat)

Table 2.4. Secondary antibodies used for immunohistochemistry

Host	Taget	Conjugated	Manufacturer	Comercial reference	Dilution
Gt	Ms	Alexa Fluor 488	Invitrogen	A-11001	1/1000
Gt	Ms	Alexa Fluor 568	Invitrogen	A-11004	1/1000
Gt	Rb	Alexa Fluor 488	Invitrogen	A-11008	1/1000
Gt	Rb	Alexa Fluor 568	Invitrogen	A-11011	1/1000
Gt	Ms IgG1	Biotin	Jackson ImmunoResearch	115-065-205	1/500
Gt	Rt	Biotin	Invitrogen	31830	1/500
-	Streptavidin	AMCA	Jackson ImmunoResearch	016-150-084	1/250
-	Streptavidin	Texas Red	Vector laboratories	SA-5006-1	1/250

Rb (rabbit), Ms (mouse), Rt (rat), Gt (goat)

Table 2.5. Primary antibodies used for western blot

Name	Host	Manufacturer	Comercial reference	Dilution	Concentration resolving gel (%)
α-AIF	Ms	Santa Cruz	sc-13116	1/200	10
α-ARGl Ms	Ms	Santa Cruz	sc-271430	1/200	7
α-B7-2 (CD-86)	Ms	Santa Cruz	sc-28347	1/500	7
α-CD206 Ms	Ms	Santa Cruz	sc-58986	1/200	7
α-CNPase	Ms	Chemicon	MAB326	1/250	10
α-DYNC1H1	Rb	Proteintech	12345-1-AP	1/500	6
α-GADPH	Rb	Cell signaling	2118S	1/1000	12
α-GFAP	Ms	Millipore	MAB360	1/1000	10
α-KIF1B	Rb	Proteintech	15263-1-AP	1/500	6
α-MBP	Rb	Abcam	ab40390	1/500	12
α-MRF	Rb	Abcam	ab854464	1/250	7
α-NF	Ms	Biolegend	SMI 312	1/500	7
α-NG2	Rb	Millipore	AB5320	1/500	7
α-OLIG2	Rb	Millipore	AB9610	1/2500	10
α-PLP	Ms	Bio-Rad	MCA839G	1/1000	12
α-POMC	Rb	Abcam	ab94446	1/500	12
α-TNFα	Ms	Santa Cruz	sc-52746	1/100	12
α-Tuj1	Ms	R&D systems	MAB1195	1/500	7
α-βACTIN	Ms	Proteintech	66009-1-lg	1/5000	7-12

Rb (rabbit), Ms (mouse)

Table 2.6. Secondary antibodies used for western blot

Host	Taget	Conjugated	Manufacturer	Comercial reference	Dilution
Gt	Ms	HRP	Santa Cruz	sc-516102	1/5000
Gt	Rb	HRP	Vector Labs	P1-1000	1/5000
Gt	Ms	HRP	Sigma-Aldrich	A4416	1/5000

Rb (rabbit), Ms (mouse), Rt (rat), Gt (goat)

3. Characterisation of anorexigenic (POMC) and orexigenic (NPY) neuronal populations in the Arc nucleus and their dynamics during ageing

3.1. Introduction

Over the past two decades, a large body of evidence has accumulated to show that the hypothalamus plays a critical role in energy homeostasis by controlling the balance between calorie intake and energy expenditure (Dietrich and Horvath 2013; Kim and Choe 2018). The hypothalamus contains a diverse collection of interconnected neurons organised in different nuclei: the dorsomedial hypothalamus (DMN), ventromedial hypothalamus (VMN), lateral hypothalamus (LH), paraventricular hypothalamus (PVN) and Arcuate nucleus (Arc). Complex but integrated interconnections between the hypothalamic nuclei control the feeding behaviour and energy expenditure (Coupe and Bouret 2013). In the Arc nucleus, the antagonistic relationship between two neuronal populations control energy homeostasis: the orexigenic (appetite-promoting) neurons expressing agouti-related peptide (AgRP) and neuropeptide Y (NPY); and the anorexigenic (appetite-suppressing) neurons expressing proopiomelanocortin (POMC) (Cone et al. 2001).

The AgRP/NPY neurons activate orexigenic behaviours through the release of neurotransmitters, AgRP and NPY, which bind to receptors in PVN and LH neurons (Betley et al. 2013; Hahn et al. 1998). In addition to their direct effect on the PVN and LH, the orexigenic neurons innervate and inhibit the activity of POMC neurons (Garcia de Yebenes et al. 1995). Studies in aged mice showed that NPY/AgRP innervation onto POMC undergoes a progressive and pronounced increase with age, associated with the age-related decrease of POMC firing (Newton et al. 2013).

The POMC neurons exert its anorexigenic effect through its projections to the PVN, VMN and LH, and by releasing α-MSH, a post-translation product of POMC that activates MC4R in target cells (Millington 2007). These neurons can release a plethora of different neurotransmitters, including GABA, glutamate and acetylcholine (Dennison et al. 2016; Jarvie and Hentges 2012; King and Hentges 2011); and express a variety of receptors, such as the insulin receptor (InsR), LepR, and 5-HT2cR (Sohn et al. 2011; Williams et al. 2010). Recent studies have described different subtypes within the POMC population, defined by a set of enriched transcripts, suggesting the possible heterogeneity of these cells (Campbell et al. 2017; Chen et al. 2017). The different POMC subtypes show different rostro-caudal distribution and project to different targets based on their position. For example, POMC neurons in the rostral Arc project mostly to

autonomic areas, whereas caudal Arc POMC neurons mainly innervate hypothalamic areas (Dennison et al. 2016; Jarvie and Hentges 2012; King and Hentges 2011).

Interestingly, in mammals, the shift in energy homeostasis is one of the hallmarks of the physiological changes during ageing (Hildrum et al. 2007). Ageing is associated with the development of metabolic syndrome components including obesity, hypertension and glucose intolerance (Purkayastha and Cai 2013; Purkayastha, Zhang, and Cai 2011; Tang et al. 2015; Zhang et al. 2008). Assumedly, age-related changes in the hypothalamic neurons controlling energy balance and metabolism, especially POMC and AgRP/NPY neurons, could underlie the decline in metabolism and energy homeostasis observed during ageing. Reportedly, the firing of the POMC neurons significantly decreases with age (Newton et al. 2013; Yang et al. 2012), and the induction of Pomc expression by exogenous leptin is impaired during ageing (Scarpace et al. 2002). Furthermore, the hypothalamic Pomc mRNA levels decline in rodents with age (Lloyd et al. 1991; Nelson, Bender, and Schachter 1988). For NPY neurons, studies in aged rats reported a decrease in NPY protein levels with age (Kowalski et al. 1992), whereas mRNA of AgRP remains stable during ageing (Wolden-Hanson, Marck, and Matsumoto 2004).

Thus, although the studies mentioned above provided useful insights about the age-related changes in the anorexigenic and orexigenic populations, they might not be sufficient to illustrate the ageing-related shift in energy homeostasis. Hence, to better understand the dynamics of appetite-regulating circuits in the aged brain, the number and distribution of POMC and NPY/AgRP neuronal populations in young, middle-aged aged and old mice was characterised in this chapter.

3.2. Aims

- Characterisation of the number of anorexigenic and orexigenic neurons in the Arc nucleus of the murine hypothalamus.
- Characterization of age-related differences in the number and/or anatomical distribution of the anorexigenic and orexigenic populations.
- Study of the physiological consequences of the changes in the number of anorexigenic and orexigenic neurons with ageing.

3.3. Results

3.3.1. Anorexigenic and orexigenic neuronal dynamics with age

Given the decline in energy metabolism reported during ageing in humans and rodents, the effects of ageing in the neuronal populations that control energy balance were investigated. However, neither the total number of those neuronal populations nor their dynamics during ageing have been defined. To quantify the total number of anorexigenic and orexigenic neurons, and any age-related differences, immunohistochemistry and specific reporter mice to each population were employed.

3.3.2. The anorexigenic population (POMC) suffer a significant decline in number in the Arc nucleus with age

To quantify the number of anorexigenic neurons in the hypothalamus, the transgenic model Pomc-GFP maintained on a C57BL/6J background was employed. Immunohistochemistry against GFP was performed in vibratome sections from young adult (2 – 6 months old), middle-aged (10 – 12 months old) and old (18 – 25 months) mice, with a minimum of 4 animals per age group. GFP expression in the mediobasal hypothalamus was detected between bregma positions -0.94 to -2.4 mm (Figure 3.1). GFP+ cells were mainly observed in the Arc nucleus, with minor contributions to the VMN, and cells with differential GFP signal were observed throughout the hypothalamus (Figure 3.1). The neuronal identity of the Pomc-expressing cells was then confirmed by co-localisation with the neuronal marker NeuN (Figure 3.2).

Next, the total number of Pomc-expressing cells per animal were quantified in 24 serial sections (60 µm thickness each), between bregma -0.94 to -2.4 mm (Figure 3.3 A – F, G). The young adults showed an average number of Pomc-expressing neurons of approximated 2132 ± 86 (n = 4) per hemisphere, 4264 ± 172 (n = 4) in total. In the middle-aged animals, the population number was reduced to 1463 ± 17 (n = 4) per hemisphere, 2926 ± 35 (n = 4) in total; a significant decline of 31.4% ($p < 0.05$). In old animals, the number of Pomc-expressing neurons was 1294 ± 69 (n = 6) per hemisphere, 2589 ± 137 (n = 6) in total. In summary, results showed attrition of the Pomc-expressing population with age. The decline is firstly observed in the middle-aged group, with further reduction in the old animals.

Additionally, the analysis of Pomc-expressing cells distribution across the rostro-caudal extent of the Arc showed regional differences (Figure 3.4). In young animals, the rostral portion of the Arc (bregma -0.94 to -1.58 mm, approximated) presented higher cell density than the caudal part (bregma -1.7 to 2.4 mm). In middle-aged animals, a reduction in Pomc-expressing neurons is observed throughout the Arc, affecting more significantly the rostral-positioned neurons. The old group followed the same pattern as the middle-aged animals, with a shaper decline in the rostral region. As a result, Pomc-expressing neurons are more uniformly distributed across the rostro-caudal Arc in middle-aged and old animals. Significant differences between age groups and rostro-caudal position in the Arc are summarized in Table 3.1.

In conclusion, results showed an age-related decline in total number of POMC neurons in the Arc nucleus, as well as changes in their distribution in the Arc nucleus. The changes are firstly observed during early ageing.

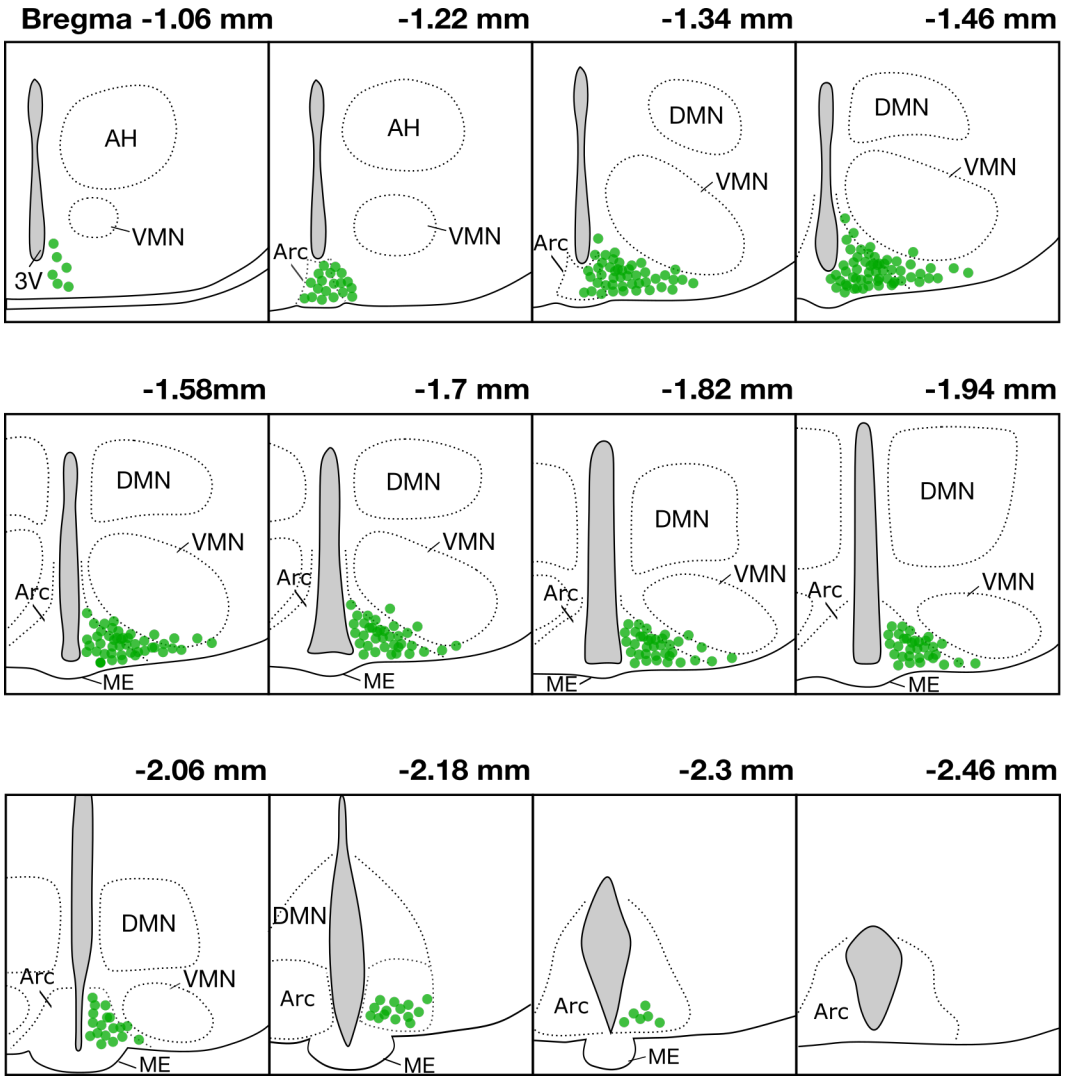


Figure 3.1. Representative distribution and density of Pomc+ cells in the hypothalamus. Approximated bregma positions are indicated. Green circles indicate the position of the groups of Pomc+ cells. Results obtained from young, middle-aged and old animals, with a minimum of 3 animals analysed per age group, were employed for the diagram design. Dashed lines outline the 3V and the limits of the different hypothalamic nuclei. 3V – third ventricle. VMN – ventromedial nucleus. AH – anterior hypothalamus. Arc – arcuate nucleus. ME – median eminence.

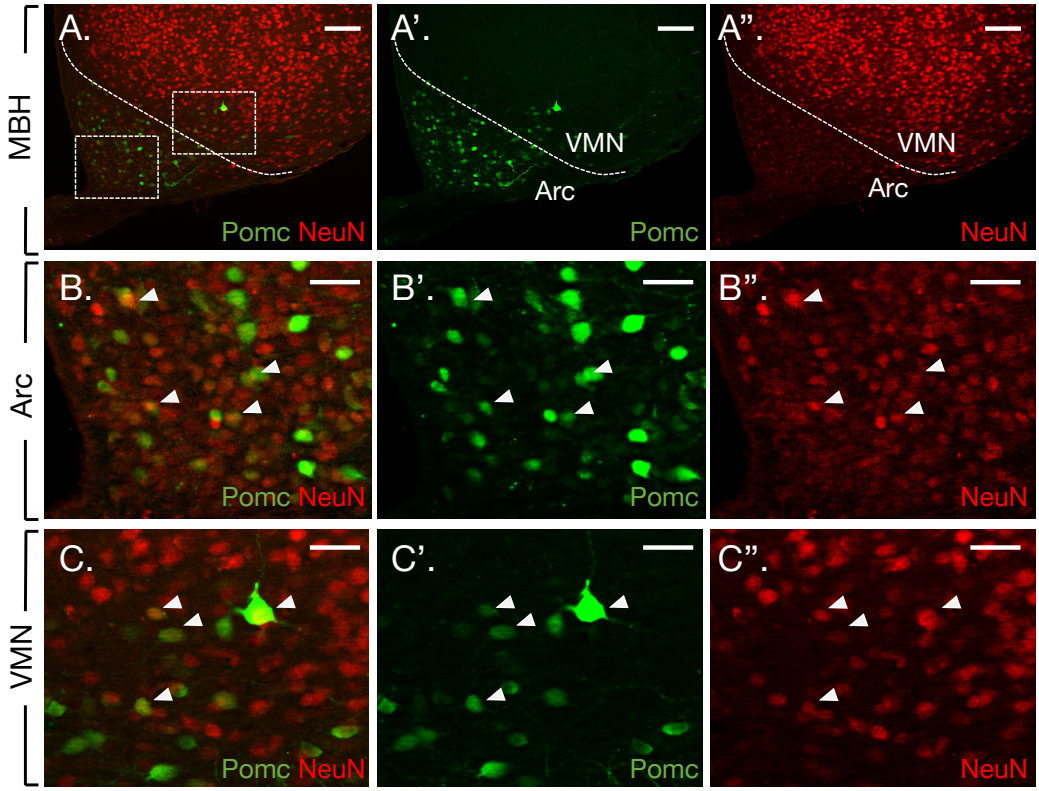


Figure 3.2. Pomc-GFP+ cells co-express the mature neuronal marker NeuN, confirming their neuronal identity. (A – A'') Representative Pomc-GFP+ brain section immunolabelled for GFP (green) and NeuN (red). NeuN expression is lower in the Arc compared to the rest of hypothalamic nuclei, such as the VMN. (B – B'') Dashed box in (A) shows a higher power of Pomc-GFP+ and NeuN+ expression in the Arc nucleus. White arrows point co-localisation of GFP+ and NeuN. (C – C'') Dashed box in (A) shows a higher power of Pomc-GFP+ and NeuN+ expression in the Arc nucleus. White arrows point co-localisation of GFP+ and NeuN expression. Approximated bregma position -1.7 mm. Images are representative of results obtained in young, middle-aged and old animals with a minimum of 3 animals analysed per age group. Dashed line indicates the limit between Arc and VMN nucleus. Scale bar (A – A") 25 µm. Scale bar (B – B" and C – C") 12 µm. MBH – mediobasal hypothalamus. Arc – arcuate nucleus. VMN – ventromedial nucleus.

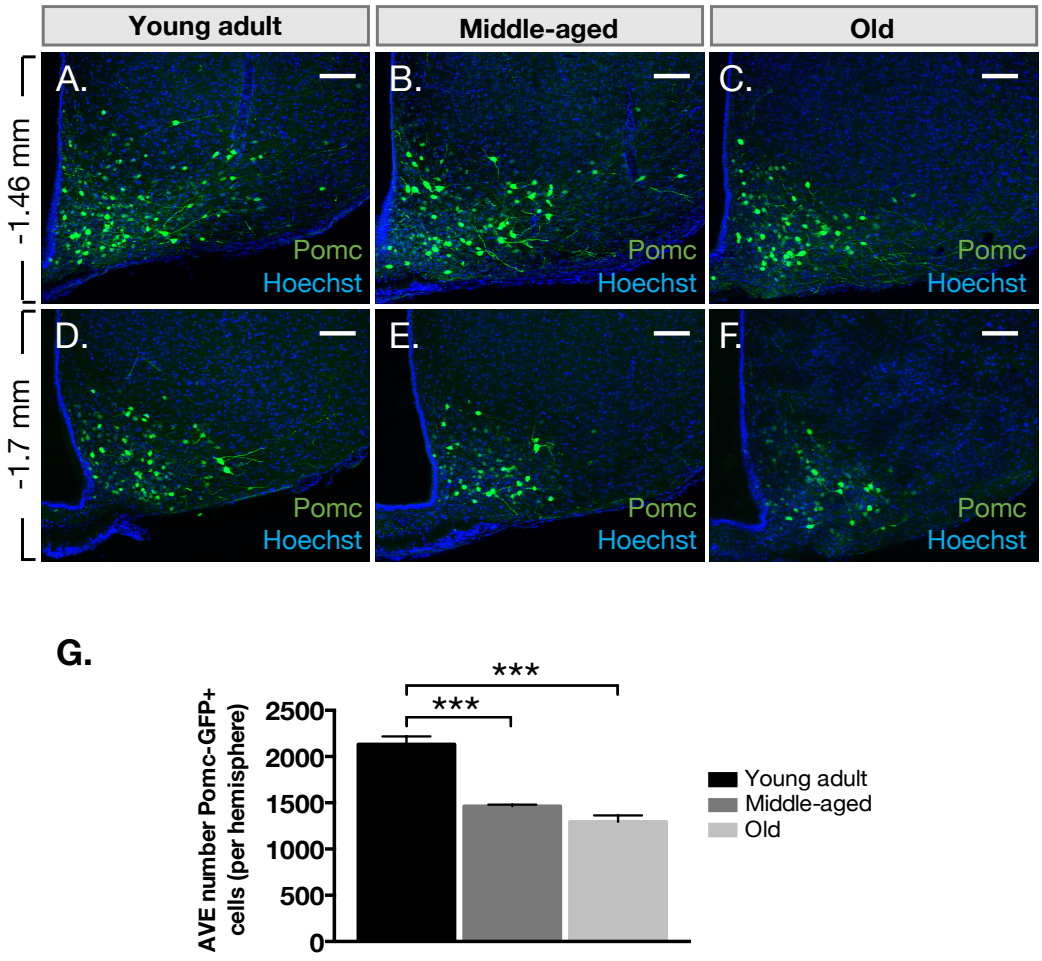


Figure 3.3. Pomc-expressing neurons show a differential distribution throughout the Arc nucleus and suffer a significative decline with age, that is more pronounced in the rostral region. (A – F) Representative images of Pomc-GFP+ brain sections stained for GFP (green) and Hoechst (blue). (A – C) Bregma -1.46 mm, from young-adult (A), middle-aged (B) and old (C) animals. (D-E) Bregma -1.7 mm, from young (D), middle-aged (E) and old (F) animals. Scale bar 50 µm. (G) Average total number of GFP+ cells per hemisphere, in young-adults (black column), middle-aged (dark grey) and old animals (light grey). Data show a significant age-related decrease in the number of GFP+ cells. Bregma positions are approximated. Data is represented as mean ±SEM, and a minimum of 4 animals per age group were analysed. Significance testing was performed using one-way ANOVA followed by Tukey post-hoc (*p<0.05, **p<0.01, ***p<0.001).

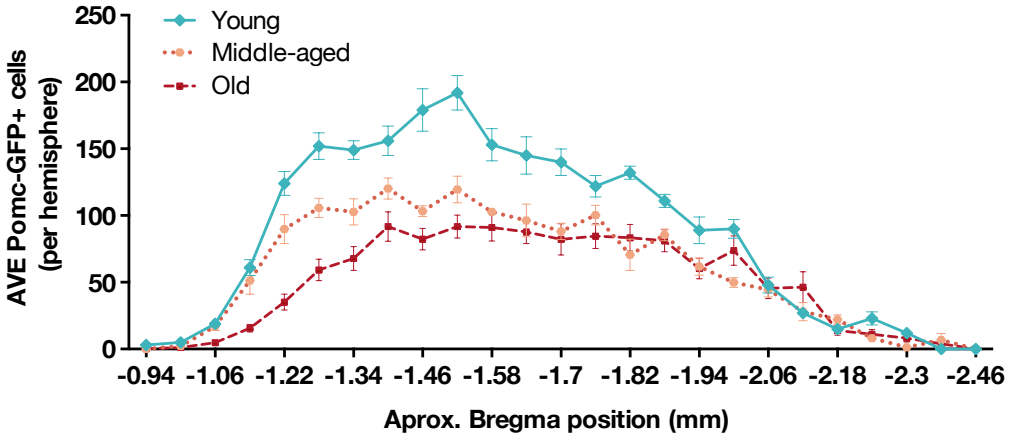


Figure 3.4. Distribution of Pomc-expressing cells within the rostro-caudal extent of the Arc nucleus. Data is represented as the average (AVE) number of GFP+ (per hemisphere) in serial sections ranging from bregma -0.94 to -2.46 mm. Data is represented as mean ±SEM, and a minimum of 4 animals per age group were quantified. Different colours represent the three age groups, young adult (blue), middle-aged (orange) and old (red).

Table 3.1. Significance testing for Figure 3.4, using two-way ANOVA followed by Tukey post-hoc test (*p<0.05, **p<0.01, ***p<0.001).

Bregma (mm)	Comparison	Significance	p-value
-1.22	Middle-aged *vs* Old	**	0.0085
-1.28	Young *vs* Old	***	< 0.0001
	Middle-aged *vs* Old	**	0.0099
-1.34	Young *vs* Middle-aged	***	< 0.0001
	Young *vs* Old	***	< 0.0001
-1.40	Young *vs* Old	***	< 0.0001
	Middle-aged *vs* Old	**	0.0012
-1.46	Young *vs* Middle-aged	***	0.0002
	Young *vs* Old	***	< 0.0001
-1.52	Young *vs* Middle-aged	***	< 0.0001
	Young *vs* Old	***	< 0.0001
-1.58	Young *vs* Middle-aged	***	< 0.0001
	Young *vs* Old	***	< 0.0001
-1.64	Young *vs* Middle-aged	***	0.0003
	Young *vs* Old	***	< 0.0001
-1.70	Young *vs* Middle-aged	***	0.0004
	Young *vs* Old	***	< 0.0001
-1.76	Young *vs* Middle-aged	**	0.0026
	Young *vs* Old	***	0.0005
-1.82	Young *vs* Middle-aged	**	0.0099
	Young *vs* Old	**	0.0016
-1.88	Young *vs* Middle-aged	***	0.0005
	Young *vs* Old	**	0.0011
-1.94	Young *vs* Middle-aged	**	0.0029
	Young *vs* Old	**	0.0011
-2.00	Young *vs* Middle-aged	**	0.0018
	Middle-aged *vs* Old	*	0.0301
-2.06	Young *vs* Middle-aged	**	0.0013
	Young *vs* Old	**	0.003

3.3.3. Hypothalamic POMC protein levels remain stable during ageing

Given that Pomc-expressing cells suffer an age-related decline, the next step was to investigate the change in POMC protein level during ageing. To do so, POMC levels were analysed in hypothalamic protein extracts from young, middle-aged and old wild-type animals (n = 6 per age group) via western blot. Anti-POMC antibody detected a single band of approximated 31 KDa (Figure 3.9, B). Densitometry analysis of the 31 KDa band showed no significant differences with age (Figure 3.9, B).

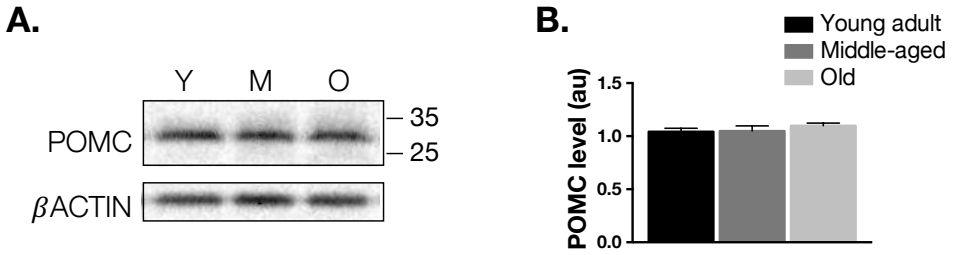


Figure 3.5. The protein levels of POMC in the hypothalamus remain constant during ageing. (A) Representative image of immunoblot for POMC protein and the loading control β-ACTIN. POMC protein runs at an approximated size of 31 KDa. Numbers indicate bands of the molecular weight marker used as a size standard (KDa) (B) Comparison of densitometric analysis of POMC levels relative to the loading control, in young adult (black), middle-aged (dark grey) and old (light grey) animals, show no age-related differences. The letters above the immunoblot lanes refer to the different age groups, young adult (Y), middle-aged (M) and old (O). Numbers indicate the bands of the molecular weight marker used as a size standard (KDa). Data is represented as mean ±SEM and a minimum of 6 animals per age group were analysed. All significance testing was performed using one-way ANOVA followed by Tukey post-hoc (*p<0.05).

3.3.4. The orexigenic population (NPY) showed no significant age-related differences in the Arc nucleus

For the quantification of Npy-expressing neurons in the hypothalamus, the transgenic mice employed was the Npy-GFP. The total number of Npy-expressing cells per animal were quantified in 24 serial sections (60 m thickness each), between bregma -0.94 to -2.4 mm. GFP was visualised in vibratome sections from young adult (2 – 6 months old), middle-aged (10 – 12 months old) and old (18 – 25 months) mice, with a minimum of 4 animals per age group. GFP+ cells were mainly observed in Arc nucleus, with minor contributions to the ME (Figure 3.6). Unlike the anorexigenic population, Npy-expressing cells were mainly located in the basal part of the Arc, near the ME and the 3V wall. Also, individual GFP+ cells showed similar fluorescent signal throughout the mediobasal hypothalamus. The neuronal identity of the Npy-expressing cells was then confirmed by co-localisation with the neuronal marker NeuN (Figure 3.7).

Next, the total number of GFP+ cells per hemisphere were counted in serial sections between bregma -0.94 to -2.4 mm. Quantification results showed no significant age-related differences in the Npy-expressing neuronal number (Figure 3.8, A – F and G). The young adult group presented an average number of Npy-expressing neurons of approximated 3001± 260 (*n* = 4) per hemisphere, 6001 ± 519 *(n* = 4) in total. In middle-aged, the population number was 2801 ± 282 (*n* = 4) per hemisphere, 560 ± 566 (*n* = 4) in total. In old animals, the number of Pomc-expressing neurons was 3492 ± 331 (*n* = 4) per hemisphere, 6983 ± 661 (*n* = 4) in total.

Also, GFP+ cells were found throughout the Arc nucleus, with a higher density in the caudal part (between bregma -1.7 to -2.06 mm). The distribution pattern was maintained with age; however, statistical analysis revealed an increase in GFP+ number in caudal positions of the Arc nucleus, specifically bregma -2.06 and -2.18 mm; Figure 3.9.

In conclusion, results showed that NPY neurons in the Arc nucleus remain generally stable in number and distribution during ageing, although a small age-related increase in number is detected in caudal position of the Arc.

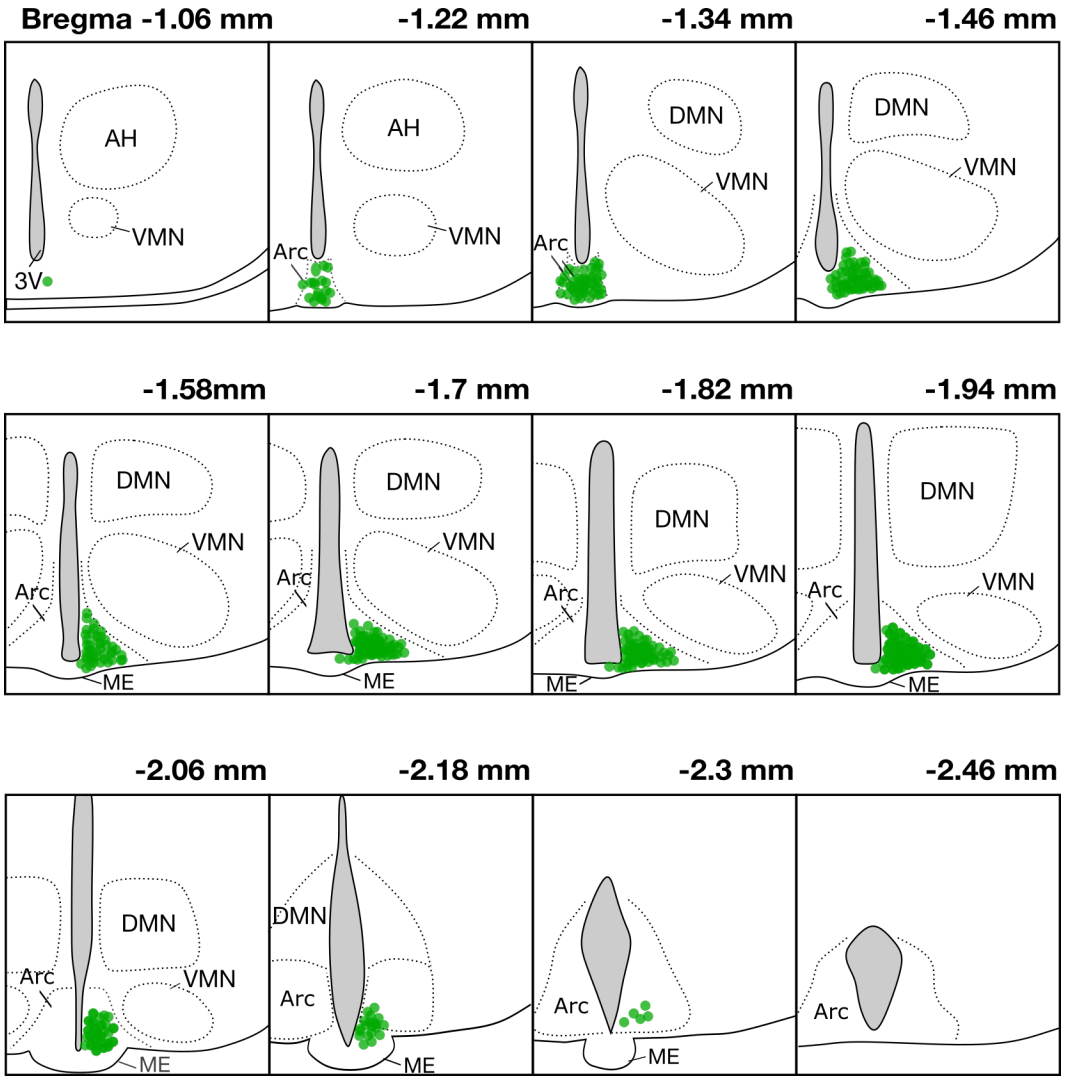


Figure 3.6. Representative distribution and density of Npy+ cells in the hypothalamus. Approximated bregma positions are indicated. Green circles indicate the position of the groups of Npy+ cells. Results obtained from young, middle-aged and old animals, with a minimum of 3 animals analysed per age group, were employed for the diagram design. Dashed lines outline the 3V and the limits of the different hypothalamic nuclei. 3V – third ventricle. VMN – ventromedial nucleus. AH – anterior hypothalamus. Arc – arcuate nucleus. ME – median eminence.

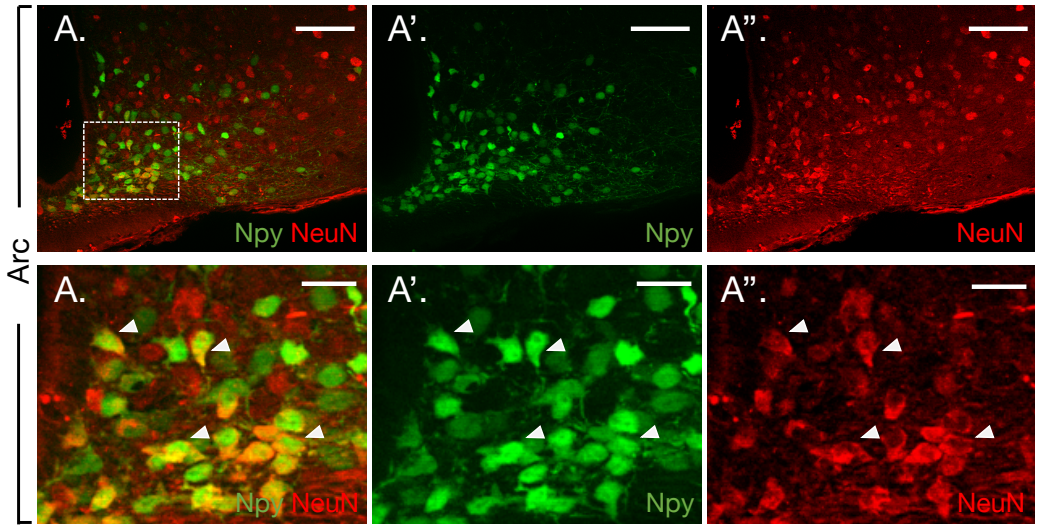


Figure 3.7. Npy-GFP+ cells co-express the neuronal marker NeuN, confirming their neuronal identity. (A – A'') Representative Npy-GFP+ brain section immunolabelled for GFP (green) and NeuN (red). (B – B'') Dashed box in (A) shows a high power of Npy-GFP+ and NeuN+ expression in the Arc nucleus. White arrows point co-localization of GFP+ and NeuN expression. Images are representative of results obtained in young, middle-aged and old animals with a minimum of 3 animals analysed per age group. Approximated bregma position -1.34 mm. Scale bar (A – A") 50 µm. Scale bar (B – B") 13 µm.

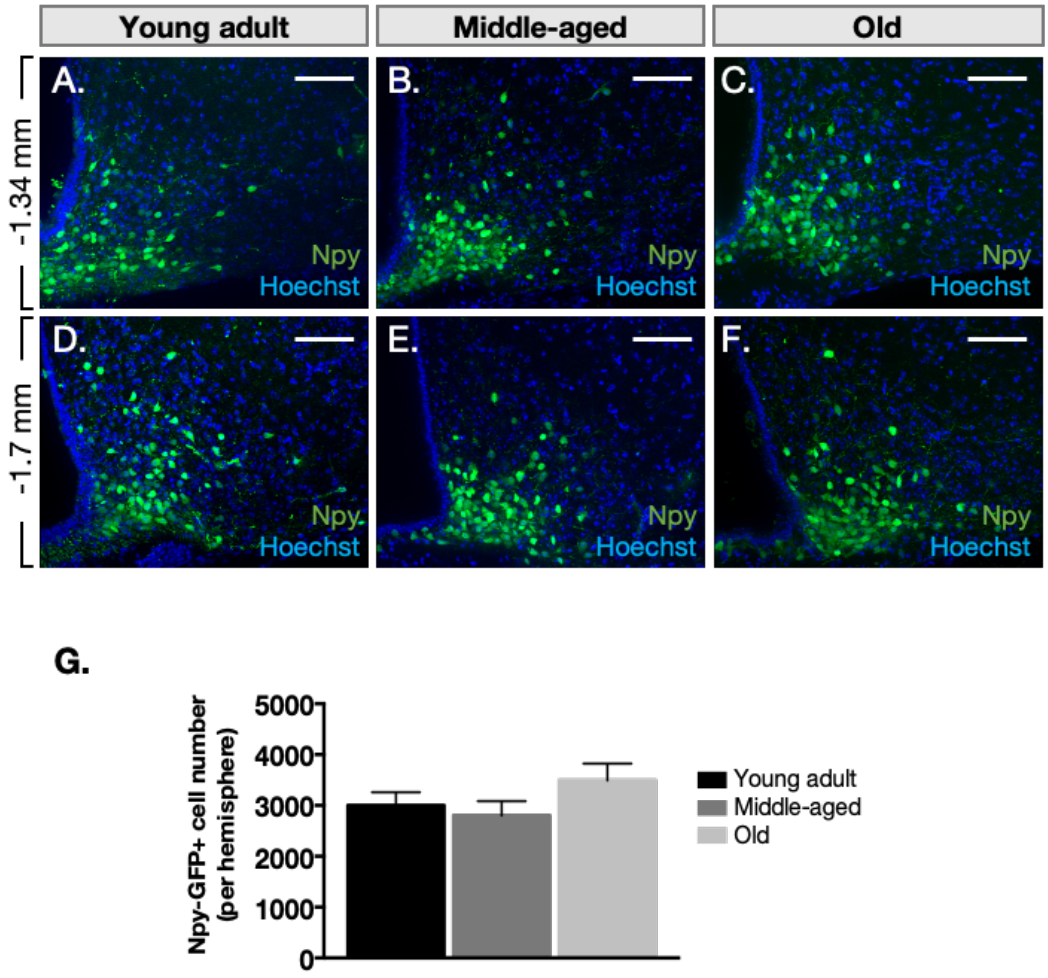


Figure 3.8. Npy-expressing neuron number in the Arc nucleus remain stable during ageing. (A – F) Representative images of Npy-GFP+ brain sections stained for GFP (green) and Hoechst (blue). (A – C) Bregma -1.34 mm, from young-adult (A), middle-aged (B) and old (C) animals. (D – E) Bregma -1.7 mm, from young (D), middle-aged (E) and old (F) animals. Scale bar 25 µm. (G) Average total number of GFP+ cells per hemisphere, in young-adults (black column), middle-aged (dark grey) and old animals (light grey). Data show non-significant differences in GFP+ cell number, however a trend towards increase is observed. Bregma positions are approximated. Data is represented as mean ±SEM and a minimum of 4 animals per age group were quantified. Significance testing was performed using one-way ANOVA followed by Tukey post-hoc test (*p<0.05).

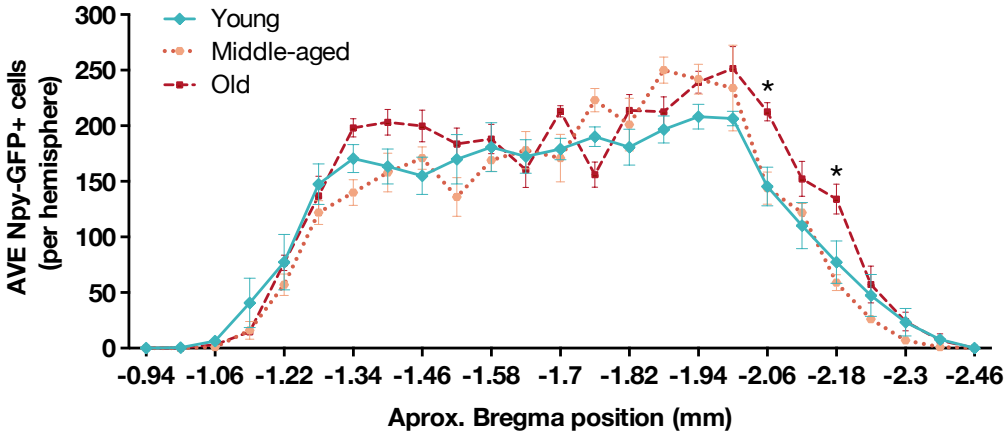


Figure 3.9. Distribution Npy-expressing cells in the Arc nucleus during ageing. Distribution of GFP+ (Npy-GFP) cells in the Arc. Data is represented as the average number of GFP+ (per hemisphere) in sections ranging from bregma -0.94 to -2.46 mm. Different colours represent the three age groups, young adult (blue), middle-aged (orange) and old (red). Data is represented as mean ±SEM, and a minimum of 4 animals per age group were quantified. Significance testing was performed using two-way ANOVA followed by Tukey post-hoc test (*$p<0.05$). Statistically significant differences for young *vs* old (bregma -2.06 and -2.18 mm) are indicated.

3.3.5. The Npy: Pomc ratio is affected by age

NPY and POMC neurons exert opposite roles, and the firing balance between both populations control feeding and metabolism in the body. Results showed that NPY outnumbered POMC population in the three age groups (**Figure 3.10, A**). The ratio between Npy- and Pomc-expressing neurons (Npy: Pomc) was calculated using the Pomc and Npy quantifications presented in the previous sections (see *section 2.8.1* for more information). Results showed that Npy: Pomc ratio suffered changes with age, 1.416 ± 0.157 in young adult mice, 1.916 ± 0.195 in middle-aged, and 3.013 ± 0.157 in old mice (**Figure 3.10, B**).

Next, Npy and Pomc distribution throughout the Arc was compared in the three age groups. In young animals, Npy- and Pomc-expressing neurons were evenly proportioned in the rostral Arc (bregma -0.94 to -1.58 mm); in the caudal Arc, Npy outnumbered Pomc (-1.7 to -2.06 mm; **Figure 3.11**, **A**). In the middle-age and old animals, Npy-expressing cells exceeded Pomc in rostral and caudal regions (**Figure 3.11**, **B – C**). In conclusion, the results suggested that Npy to Pomc ratio in the Arc nucleus suffer an age-related change.

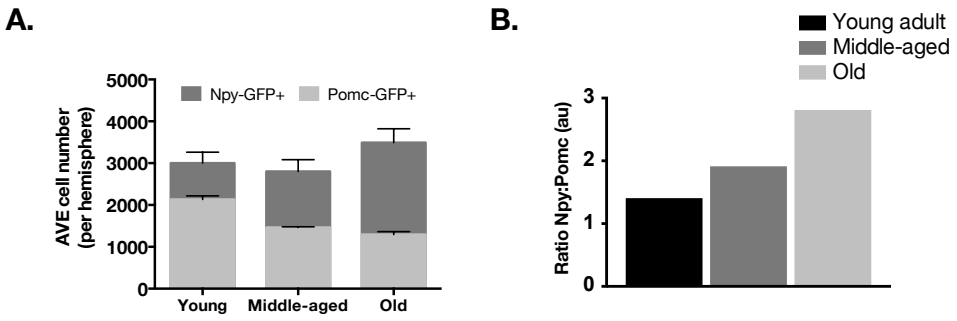


Figure 3.10. The orexigenic (Npy+) and anorexigenic (Pomc) neuronal populations change in number with age affecting the Npy/Pomc ratio, important for the control of the energy balance. (A) Average Npy-GFP+ (dark grey) and Pomc-GFP+ (light grey) cells (per hemisphere) comparison per age group. Data is represented as mean ± SEM and minimum of 4 animals per transgenic and age group were quantified. (B) Ratio Npy:Pomc represented as average total number of Npy-GFP+ cells divided by the average total number of Pomc-GFP+ (obtained in previous sections of this chapter); for young adult (black), middle-aged (dark grey) and old (light grey) animals.

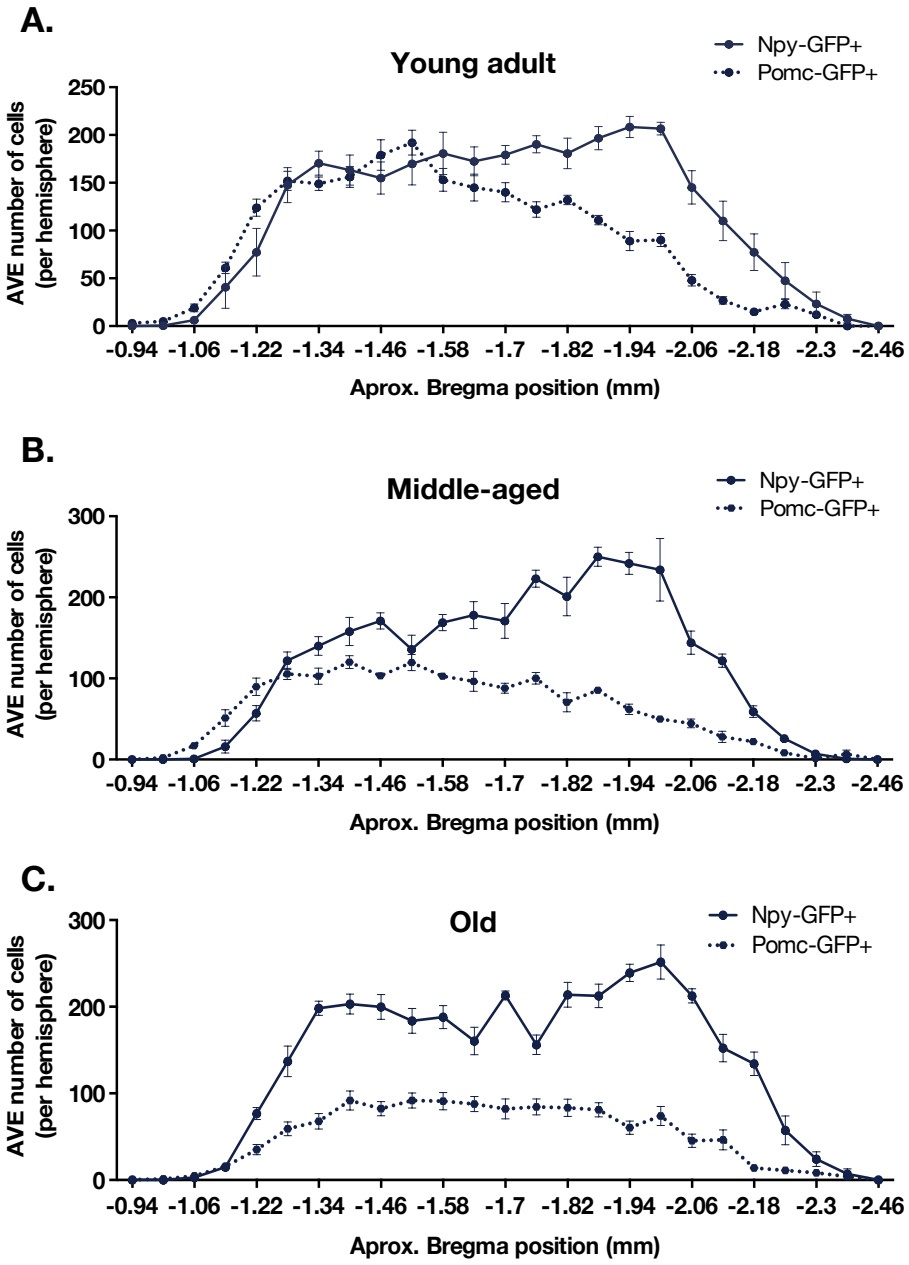


Figure 3.11. Pomc-and Npy-expressing cells show a differential distribution within the Arc and, as the age progresses, Npy-expressing cells outnumbers Pomc and become the majoritarian population thorough the Arc. (A - C) Graphs show the distribution of Npy-GFP+ (solid line) and the Pomc-GFP+ (dashed line) cells in the Arc, represented as the average number of GFP+ (per hemisphere) in the bregma positions ranging from -0.94 to -2.46 mm, in young adult (A), middle-aged (B) and old (C) animals. In the young groups, Npy-GFP+ population show a more even distribution in the Arc than Pomc-GFP+ cells that are more predominant in the rostral positions of the Arc (bregma -1.22 to -1.58 mm). During ageing, the Npy population maintain their distribution; however, the Pomc-GFP+ population decline, mostly affecting the rostral positioned cells and appear uniformly distributed in the Arc of old animals. AVE – average. Data is represented as mean ± SEM and a minimum of 4 animals per age group were quantified.

3.3.6. Programmed cell death is absent in the ageing hypothalamus

3.3.6.1. The Decline of Pomc-expressing neurons is not due to apoptosis

Given that the results indicated that Pomc-expressing neurons decline with age, I decided to investigate if (1) apoptosis occurs in the hypothalamus and/or increases with age; and (2) apoptotic events target the anorexigenic population. To do so, immunohistochemistry for the activated form of the caspase 3 (CC3) and TUNEL assay were employed.

Firstly, to identify apoptotic events, the apoptosis marker cleaved caspase 3 (CC3), the activated form of the caspase 3 was used. The caspase 3 is the final effector of the apoptotic cascade, either receptor or mitochondrial-mediated, and has been widely used to detect apoptosis (Stadelmann et al. 1999). Immunohistochemistry for CC3 was performed in hypothalamic brain sections from young adult (n = 4), middle-aged (n = 6), and old mice (n = 3). In order to avoid false positives, the chromatin (Hoechst staining) and the nucleus morphology was analysed in the CC3+ cells, and only cells showing double staining (CC3/Hoechst) were considered real apoptotic events. Results failed to show CC3 staining the Arc; however, sporadic CC3+ cells were detected in other regions of the hypothalamus, including the VMN (**Figure 3.12**, **A – A'''** and **B – B'''**). Additionally, other regions known to suffer apoptotic events were studied, and positive cells were found in the lateral ventricles (LV), thus confirming the validity of the method (**Figure S 2**).

Secondly, TUNEL assay was used to examine apoptosis via DNA fragmentation. The assay detects the DNA strands breaks and enzymatically labels the free 3'-OH termini with modified nucleotides (digoxigenin-dNTP), further detected by a digoxigenin/anti-digoxigenin fluorescent system. Only TUNEL+ cells that showed co-staining with the nuclear marker Hoechst were considered. The TUNEL assay was performed in hypothalamic brain sections from young adult (n = 4), middle-aged (n = 4) and old mice, n = 2). Results showed very rare TUNEL+ cells in the Arc nucleus and in the periventricular region of the 3V (**Figure 3.12**, **C – C'''** and **D – D'''**). As a control region, TUNEL+ cells were observed surrounding the LV (**Figure S 2**).

In conclusion, very few cells were detected via CC3 immunohistochemistry and TUNEL assay in the hypothalamus of young, middle-aged and old animals; thus, suggesting that apoptosis may not play a significant role in Pomc cell number decline reported earlier in this chapter.

3.3.6.2. The age-related decline in Pomc-expressing neurons is not due to necroptosis

Following the apoptosis studies, the next step was to study if necroptosis activation mediates the decline in Pomc-expressing cells observed with age. To do so, the expression of the necroptosis marker MLKL (mixed lineage kinase domain-like) was investigated via immunohistochemistry in hypothalamic brains sections of young adult (n = 3), middle-aged (n = 2) and old animals (n = 3).

Results showed sporadic MLKL+ cells in the mediobasal hypothalamus of the three groups of age. The identity of the sporadic MLKL+ cells was assessed via co-localisation with different markers, including the neuronal marker NeuN and the microglial marker Iba1. However, only the Iba1 marker showed co-localisation with MLKL+ cells (**Figure 3.13**, **A – A'''**). Additionally, in some cases, MLKL staining resembled small vessels, based on the morphological characteristics of the brain vasculature (**Figure 3.13**, **B – B'''**). As a control region, the corpus callosum was analysed, and MLKL+/Iba1+ cells were observed (**Figure S 3**).

In conclusion, necroptotic events (MLKL+) in the Arc nucleus are sporadic in the three age groups studied and seem to target only microglial cells and microvascular cells.

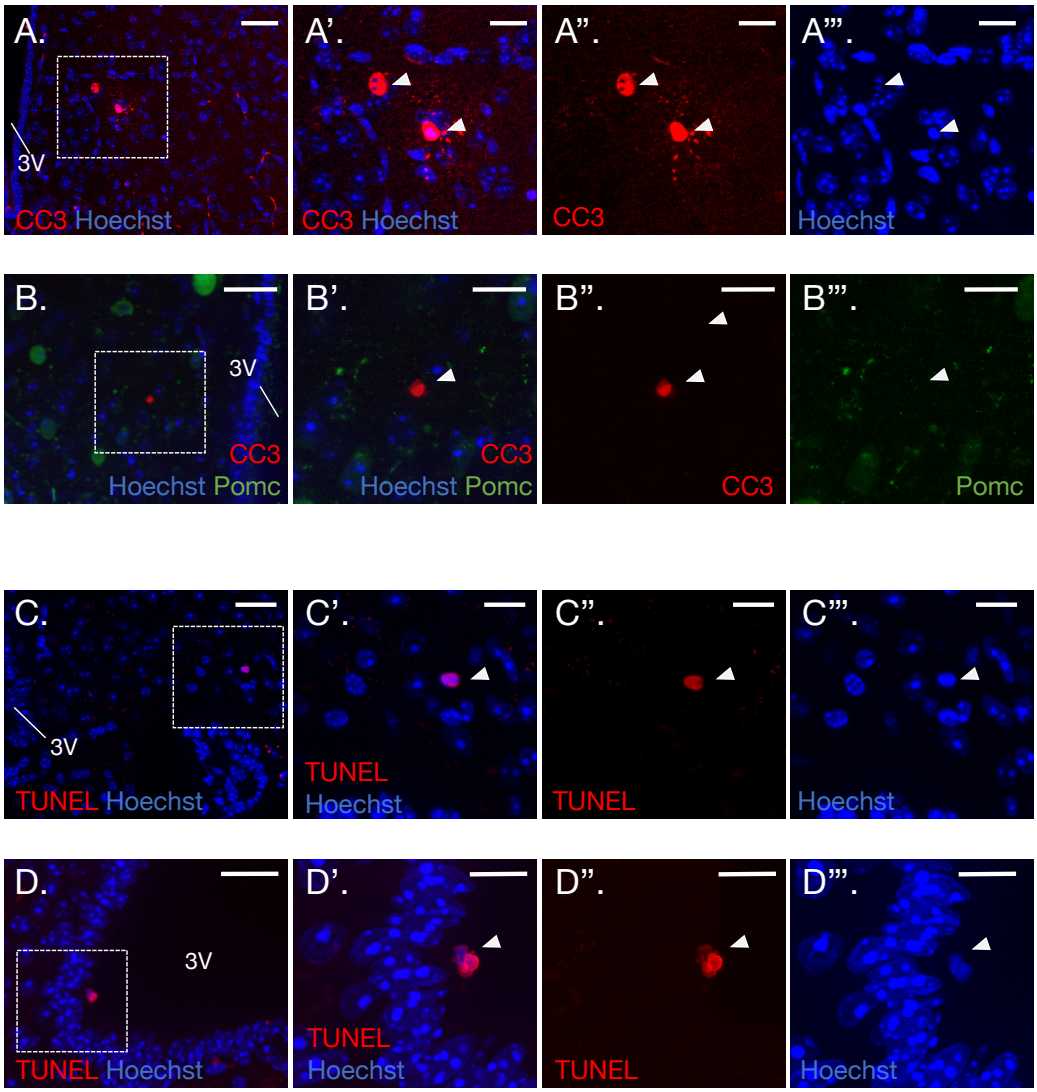


Figure 3.12. Analysis of CC3 immunostaining and TUNEL assays revealed rare apoptotic events in the hypothalamus. (A – A''') CC3 immunostaining (green) and Hoechst (blue) in the VMN nucleus of the hypothalamus (bregma -1.43 mm). (A', A'', A'') Dashed box in (A) shows a higher power and features two CC3+ cells and the apoptotic morphology of their nuclei (white arrows). (B – B''') CC3 (red), Pomc-GFP (green) and Hoechst (blue) detection in the periventricular region of the hypothalamus (bregma -1.06 mm). (B', B'', B'') Dashed box in (B) features one CC3+ cell that failed to show co-localization with Pomc-GFP+ expression (white arrow). (C – C''') TUNEL+ cell (red) and Hoechst (blue) in the Arc nucleus of the hypothalamus (bregma -1.94 mm). (C', C'', C'') Dashed box in (C) features a TUNEL+ cell and the apoptotic nucleus (white arrow). (D – D''') TUNEL+ cells (red) and Hoechst (blue) close to the 3V wall nucleus of the hypothalamus (bregma -1.82 mm). (D', D'', D'') Dashed box in (D) features a TUNEL+ cell and the apoptotic nuclei (white arrow). Scale bar (A, B, C, D) 25 µm. Scale bar (A' – A''', B' – B''', C' – C''', D' – D''') 15 µm. 3V – third ventricle.

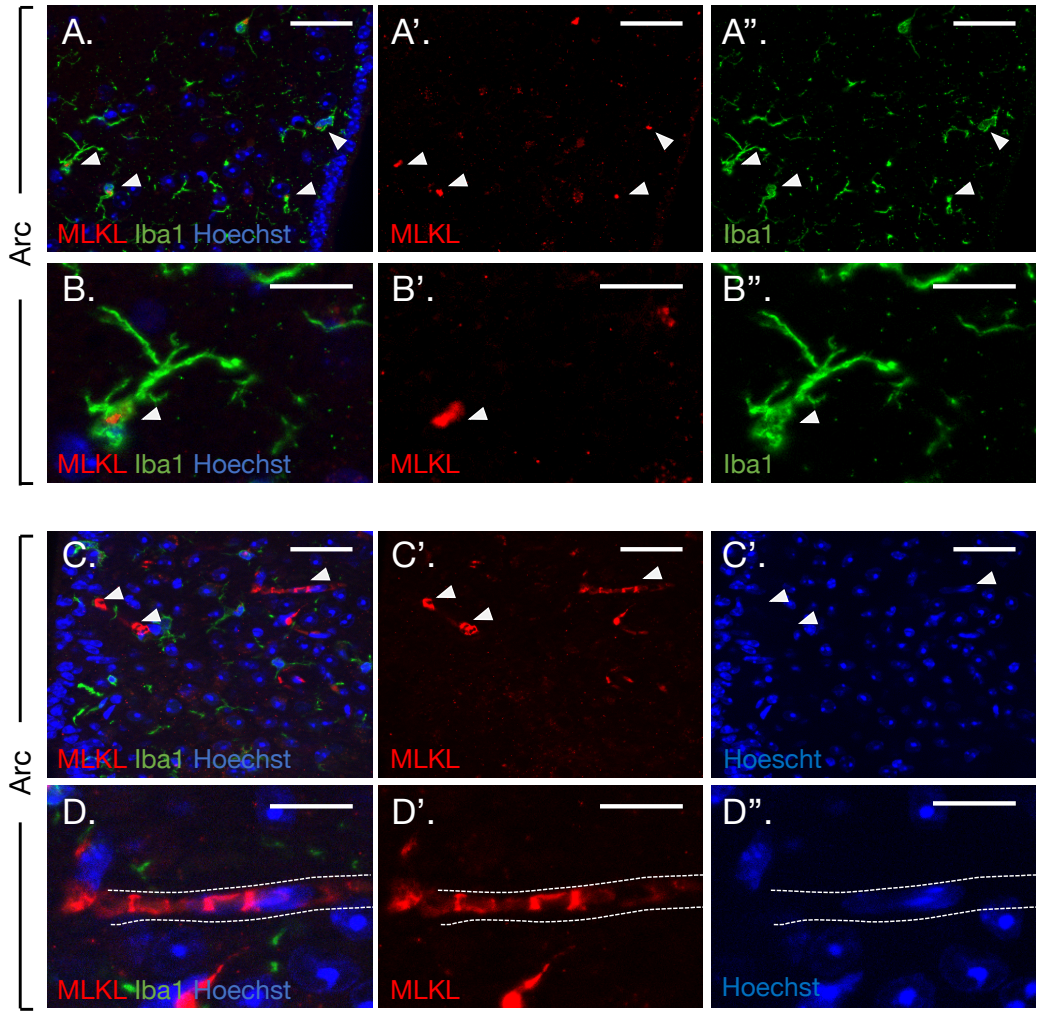


Figure 3.13. The necroptosis marker MLKL showed co-localization with microglia (Iba1+) and suggest association to blood vessels in the Arc nucleus. (A – B'') MLKL (red), Iba1 (green) and Hoechst (blue) staining in the Arc nucleus of the hypothalamus (bregma -1.7mm). White arrows indicate MLKL+/Iba1+ cells. (C – D'') MLKL (red), Iba1 (green) and Hoechst (blue) staining in the Arc nucleus of the hypothalamus (bregma -1.94 mm). White arrows indicate MLKL+ cells that seem to be part of a blood vessel due to its morphology and proximity between each other. (D – D''') High power of (C – C") shows MLKL+ cells associated to a blood vessel delineated by dashed lines. Scale bar (A – A'', C – C'') 50 µm. Scale bar (B – B'', D – D'') 12 µm. Bregma positions are approximated. Scale bar 25 µm. 3V – third ventricle. Arc – arcuate nucleus.

3.4. Discussion

As part of the ageing process, changes in the energy balance have been reported in mammals (Roberts 2000), likely caused by the progressive impairment of the hypothalamic circuits that control body homeostasis. Previous studies reported changes in the anorexigenic (POMC) and orexigenic (NPY/AgRP) populations with age, although they might not be sufficient to illustrate the age-related shift in energy homeostasis. The coordinated regulation of neuronal circuit involving POMC and NPY/AgRP is essential for maintaining energy balance; however, the number and distribution of both neuronal populations have not been explored, nor their dynamics, during ageing.

In this study, using the transgenic models Pomc-GFP and Npy-GFP, the number and distribution of both neuronal populations was quantified in serial hypothalamic sections across the mouse lifespan. The detailed characterization of both appetite-regulating populations showed that the anorexigenic POMC, but not the orexigenic NPY, suffer a significant decline with age.

Finally, cell death studies confirmed that the POMC neuronal decline is not due to apoptosis and/or necroptosis, suggesting that age-related inhibition of POMC expression in a subset of anorexigenic neurons may underlie the differences observed with age.

3.4.1. Population dynamics: Pomc and Npy

3.4.1.1. Technical considerations

In this study, POMC and NPY neurons counting was performed in 24 serial sections of 60 µm ranging from bregma -0.94 to -2.4 mm, to cover the entire Arc nucleus. This counting system method allowed to obtain an unbiased quantification of the entire neuronal population, taking into account any possible regional differences. Different transgenic animals were used specific to each population: Pomc-GFP and Npy-GFP. It should be noted that results are based on GFP transgene expression under the control of the POMC or NPY promoter, respectively.

To quantify the number and distribution of the anorexigenic neurons, the transgenic Pomc-GFP was employed. The validity of the model was confirmed by immunohistochemistry against POMC, with > 99% co-expression with EGFP+ neurons in the Arc. Co-localisation of EGFP with TH (tyrosine hydroxylase) or NPY was not observed (Cowley et al. 2001). Also, previous studies confirmed the validity of the transgenic model for studying rapid changes in Pomc expression (Niikura et al. 2013).

To quantify the number and distribution of the orexigenic neurons, the transgenic Npy-GFP was employed. The fidelity of GFP expression in orexigenic neurons was previously confirmed via single cell RT-PCR and immunohistochemistry against AgRP and NPY, taking into account that 95% of NPY neurons co-express AgRP (Broberger et al. 1998; van den Pol et al. 2009).

3.4.1.2. Decline of a selected subpopulation of POMC neurons and may cause physiological changes associated with ageing

In the current study, the number of POMC neurons was quantified in hypothalamic coronal sections of the Pomc-GFP reporter mice. Results revealed that the Arc nucleus of young mice hosts a total of 4262 (SEM ± 172) POMC neurons, which declined by more than 30% during ageing. However, the age-related neuronal decline is not accompanied by a decreased POMC expression at RNA (see Table 4.10 in *Chapter 4*) and protein levels, as western blot analysis revealed. These findings suggest a possible adaptive response to maintain POMC expression stable during ageing. However, it is also plausible that the results reflect the relativity insensitivity or the dilution effect of the western blot at detecting subtle protein expression changes.

The results in this study contrast with previous studies in rodents, that observed a decline in the expression of POMC and POMC-derived peptides with age (Barnea, Cho, and Porter 1982; Lloyd et al. 1991; Nelson et al. 1988). The disparity in results across studies could be due to: (1) the sex of the animals used, as previous studies were done only in females, and the present study is done in both females and males; (2) The methods of quantification used, *in situ* hybridisation/immunohistochemistry *vs* western blot, that differ in sensitivity to quantify total expression levels (Dittadi et al. 1993).

In terms of function, POMC neurons are critical regulators of metabolism by controlling food intake and energy expenditure. Experimental ablation of these cells is sufficient to cause excess weight gain and glucose intolerance in adult mice (Gropp et al. 2005; Zhan et al. 2013). However, the physiological consequences of their age-related decline are still unclear. Some insights can be inferred from studies in diet-induced obese mice, where weight gain is associated with a reduction of POMC neurons in the Arc by ~12% (Li et al. 2012) or ~25% (Thaler et al. 2012). In this context, it is noteworthy that POMC cells play an essential role to protect against obesity; thus, is it possible that loss of POMC neurons promotes the weight gain and increased adiposity observed in aged rodents and humans (Pappas and Nagy 2019).

Following the neuronal decline, the distribution of Pomc-expressing cells also showed age-related anatomical differences. Several reports suggested that POMC neurons in different regions of the Arc nucleus have distinct functions. POMC neurons can co-express a plethora of different transmitters including nociceptin (Maolood and Meister 2010), CART (N Vrang et al. 1999), acetylcholine (Meister et al. 2006), GABA and glutamate (Dicken, Tooker, and Hentges 2012; Jarvie and Hentges 2012; Wittmann, Hrabovszky, and Lechan 2013), adding to the ability of these neurons to regulate a wide range of physiological processes. Heterogeneity in transmitter phenotype often correlates well with the cell's location within the rostro-caudal extent of the Arc nucleus (Jarvie and Hentges, 2012; Wittmann, Hrabovszky and Lechan, 2013). In addition to co-transmitters release, receptor expression and responsiveness to leptin, insulin and serotonin seem to be dependent on their anatomical location within the Arc nucleus (Sohn et al. 2011; Williams et al. 2010). For example, rostral, but not caudal, POMC-positioned cells are activated by leptin administration (Williams et al. 2010).

In line with their functional heterogeneity, Arc POMC neurons send projections throughout the brain to affect a wide array of functions. POMC projections are found in brain areas involved in general homeostasis, including hypothalamic nuclei (PVN, SON and LH) and the brainstem (BST), as well as areas that mediate reward, such as the ventral tegmental area (VTA). Innervation by POMC has also been reported the amygdala, zona incerta, the periaqueductal gray (PAG) and dorsal vagal complex (DVC) (King and Hentges 2011; Lima et al. 2016; Wang et al. 2015; Wei et al. 2018; Zheng et al. 2005, 2010). Interestingly, only a small portion of POMC neurons project to any individual target site, suggesting that relatively few POMC neurons may mediate potent

and specific physiological responses (King and Hentges 2011). Also, neurons in the rostral or caudal regions of the Arc seem to innervate different target regions. For example, POMC neurons innervating the DVC and PAG are mostly located in the rostral Arc (King and Hentges 2011; Zheng et al. 2005).

Taking all together, expression of select receptors, co-expression of specific transmitters and projection to target sites seems to be dependent on the rostro-caudal position of the POMC neurons in the Arc. At the same time, relatively few POMC neurons seem to have potent effects on their target regions. The current study reported an age-related decline in POMC neurons, affecting mostly to neurons located in rostral positions of the Arc. Therefore, loss of a selected subpopulation of POMC may cause significant consequences and underlie some of the physiological changes associated with ageing.

3.4.1.3. Npy neuronal population in the Arc showed subtle changes with ageing

In the current study, the number of NPY neurons was quantified in hypothalamic coronal sections of the Npy-GFP mouse. Results revealed that the Arc nucleus of young mice hosts a total of 6001 ± 519 neurons in young adult, 5601 ± 566 in middle-aged, and 6983 ± 661 in old mice. In relative similarity, previous reports estimated the ARC contained 7000-8000 NPY neurons (Lemus et al. 2015). In terms of distribution, NPY neuronal number varies along the rostro-caudal extent of the Arc with higher cell counts in the caudal region, in line with previous findings (Lemus et al. 2015). Analysis failed to show age-related differences in the total NPY count; however, a small increase in NPY neurons with age was observed in caudal positions of the Arc.

NPY neurons in the Arc control energy balance by sensing and integrating metabolic signals to induce food intake. As reported for POMC neurons, different NPY subpopulations have been identified based on their responsiveness to peripheral signals, such as ghrelin, leptin, insulin and glucose (Kohno and Yada 2012), suggesting functional heterogeneity. In regard to their projection patterns, studies showed that small subsets of NPY neurons project to specific brain regions (Atasoy et al. 2012; Betley et al. 2013; Wu, Boyle, and Palmiter 2009), where they directly inhibit downstream targets including POMC neurons (Garcia de Yebenes et al. 1995). Interestingly, NPY innervation onto POMC undergoes a progressive and pronounced increase with age, associated with the decrease in POMC activity (Newton et al. 2013).

Therefore, although the changes observed in the orexigenic population are very subtle, variation of a small portion or a specific subtype of NPY neurons could underlie some of physiological responses associated to ageing. However, it is more plausible that changes in NPY neuronal morphology, connectivity or transcriptome, rather than number, are responsible for the decline in metabolism and energy homeostasis observed during ageing.

3.4.1.4. Npy to Pomc ratio change with age may produce an uneven power balance affecting the control of energy balance

The anorexigenic neurons that express POMC and the orexigenic neurons co-expressing NPY/AgRP in the Arc nucleus of the hypothalamus are known to exert opposing actions on feeding; thus, the regulation of energy homeostasis involves a dynamic balance between the two neuronal populations (Cone et al. 2001). One finding of this study is that NPY neurons outnumbered POMC neurons in the three age groups, and the ratio of NPY to POMC neurons increased from 1.4:1 in young adults to 3:1 in old animals. The coordinated regulation of neuronal circuit involving these neurons is essential in properly maintaining energy balance, and any disturbance therein may result in metabolic disbalance. Thus, the age-related changes in the ratio of Npy and Pomc neurons may produce and uneven balance power affecting the control of energy balance.

3.4.2. The decline of POMC population is not due specific apoptosis or necroptosis of the anorexigenic population

The present study identified a decline in the anorexigenic population with age, although the mechanism underlying the neuronal loss is still unclear. Potential causes may involve the selective cell death of POMC neurons, as a result of activation of apoptotic and necroptotic mechanisms with age.

Apoptosis can be triggered through several signalling pathways and involves a family of cysteine-dependent aspartate-directed proteases, known as caspases (Degterev, Boyce, and Yuan 2003). The caspase-3 is the final effector of the apoptotic cascade,

and the detection of its activated form CC3 is a reliable marker for apoptosis activation (Stadelmann et al. 1999). However, in the CNS, caspase-3 can also play non-apoptotic roles, including differentiation of glial cells (Oomman et al. 2006) and cytoskeletal remodelling in neurons and astrocytes (Acarin et al. 2005; Guyenet et al. 2013). Therefore, along with the CC3, the TUNEL assay was chosen as an alternative method to confirm apoptosis. The TUNEL assay detects apoptosis via DNA fragmentation, even where chromatin condensation has begun and there are only a few DNA strand breaks (Kuan et al. 2004; Kyrylkova et al. 2012). Experiments in this chapter found little evidence of CC3 and TUNEL staining within the Arc nucleus across the mouse lifespan; however, CC3+ and TUNEL+ cells were observed in other brain regions confirming the validity of the results. Caspase-independent cell death was also investigated in the ageing hypothalamus via immunohistochemistry for MLKL, the terminal effector of the necroptosis pathway (Czabotar and Murphy 2015). Few cells undergoing necroptosis (MLKL+) were observed in the ageing hypothalamus and co-localisation studies indicated that MLKL+ cells belong to the microglial lineage.

Taking all together, the scarce amount of apoptotic and necroptotic events observed in the ageing hypothalamus suggested that programmed cell death events may not be responsible for the full magnitude of the POMC decline. In line with this, previous studies also failed to establish cell death as the mechanism underlying the POMC neuron loss in diet-induced obese mice (Guyenet et al. 2013; McNay et al. 2012). It is also possible that because cell death is a dynamic process, cells can be found in different stages, making them difficult to detect using the available techniques.

One potential mechanism underlying the POMC neuronal decline could be that ageing is influencing changes in Pomc expression. The promoter of the Pomc gene contains multiple CpG islands that can be either methylated or unmethylated under different nutritional and environmental factors (Marco et al. 2013; Plagemann et al. 2009; Stevens et al. 2010). Also, studies in animal models showed that the pattern of epigenetic markers changes over lifespan (Roth, 2012). Therefore, it is possible that age-related epigenetic modifications, but not cell death, underlie the decline in POMC neurons reported in this study.

4. RNA-seq analysis of the young and aged hypothalamus

4.1. Introduction

The hypothalamus plays a key role in homeostatic and metabolism regulation (Dietrich and Horvath 2013; Kim and Choe 2018) and, as part of the ageing process, changes in the energy balance have been reported in mammals (Roberts 2000). Consequently, an understanding of the impact of ageing on the hypothalamus may provide important insights into the systemic ageing. Traditional histological studies using immunohistochemistry or *in situ* hybridisation have been widely used to study different processes and cell types in the hypothalamus; however, they are limited by the number of proteins or transcripts that can be simultaneously analysed and by a strong bias towards known markers. Consequently, the development of large-scale studies of gene expression has led to significant advances in understanding the hypothalamic functions, including the effects of ageing.

A classical approach to study the changes in gene expression has been the DNA microarrays. In 2001, Jiang and colleagues employed a high-density oligonucleotide array to examine gene expression changes in the hypothalamus with age. The main results of the study showed downregulation of genes related to neuronal signalling, plasticity and structure with age. Moreover, they found proteases upregulated during in the aged hypothalamus, several of which were involved in the processing and degradation of neuropeptides that could affect the neuroendocrine system (Jiang et al. 2001). However, the limitations of the method, with only 13000 genes analysed, made the study incomplete.

Recently, the emergence of RNA sequencing (RNA-seq) provided an alternative approach for high throughput gene expression studies. Through RNA-seq, gene expression, and gene interactions at any time point or in a particular tissue can be investigated (Ozsolak and Milos 2011). This method offered higher specificity and sensitivity compared to microarrays, facilitating the detection of a broader range of differentially expressed genes, especially genes with low expression (Bottomly et al. 2011; Zhao et al. 2014). Moreover, advances in the RNA-seq technology enabled the profiling of individual cells via single-cell RNA-seq, facilitating the transcriptional cataloguing of cell types in many tissues (Han et al. 2018; Regev et al. 2017; Schaum et al. 2018)

Interestingly, two different studies using the single-cell RNA-seq approach have systematically catalogued the cell types from the adult murine hypothalamus, identifying 34 neuronal and up to 36 different non-neuronal populations (Campbell et al. 2017; Chen et al. 2017). These studies have provided a comprehensive RNA-seq database of the different cell types in the adult hypothalamus; however, the effect of ageing on these cells remained largely unexplored.

In 2019, Boisvert and colleagues investigated the effect of ageing in the astrocyte population of the hypothalamus and other brain regions. They employed the Astrocyte-Ribotag mouse model for purification of astrocyte-enriched mRNA from the adult and aged brain to provide an RNA-seq database of gene expression (Boisvert et al. 2018; Sanz et al. 2015). The study confirmed previous histological data, with aged astrocytes exhibiting gene expression changes consistent with the reactive phenotype, such as upregulation of GFAP (Ike et al. 2004; Sofroniew and Vinters 2010), but also provided new information regarding the biological processes affected in the aged hypothalamic astrocytes. Results confirmed altered expression of synapse-regulating genes, supporting an active role for astrocytes in eliminating synapses in the ageing brain; increased expression of immune pathways, including the complement system, cytokines, and MHC; and decreased expression of genes involved in cholesterol synthesis (Boisvert et al. 2018).

In conclusion, the results mentioned above provided useful insights into the processes affected in the hypothalamus with ageing, however, the full picture remains unknown. Consequently, to investigate the biological processes affected in the hypothalamus with age, the analysis of the hypothalamic transcriptome of young and aged animals was performed in this chapter using RNA-seq.

4.2. Aims

To identify and gain insights into the effect of ageing in the hypothalamic transcriptome, RNA-seq was performed on mRNA extracted from the hypothalamus of young and old mice. The results of RNA-seq were then analysed, and gene ontology (GO) analysis was performed to reduce complexity and highlight biological processes affected in the aged hypothalamus.

4.3. Results

To better understand the processes involved in ageing, changes in gene expression were examined in the aged hypothalamus. To do so, hypothalami from young and old male mice were collected (n = 5 per age group), and mRNA was isolated. The samples were sent to Novogen (Cambridge, UK), where the RNA-seq differential gene expression experiment was performed. Each sample had: a sequencing depth of at least 20 million reads which is sufficient to accurately call differentially expressed genes (Liu, Zhou, and White 2014). The mapping rate is consistently high in all samples and the read numbers per sample were very similar (Table 4.1). Therefore, we can be confident in the outcome of the analysis. Differential gene expression analysis was carried out by Dr Simon Moxon using through Kallisto (Bray et al. 2016) and Sleuth (Pimentel et al. 2017).

Table 4.1. Information samples used for RNA-seq analysis

Sample name	Age (months)	Age group	Mapped reads	Proccessed reads	Mapped fraction
s1500	3	Young	18213349	20268284	0.8986
s2175	3	Young	23055413	25340676	0.9098
s2323	3	Young	21098009	23417224	0.901
s2399	3	Young	20870255	23233870	0.8983
s8278	4	Young	24859990	27550149	0.9024
s8282	24	Old	18124777	20056008	0.9037
s9001	25	Old	25177861	27739333	0.9077
s9002	26	Old	22615759	25199938	0.8975
s9003	26	Old	19618635	21778336	0.9008
s9004	25	Old	23073158	25647448	0.8996

Results revealed 2467 genes with differential expression (q-value < 0.05) in the aged group relative to the young animals, with 1131 of them downregulated and 1334 upregulated.

To classify the upregulated and downregulated genes identified by RNA-seq, gene ontology (GO) analysis was performed to reduce complexity and highlight biological processes enriched in the transcriptomic data. To do so, the online tool g:profiler (Raudvere et al. 2019) was used to analyse the list of upregulated and downregulated genes.

4.3.1. Upregulated genes in the aged hypothalamus

First, the upregulated gene list was analysed using g:profiler. The GO results highlighted multiple biological processes involved in the immune response. In fact, from the 172 GO modules enriched in the analysis, at least 108 of them were involved in immune response and neuroinflammation (Figure 4.1 – A, B). Additionally, GO enrichment analysis highlighted modules that appeared to represent the oligodendrocytes (OL), and included genes involved in myelination and OL differentiation (Figure 4.1 – C, D).

4.3.1.1. Upregulated expression of immune response genes in the aged hypothalamus

The immune genes and pathways upregulated in aged hypothalamus included the complement system, the major histocompatibility complex (MHC) and cytokine production (Table 1-3). The complement upregulated genes included C1qa, C1qb, C1qc, C1qbp, C1ra, C3, C3ar1 and C4b (Table 4.2). The MHC upregulated genes included B2m, H2-D1, H2-K1, H2-K2, H2-Q4, H2-Q6, H2-Q7, H2-M3, H2-T22, H2-T23 and Tap1 (Table 4.3). Production of cytokines was also increased in the aged hypothalamus, including the pro-inflammatory cytokines Tnf and Ccl3 (Table 4.4).

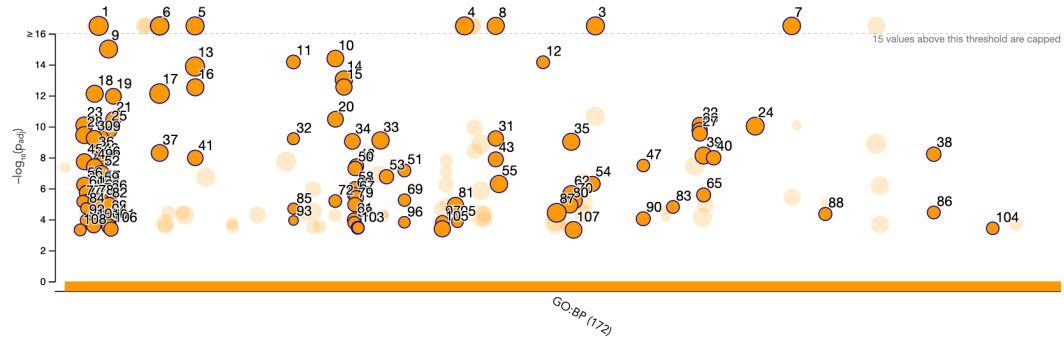
Given that microglia and astrocytes are key mediators of CNS immune response, genes considered markers of astrocytes and microglia were investigated. Transcriptomic results showed increased in RNA levels of Iba1, Trem2, Tmem119, Cx3cr1 and P2ry12,

expressed by microglial cells; and Gfap, S100β and Serpina3n, expressed by astrocytes (Table 4.5).

4.3.1.2. Upregulated expression of myelin-related genes in the aged hypothalamus

In addition to immune response, GO analysis also showed an enrichment of genes involved in myelination and terminal OL differentiation (Table 4.6). Genes essential for myelin production were upregulated in the aged hypothalamus, including Mbp and Plp1, Mog, Mag and Cnp (Aggarwal, Yurlova and Simons, 2011), as well as the transcriptional factor MYRF that directly promotes the expression of these genes (Bujalka et al. 2013). The RNA levels of Cd82, Trf and Opalin, expressed in mature-myelinating OL, were also increased in the hypothalamus with age (Goldman and Kuypers 2015).

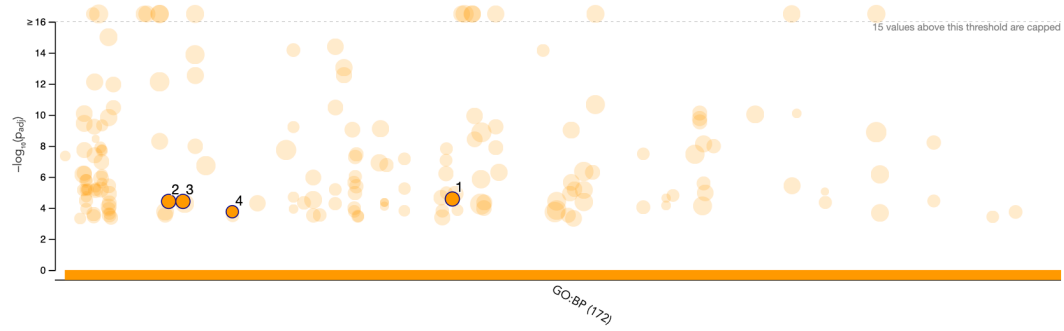
A.



B.

Enriched GO terms	GO ID
Immune system process	GO:0002376
Defense response	GO:0006952
Antigen processing and presentation	GO:0019882
Cytokine production	GO:0001816
Microglial activation involved in immune response	GO:0002282
Inflammatory response	GO:0006954

C.



D.

Enriched GO terms	GO ID
Myelination	GO:0042552
Ensheathment of neurons	GO:0007272
Axon ensheathment	GO:0008366
Oligodendrocyte development	GO:0014003

Figure 4.1. GO term enrichment for upregulated genes in the aged hypothalamus. (A and C) Manhattan plots illustrating GO term enrichment for upregulated genes in the aged hypothalamus. The x-axis represents functional terms that are grouped by biological process (BP), represented by orange circles. The y-axis shows the adjusted enrichment p-values in negative log10 scale. (A) Selected GO terms involved to immune response and inflammation (dark orange circles). (B) Highlighted enriched GO terms for plot A involved in immune response and their corresponding GO identification (ID) numbers. (C) Selected GO terms involved in myelination (dark orange circles). (D) Top enriched GO terms for plot C involved in myelin production and their corresponding GO identification (ID) numbers.

Table 4.2. Complement system genes upregulated in the aged hypothalamus

Gene	Description	Ensembl Gene ID	Log2 Fold-change (0 – 2.5)	q-value
C4b	Complement component 4B	ENSMUSG00000073418	2.007546385	1.93E-36
C3ar1	Complement component 3a receptor 1	ENSMUSG00000040552	1.503129013	5.40E-13
C1qa	Complement component 1, q subcomponent, α polypeptide	ENSMUSG00000036887	1.162447146	3.00E-93
C1qb	Complement component 1, q subcomponent, β polypeptide	ENSMUSG00000036905	1.064616806	1.16E-28
C1qc	Complement component 1, q subcomponent, C chain	ENSMUSG00000036896	1.063741745	2.03E-28
C3ar1	Complement component 3a receptor 1	ENSMUSG00000040552	1.063741745	1.02E-10
C1ra	Complement component 1, r subcomponent A	ENSMUSG00000055172	1.046848276	0.008219
C3	Complement component 3	ENSMUSG00000024164	0.948507801	0.026414
C1qbp	Complement component 1, q subcomponent binding protein	ENSMUSG00000018446	0.239056656	0.001580

Table 4.3. MHC genes upregulated in the aged hypothalamus

Gene	Description	Ensembl Gene ID	Log2 Fold-change (0 – 2.5)	q-value
H2-Q7	Histocompatibility 2, Q region locus 7	ENSMUSG00000060550	2.143994466	3.77E-12
H2-Q6	Histocompatibility 2, Q region locus 6	ENSMUSG00000073409	2.059270566	5.11E-18
H2-K2	Histocompatibility 2, K2, K region	ENSMUSG00000067203	1.654193747	3.86E-05
H2-Q4	Histocompatibility 2, Q region locus 4	ENSMUSG00000035929	1.516117277	3.52E-19
Tap1	Transporter 1, ATP-binding cassette, B	ENSMUSG00000037321	1.360246828	3.64E-09
H2-K1	Histocompatibility 2, K1, K region	ENSMUSG00000061232	1.316655004	9.17E-31
H2-D1	Histocompatibility 2, D region locus 1	ENSMUSG00000073411	1.155707001	2.86E-27
H2-T23	Histocompatibility 2, T region locus 23	ENSMUSG00000067212	1.103738226	2.32E-24
B2m	Beta-2 microglobulin	ENSMUSG00000060802	1.042919809	1.11E-100
H2-M3	Histocompatibility 2, M region locus 3	ENSMUSG00000016206	0.693164289	1.45E-05
H2-T22	Histocompatibility 2, T region locus 22	ENSMUSG00000056116	0.479036072	3.15E-09

Table 4.4. Cytokines upregulated in the aged hypothalamus

Gene	Description	Ensembl Gene ID	Log2 Fold-change (0 – 2.5)	q-value
Ccl3	Chemokine (C-C motif) ligand 3	ENSMUSG00000000982	2.172975208	0.000810
Ccl12	Chemokine (C-C motif) ligand 12	ENSMUSG00000035352	1.983459241	0.007173
Ccl6	Chemokine (C-C motif) ligand 6	ENSMUSG00000018927	1.500654046	4.42E-06
Tnf	Tumor necrosis factor	ENSMUSG00000024401	1.110886950	0.000158
Cxcl13	Chemokine (C-X-C motif) ligand 13	ENSMUSG00000023078	0.974190564	2.97E-09
Cxcl16	Chemokine (C-X-C motif) ligand 16	ENSMUSG00000018920	0.974190564	0.001018
Il16	Interleukin 16	ENSMUSG00000001741	0.806657184	0.022400
Il33	Interleukin 33	ENSMUSG00000024810	0.691608164	1.21E-23
Cxcl14	Chemokine (C-X-C motif) ligand 14	ENSMUSG00000021508	0.144924121	0.044403

Table 4.5. Upregulated microglial and astrocytes markers in the aged hypothalamus

Gene	Description	Ensembl Gene ID	Log2 Fold-change (0 – 2.5)	q-value
Trem2	Triggering receptor expressed on myeloid cells 2	ENSMUSG00000023992	1.316341702	6.55E-25
Aif1	Allograft inflammatory factor 1 (Iba1)	ENSMUSG00000024397	1.145589307	2.09E-07
Serpina3n	Serine protease inhibitor A3N	ENSMUSG00000021091	0.969527618	2.51E-33
Gfap	Glial fibrillary acidic protein	ENSMUSG00000020932	0.622147905	3.94E-17
Tmem119	Transmembrane protein 119	ENSMUSG00000054675	0.485306598	4.08E-05
Cx3cr1	Chemokine (C-X3-C motif) receptor 1	ENSMUSG00000052336	0.447714041	5.06E-05
S100b	S100 protein, beta polypeptide, neural	ENSMUSG00000033208	0.343824528	0.000449
P2ry12	Purinergic receptor P2Y, G-protein coupled 12	ENSMUSG00000036353	0.280659196	0.007173

Table 4.6. Upregulated myelin-related genes in the aged hypothalamus

Gene	Description	Ensembl Gene ID	Log2 Fold-change (0 – 2.5)	q-value
Pmp22	Peripheral myelin protein 22	ENSMUSG00000018217	0.607378173	2.18E-10
Mbp	Myelin basic protein	ENSMUSG00000041607	0.490941737	8.03E-15
Trf	Transferrin	ENSMUSG00000032554	0.490207863	3.57E-17
Opalin	Oligodendrocytic myelin paranodal and inner loop protein	ENSMUSG00000050121	0.488732173	0.036709
Nkx6-2	NK6 homeobox 2	ENSMUSG00000041309	0.458867079	4.30E-06
Mobp	Myelin-associated oligodendrocytic basic protein	ENSMUSG00000032517	0.458818701	6.60E-20
Cd82	CD82 antigen	ENSMUSG00000027215	0.327519208	0.000952
Cd9	CD9 antigen	ENSMUSG00000030342	0.317156812	2.98E-06
Sox10	SRY (sex determining region Y)- box 10	ENSMUSG00000033006	0.279819959	3.26E-05
Cnp	2',3'-cyclic nucleotide 3' phosphodiesterase	ENSMUSG00000006782	0.244301843	1.15E-06
Mag	Myelin-associated glycoprotein	ENSMUSG00000036634	0.236672146	0.006112
Myrf	Myelin regulatory factor	ENSMUSG00000036098	0.214081203	0.000628
Plp1	Proteolipid protein 1	ENSMUSG00000031425	0.21005480	0.002356
Mog	Myelin-associated glycoprotein	ENSMUSG00000076439	0.194190652	0.015457

4.3.2. Downregulated genes in the aged hypothalamus

Second, the downregulated genes were analysed following the same approach (g:profiler). GO results highlighted the biological processes involved in neuronal morphogenesis and intracellular transport. From the 295 biological processes enriched in the GO analysis, we identified at least 26 involved in neuronal cytoskeleton organisation (Figure 4.2 – A, B) and six associated with intracellular transport (Figure 4.2 – C, D).

4.3.2.1. Neuronal structural genes are downregulated in the aged hypothalamus

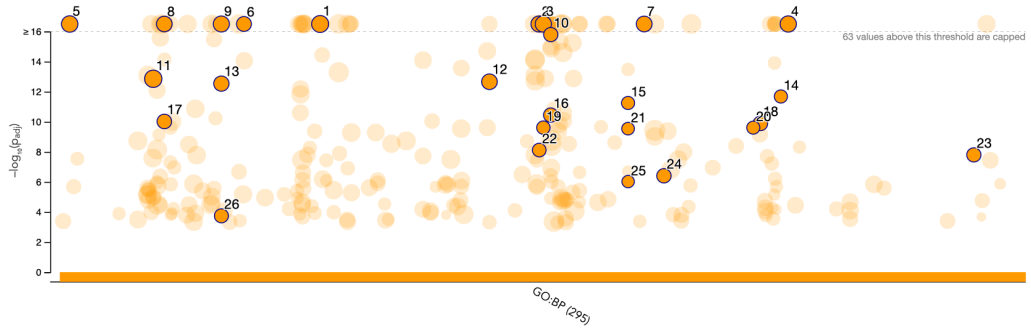
The GO analysis revealed an enrichment in genes involved in the architecture of the neurons and the regulation of neuronal projection development. Results showed downregulation of neuronal cytoskeleton elements including the Tuba1a and Tubb2b, part of the tubulin family of proteins that form and organise cell structures called microtubules; the intermediate filament Nfm, found in the neuronal cytoplasm; and the structural microtubule-associated proteins Map1a, Map1b, Map2, Mapt, Map6 and Map10, critical for the organisation and stabilisation of neuronal microtubules (Kapitein and Hoogenraad, 2015; Table 4.7).

Besides, genes involved in axonal guidance were also downregulated in the aged hypothalamus (Table 4.8). Results showed downregulation of Sema3d, Sema5a and Sema6b, members of the semaphorin family involved in axon growth and guidance, and the semaphorin receptors, neuropilins Nrp1 and Nrp2 and plexins Plxna1, Plexa2 and Pxna4 (Mann, Chauvet, and Rougon 2007; Russell and Bashaw 2018). Additionally, the axon guidance cues Slit2 and Efnb1, and the netrin receptor Unc5c (Russell and Bashaw 2018) showed decreased RNA levels in the aged hypothalamus. Finally, the neurotrophin Bdnf, that regulates neuronal growth and plasticity in adulthood (Tapia-Arancibia et al. 2004), was also downregulated in the aged hypothalamus.

4.3.2.2. Intracellular transport genes are downregulated in the aged hypothalamus

The GO results showed enrichment in genes involved in the intracellular and microtubule-based transport. Results showed an age-related downregulation of microtubule motor kinesin and dynein proteins, that drive the movement of organelles, vesicles, RNA granules, and proteins along the axon in neurons. Downregulated genes included members of the kinesin family, that drive anterograde transport outward from the soma: Kif2a, Kif26b, Kif3c, Kif3a, Kif3b and Kif21a and Kif1b; and Dync1H1, Dync1LI and Dync2H1, encoding different subunits of the cytosolic dynein that drives retrograde transport back from the distal axon (Maday *et al.*, 2014; Table 4.9). Importantly, these proteins are not only essential for neuronal transport but are also involved in myelination by transporting the different myelin components to developing the myelin sheath (Herbert et al. 2017).

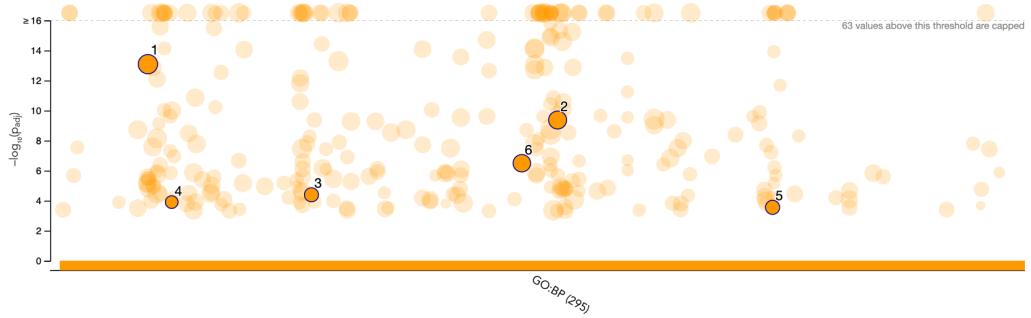
A.



B.

Enriched GO terms	GO ID
Neuron projection development	GO:0031175
Neuron projection morphogenesis	GO:0048812
Dendrite development	GO:0016358
Axon development	GO:0061564
Axonogenesis	GO:0007409
Cytoskeleton organization	GO:0007010

C.



D.

Enriched GO terms	GO ID
Transport	GO:0006810
Regulation of the transport	GO:0051049
Cytoskeleton-dependent intracellular transport	GO:0030705
Axo-dendritic transport	GO:0008088
Microtubule-based transport	GO:0099111
Intracellular transport	GO:0046907

Figure 4.2. GO term enrichment for downregulated genes in the aged hypothalamus. (A and C) Manhattan plots illustrating GO term enrichment for downregulated genes in the aged hypothalamus. The x-axis represents functional terms that are grouped by biological process (BP), represented by orange circles. The y-axis shows the adjusted enrichment p-values in negative log10 scale. (A) Selected GO terms involved to neuronal cytoskeleton organization (dark orange circles). (B) Top enriched GO terms for plot (A) involved in neuronal cytoskeleton organization and their corresponding GO identification (ID) numbers. (C) Selected GO terms involved in transport (dark orange circles). (B) Top enriched GO terms for plot (A) involved in transport and their corresponding GO identification (ID) numbers.

Table 4.7. Neuronal cytoskeleton genes downregulated in the aged hypothalamus

Gene	Description	Ensembl Gene ID	Log2 Fold-change (-0.5 to 0)	q-value
Map1b	Microtubule-associated protein 1B	ENSMUSG00000052727	-0.41906055	6.11E-09
Map10	Microtubule-associated protein 10	ENSMUSG00000050930	-0.345951694	0.012863
Map1a	Microtubule-associated protein 1 A	ENSMUSG00000027254	-0.316210624	4.48E-07
Map2	Microtubule-associated protein 2	ENSMUSG00000015222	-0.308426493	7.49E-07
Map6	Microtubule-associated protein 6	ENSMUSG00000055407	-0.245507946	8.22E-05
Tubb2b	Tubulin, beta 2B class IIB	ENSMUSG00000045136	-0.241513988	5.20E-06
Nefm	Neurofilament, medium polypeptide	ENSMUSG00000022054	-0.189661278	0.000216
Mapt	Microtubule-associated protein tau	ENSMUSG00000018411	-0.151364805	0.017104
Tuba1a	Tubulin, alpha 1A	ENSMUSG00000072235	-0.130554880	0.020644

Table 4.8. Axon guidance-related genes downregulated in the aged hypothalamus

Gene	Description	Ensembl Gene ID	Log2 Fold-change (-0.5 to 0)	q-value
Slit2	Slit homolog 2 (Drosophila)	ENSMUSG00000031558	-0.441176060	0.000176
Bdnf	Brain derived neurotrophic factor	ENSMUSG00000048482	-0.435612844	1.59E-07
Plxna4	Plexin A2	ENSMUSG00000029765	-0.395778259	4.41E-08
Sema3d	Semaphorin 3D	ENSMUSG00000040254	-0.368477194	0.037944
Plxna2	Plexin A2	ENSMUSG00000026640	-0.325646994	4.88E-05
Unc5c	Unc-5 netrin receptor C	ENSMUSG00000059921	-0.322489367	1.07E-05
Sema5a	Semaphorin 5A	ENSMUSG00000022231	-0.317199224	4.55E-07
Slit1	Slit homolog 1 (Drosophila)	ENSMUSG00000025020	-0.316489949	0.002399
Nrp2	Neuropilin 2	ENSMUSG00000025969	-0.2719596	8.63E-05
Nrp1	Neuropilin 1	ENSMUSG00000025810	-0.254873551	0.000584
Plxna1	Plexin A1	ENSMUSG00000030084	-0.243546084	0.032184
Efnb1	Ephrin B1	ENSMUSG00000031217	-0.224865588	0.034956
Sema6d	Semaphorin 6D	ENSMUSG00000027200	-0.148115723	0.015836

Table 4.9. Intracellular transport genes downregulated in the aged hypothalamus

Gene	Description	Ensembl Gene ID	Log2 Fold-change (-0.5 to 0)	q-value
Dync1H1	Dynein cytoplasmic 1 heavy chain 1	ENSMUSG00000018707	-0.382704803	0.000565
Dync2H1	Dynein cytoplasmic 2 heavy chain 1	ENSMUSG00000047193	-0.334862001	0.004433
Kif3c	Kinesin familty member 3C	ENSMUSG00000020668	-0.226288838	5.09E-05
Kif3a	Kinesin familty member 3A	ENSMUSG00000018395	-0.214550537	0.000124
Kif3b	Kinesin family member 3B	ENSMUSG00000027475	-0.203014437	0.000914
Kif26b	Kinesin family member 26B	ENSMUSG00000026494	-0.185460094	0.027210
Kif1a	Kinesin family member 1A	ENSMUSG00000014602	-0.184961501	0.021969
Kif21a	Kinesin family member 21A	ENSMUSG00000022629	-0.177645354	0.001007
Kif2a	Kinesin family member 2A	ENSMUSG00000021693	-0.169410524	0.014201
Kifap3	Kinesin-associated protein 3	ENSMUSG00000026585	-0.165267676	0.000907
Dync1Li1	Dynein cytoplasmic 1 light intermediate chain 1	ENSMUSG00000032435	-0.162547379	0.007173
Kif1b	Kinesin family member 1B	ENSMUSG00000063077	-0.124519737	0.045217

4.3.3. Genes with no differential expression

In addition to the upregulated and downregulated genes, the RNA-seq analysis revealed no age-related changes in the expression of the major hypothalamic neuropeptides (Table 4.10). These include the orexigenic neuropeptides AgRP and Npy; and anorexigenic peptide Pomc and Cartp. In addition, no changes were observed for Hcrt (also known as orexin) and Pmch, expressed by neurons located in the LH. Moreover, the enkephalin Penk and the Substance P (also known as Tac1), both synthesized in the VMN, also showed no differences with age. Finally, other hypothalamic neuropeptides with no age-related differences included Avp, Crh, Gad1, Nts, Oxt, Sst, and Trh.

Table 4.10. Hypothalamic neuropeptide genes with no differential expression with age

Gene	Description	Ensembl Gene ID	q-value
Pomc	Pro-opiomelanocortin-alpha	ENSMUSG00000020660	0.783506
Cartp	CART prepropeptide	ENSMUSG00000021647	0.886421
Npy	Neuropeptide Y	ENSMUSG00000029819	0.815756
Agrp	Agouti related neuropeptide	ENSMUSG00000005705	0.645035
Gad1	Glutamate decarboxylase 1	ENSMUSG00000070880	0.963289
Sst	Somatostatin	ENSMUSG00000004366	0.892759
Nts	Neurotensin	ENSMUSG00000019890	0.189772
Trh	Thyrotropin releasing hormone	ENSMUSG00000005892	0.333884
Oxt	Oxytocin	ENSMUSG00000027301	0.784253
Avp	Arginine vasopressin	ENSMUSG00000037727	0.083454
Penk	Preproenkephalin	ENSMUSG00000045573	0.094938
Crh	Corticotropin releasing hormone	ENSMUSG00000049796	0.837774
Pmch	Pro-melanin-concentrating hormone	ENSMUSG00000035383	0.063059
Tac1	Tachykinin 1	ENSMUSG00000061762	0.419022
Hcrt	Hypocretin	ENSMUSG00000045471	0.386745

4.3.4. Transcriptomic conclusions

- Upregulation of immune and inflammatory response molecules, as well as myelin-related genes, in the aged hypothalamus.
- Downregulation of genes involved in neuronal cytoskeleton organisation, microtubule-based transport and axonal growth in the aged hypothalamus.
- No differential expression of the major hypothalamic neuropeptides with age.

4.4. Discussion

A better understanding of the molecular effects of ageing in the hypothalamus may help to reveal mechanisms underlying the age-related decline in body homeostasis. Therefore, in this study, bulk RNA-seq analysis of the hypothalamus from young and old mice revealed four biological processes significantly altered with age: immune response, myelination, neuronal structure and intracellular transport. In contrast, no differences in gene expression between the adult and aged brain was found for the major hypothalamic neuropeptides.

4.4.1. Upregulated genes are associated with immune response and myelin formation

Inflammation in the hypothalamus has been reported during ageing, however, most of the studies are restricted to the astrocyte population (Boisvert et al. 2018; Santos, Bobermin, et al. 2018; Santos, Roppa, et al. 2018; Wang et al. 2006). Results in this chapter represent a more comprehensive investigation of the age-related immune response in the aged hypothalamus. Major changes detected included the upregulation of the complement pathway, MHC genes and inflammatory cytokines. The complement pathway and MHC genes play important roles in neuroinflammation, but growing evidence also suggested their involvement in key homeostatic functions, including the maintenance of synaptic plasticity, neurogenesis and clearance of cellular debris (Alawieh, Elvington, and Tomlinson 2015). Recently, the complement pathway has been implicated in synaptic pruning in neurological disorders; for example, C4 is upregulated in schizophrenia, and complement is involved in eliminating synapses in AD (Hong et al. 2016; Sekar et al. 2016). Interestingly, the complement-dependent synapse pruning involves synapses tagging by astrocytes followed by microglia phagocytosis, showing crosstalk between the two glial cell types (Liddelow et al. 2017).

Moreover, the present study identified upregulation of cytokines with different roles in regulating the pathophysiology and the inflammatory responses in the CNS. These included the pro-inflammatory mediator Tnfα, previously associated with hypothalamic inflammation and systemic ageing (Zhang et al. 2013). However, changes in cytokine expression are not restricted to Tnfα, and results also showed upregulation of Ccl3 and

Il16, involved in the disease progression and axonal degeneration reported in MS (Skundric 2015; Sørensen et al. 2004). Another inflammatory mediator upregulated is Il-33, that enhance chemokines and nitric oxide production, as well as phagocytosis by microglia; and its involvement in the pathophysiology of different neurological disorders has been reported (Abd Rachman Isnadi et al. 2018; Hudson et al. 2008). In the hypothalamus, increased Il-33 levels are detected in diet-induced inflammation (Huang et al. 2019).

In the CNS, most of the immune-related genes are expressed by astrocytes and microglia (Gasque, Fontaine, and Morgan 1995; Morgan and Gasque 1997), although some neuronal contribution cannot be ruled out (Freidin, Bennett, and Kessler 1992). Accordingly, analysis of the microglial and astrocyte transcriptome from discrete brain regions indicated that immune-related genes are particularly sensitive to ageing (Boisvert et al. 2018; Grabert et al. 2016). In the present study, age-related upregulation of astrocytes reactive markers, including Gfap, S100β and Serpina3n were observed confirming previous reports (Boisvert et al. 2018; Santos, Bobermin, et al. 2018; Santos, Roppa, et al. 2018; Wang et al. 2006). Besides astrocyte makers, increased expression of microglial homeostatic genes, such as Iba1, Trem2, Tmem119, Cx3cr1 and P2ry12 was also detected. Upregulated of homeostatic genes in microglia has been reported upon activation (von Bernhardi, Eugenín-von Bernhardi, and Eugenín 2015; Tang and Le 2016); thus, suggesting that hypothalamic microglia may acquire an activated phenotype with age. The detailed investigation of hypothalamic microglia and astrocytes dynamics with age is presented in *Chapter 5*.

In summary, the upregulated genes reflect a neuroinflammatory response in the ageing hypothalamus, possibly due to astrocyte and microglia activation. Interestingly, low doses of the anti-inflammatory drug ibuprofen have been shown to reduce astrocyte and microglial reactivity, leading to a protective effect against age-related cognitive decline in rodents (Rogers et al. 2017). Moreover, ageing delay and lifespan extension have been achieved in mice through preventing against hypothalamic NF-κB activation (Zhang et al. 2013); thus, providing evidence of a link between the hypothalamus and ageing development (Tang and Cai 2013; Tang et al. 2015; Zhang et al. 2013).

Following the neuroinflammatory response, a novel finding of this study was the upregulation of genes involved in myelination and neuronal ensheathment. In mammals,

myelin degeneration is a hallmark of the ageing brain and is highly correlated by a decline in the levels of myelin components (Peters, Moss and Sethares, 2000; Sandell and Peters, 2001; Peters, 2002; Bowley et al., 2010; Xing et al., 2012; Wang et al., 2020).

To date, however, the myelination pattern and the precise myelin dynamics in the hypothalamus throughout life are not well characterised. Therefore, the present chapter provides the first detailed investigation of the expression of myelin-related genes in the adult and aged hypothalamus. Results showed an increase in the expression of the major abundant myelin proteins Mbp and Plp1, that hold structural function by stabilising and compacting myelin membranes (Baron and Hoekstra 2010). Additional myelin proteins upregulated included Mog, Mag, Mobp and Cnp, Pmp22 and Cd9, although these lack a primarily structural role and their function is relatively unknown (Morell and Quarles 1999; Nakamura, Iwamoto, and Mekada 1996; Ohsawa et al. 2006).

Unsurprisingly, the transcriptional factor Myrf, that specifically activates the expression of myelin-related genes such as Mbp, Plp, Mog and Mag were also upregulated in the aged group (Bujalka et al. 2013). In spite its role in activating myelin gene expression, MYRF is necessary to promote and maintain terminal OL differentiation, and genetic ablation resulted in a delayed but severe CNS demyelination (Emery et al. 2009; Koenning et al. 2012). In addition, other transcriptional factors upregulated were Sox10 and Nkx6.2, that appear to have similar functions in the control of OL differentiation, and targeted mutations of these two genes caused retarded OL maturation and myelin gene expression (Claus Stolt et al. 2002; Qi et al. 2001). Moreover, upregulation of Cd82, Trf and Opalin, expressed in the terminal stages of the OL differentiation, was also detected (Goldman and Kuypers 2015; Tripathi et al. 2017). From the markers mentioned above, Opalin is considered the most terminal marker of OL differentiation, given that its expression starts at the onset of myelination, after the appearance of other myelin genes (Golan et al. 2008).

The relative amounts and timely expression of the different myelin components are key for the normal myelination and myelin remodelling throughout various stages of life (Zeller et al. 1985). Impaired axonal transport and accumulated organelles including mitochondria are observed in myelinated axons of PLP-overexpressing mice (Edgar et al. 2010; Ip et al. 2012). Moreover, myelin-related gene expression and myelin structure are altered in neurodegenerative disorders, such as MS and AD, where age is a major

risk factor (Allen et al. 2018; Gendelman et al. 1985). Therefore, in line with the results presented in this chapter, the myelin sheath formation and the terminal OL differentiation seem to be altered in the aged hypothalamus. To validate these results and to fully understand the precise dynamics of myelination throughout life, a detailed characterization of the hypothalamic myelination pattern across the mouse lifespan is presented in *Chapter 7*.

4.4.2. Downregulated genes are associated with the neuronal cytoskeleton, intracellular transport and axonal growth

The neuronal cytoskeleton has been proposed as a modulator of ageing in processes, and their dysfunction has been associated to multiple neurogenerative disorders, such as AD, Amyotrophic lateral sclerosis (ALS) and PD (Iqbal, Grundke-Iqbal, and Wisniewski 1986; Salvadores et al. 2017). The neuronal cytoskeleton is mainly composed of three elements: the actin microfilaments, the intermediate filaments, and the microtubules (Mclean and Robertson 2011). Considering the important functionality of cytoskeletal proteins in neurons, changes in the expression of some of the components could lead to important functional consequences in the normal functioning of the hypothalamic circuitry.

In neurons, neurofilaments (NF) represent the most abundant intermediate filaments are composed by NFL, NFM, and NFH subunits (for light, medium, and heavy, respectively) (Cooper and Hausman 2007); and the ratio of NF subunits is critical for proper filament formation (Mclean and Robertson 2011). However, the results presented in this chapter identified downregulation of Nfm, but not Nfl and Nfh, suggesting that the ratio of subunits changes with age. Alterations in the NF subunits ratio include the formation of neuronal cytoplasmic inclusions, disruption of intracellular transport and axonal degeneration (Szaro and Strong 2010; Xiao, McLean, and Robertson 2006).

In regard to other cytoskeleton components, this study reported an age-related decline in Tuba1a and Tubb2b. Tuba1a and Tubb2b encode α- and β- tubulin isoforms that, in turn, compose microtubules (Aiken et al. 2017; Gloster et al. 1994). Microtubules are required for the maintenance of neuronal shape and stability, axonal and neurite growth, and also form the cytoskeletal "highways" upon which trafficking proteins move (Conde and Cáceres 2009; Kapitein and Hoogenraad 2015). Thus, the reduced tubulin levels

during ageing would be detrimental to these processes and the normal functioning of the hypothalamic neuronal cell types. In fact, previous studies identified trafficking defects and synaptic impairment in mice lacking TUBA1A (Buscaglia et al. 2020), and mutations in TUBB2B have been shown to cause errors in axon guidance and axonal damage in humans (Jaglin et al. 2009). In addition, microtubules interact with a group of proteins known as microtubule-associated proteins (MAPs), that influence their stability and interactions with other cellular components (Kapitein and Hoogenraad, 2015). Downregulation of several MAP family members were detected in the aged hypothalamus, including Mapt (also known as tau). Tau is the major MAP in mature neurons and, its abnormal accumulation underlies the physiopathology of AD and other related tauopathies (Iqbal et al. 2010). Also, *in vitro* studies indicated that tau modulates the interaction of motor proteins with microtubules (Dixit et al. 2008)

Following the reduction in the expression of neuronal cytoskeleton components, downregulation of axon growth and guidance molecules was also observed. The CNS axons have a minimal natural ability to regenerate after injury (Huebner and Strittmatter 2009), and an age-dependent decline in axon growth potential has been recently reported across different brain regions of the adult mouse (Geoffroy et al. 2016). Thus, axonal growth and the regenerative capacity of hypothalamic neurons seem to decrease substantially with age, making them more vulnerable to negative environmental influences such as increased inflammation.

Since trafficking defects are associated with microtubule and MAP dysfunction, it is not surprising that the transcriptomic data also revealed downregulation of microtubule-associated motor proteins. These included several members of the kinesin and dynein families, essential for anterograde and retrograde axonal transport, respectively. (Howard 1996; Ligon et al. 2004). Previous observations also suggested that the axonal transport is reduced with age in rodent neurons (Brunetti et al. 1987; Geinisman, Bondareff, and Telser 1977; Li et al. 2003; Stromska and Ochs 1982; Takihara et al. 2015; Uchida et al. 2001). The neuronal function relies heavily on the intracellular transport of essential cargos within axons and dendrites; for example, mobilisation of mitochondria to regions of high energy demand, trafficking of mRNA and ribosomal subunits for local translation and delivery of signalling endosome-mediated delivery of survival factors (Mattedi and Vagnoni 2019). Thus, an interruption in the bi-directional

axonal transport could have detrimental consequences for a wide array of neuronal functions.

Besides their role in neuronal intracellular transport, dynein and kinesin proteins are also involved in the myelination process. Previous studies in zebrafish identified the kinesin Kif1b is essential for the anterograde transport of Mbp, and Kif1b mutants showed myelin defects (Lyons et al. 2009). Similar to Kif1b, the retrograde motor complex dynein/dynactin is also necessary for normal myelination in zebrafish and mammals (Herbert et al. 2017).

In summary, an increased the axonal atrophy with age could be expected in the neuronal tracts present in the hypothalamus due to the reduced expression of cytoskeletal proteins and axonal transport, as well as the decreased expression of genes involved in axonal growth and guidance.

4.4.3. General conclusions

Overall, the ageing hypothalamus alters its gene expression to generate a detrimental environment for neuronal and glial function, due to exacerbated inflammatory and neurotoxic responses. This inflammatory response may be mediated by microglia and astrocyte activation, although it is still unclear how their activation is initially triggered. It has been proposed that age-related changes in the hypothalamic circuitry, including inflammation, could underlie the decline in metabolism and energy homeostasis observed during ageing. However, it is possible that changes in neuronal connectivity, rather than altered expression of the hypothalamic neurotransmitters, are behind the energy shift reported in aged individuals. To sum up, the data presented here support previous studies and provides new clues about the functional changes in the hypothalamus with age.

5. Age-related inflammation in the hypothalamus

5.1. Introduction

Chronic inflammation is a hallmark of many age-related neurodegenerative diseases as well as metabolic syndrome disorders (López-Otín et al. 2013). Recent research indicates that hypothalamic inflammation participates in the development of metabolic syndrome components including obesity, hypertension and glucose intolerance, all these often associated to ageing (Purkayastha and Cai 2013; Purkayastha et al. 2011; Tang et al. 2015; Zhang et al. 2008). Consequently, in the last years, it has been proposed the role of hypothalamic inflammation in the ageing development and lifespan control (Tang and Cai 2013; Tang et al. 2015; Zhang et al. 2013). Within the hypothalamus, microglia and astrocytes mediate immunity and inflammation by producing and releasing a range of inflammatory mediators (Clarke et al. 2018; Colombo and Farina 2016; Valdearcos et al. 2017).

In their immune role, microglia respond to abnormalities in the parenchyma by triggering their activation which, in turn, can influence astrocyte activation and recruitment through their secretory profile (Liddelow and Barres 2017). Microglial activation can be broadly categorised in two main types: M1 and M2, with different roles in neuroinflammation. The M1 phenotype exerts neurotoxic proprieties, and M2 has a phagocytic/ neuroprotective role in the inflammatory response (von Bernhardi et al. 2015; Gordon 2003; Tang and Le 2016). Some specific markers have been described for both subtypes: The M1 phenotype expresses markers such as CD86 (cluster of differentiation 86), and also produce inflammatory cytokines such as TNFα, IL-1β, and IL-6; the M2 phenotype typical markers are Arg1 (Arginase I), CD206 (cluster of differentiation 206), and Chi3l3 (chitinase-like 3) (Tang and Le 2016).

Immunohistochemistry studies for pro-inflammatory factors revealed that microglial cells in the mediobasal hypothalamus (MBH) increase with age, concomitantly with TNFα expression and activation of NF-κB. TNFα is a pro-inflammatory cytokine that leads to the activation of transcription factor NF-κB. NF-κB is the master switch and central regulator of inflammation, immune response and cell death (Hayden and Ghosh 2008). In the MBH, TNFα upregulation and NF-κB activation were mostly limited to microglia in middle-aged animals but became prevalent across the MBH in aged animals, producing a neurotoxic effect to neighbouring cells, including neurons. Interestingly, suppression

of MBH microglial NF-κB activation prevented the microglia increase, exerting an anti-ageing effect (Zhang et al. 2013).

Together with microglia, astrocytes provide immune defence to the CNS by producing and releasing a range of inflammatory mediators (Pekny and Pekna 2014; Sofroniew 2009). In addition to immune defence, growing evidence has highlighted a critical role of astrocytes in orchestrating hypothalamic functions by participating in synaptic plasticity, metabolic and trophic support to neurons, and nutrient sensing (Fuente-Martín *et al.*, 2012; Fuente-Martín *et al.*, 2016). Interestingly, hypothalamic astrocytes increase in number and can acquire a pro-inflammatory phenotype with age (Santos, Bobermin, et al. 2018; Santos, Roppa, et al. 2018; Wang et al. 2006). Also, TGFβ (transforming growth factor beta) production and release by astrocytes are increased in the hypothalamus of aged mice, and TGFβ excess was further shown to induce hypothalamic RNA stress response and activation of NF-κB in MBH neurons (Yan et al. 2014). Besides their pro-inflammatory phenotype, aged astrocytes also exhibit changes in their neurochemical properties, including changes in the regulation of glutamatergic homeostasis, glutathione biosynthesis, glucose metabolism and amino acid profile

Activation of inflammatory pathways in hypothalamic cells seems to be restricted to astrocytes and microglia cells during early ageing but becomes prevalent in neurons with age. Thus, inhibition of NF-κB activation in hypothalamic neurons increased mice lifespan while decreasing ageing-related changes in histological markers such as muscle size and skin thickness. In contrast, the over-activation of NF-κB by the constitutive activation of IKKβ (inhibitor of nuclear factor kappa-B kinase subunit beta) showed the opposite effects (Zhang et al. 2013).

The results mentioned above provide information on hypothalamic glia-neuron crosstalk by which microglia and astrocyte inflammatory activation leads to cytokine release and neuronal inflammation in the aged hypothalamus. However, the effects of ageing in the microglial and astrocytic populations are still in the early stages of characterisation. Consequently, in this chapter, to further understand ageing-related changes in hypothalamic microglia and astrocyte populations were characterised across the murine lifespan.

5.2. Aims

Given that age-related neuroinflammation is mediated by microglia and astrocytes, both populations were investigated in the ageing hypothalamus. To do so, immunohistochemistry for Iba1 or GFAP was employed to visualise microglia or astrocytes in the hypothalamus, respectively. Additionally, expression of specific glial markers was detected via western blot. The aims included:

- Determine age-related changes in distribution, number and/or morphology in hypothalamic microglia and astrocytes.
- Evaluate the activation state of microglial cells in the hypothalamus during the ageing process.

5.3. Results

5.3.1. Hypothalamic microglia

5.3.1.1. Microglia morphology, but not distribution, is altered in the mediobasal hypothalamus during ageing

Given that microglia mediate immunity and inflammation in the hypothalamus, the distribution and morphology of microglial cells were investigated in the ageing hypothalamus. To do so, Immunohistochemistry for Iba1 was performed on brain sections from young adult (n = 4), middle-aged (n = 4) and old mice (n = 3); between bregma positions -1.22 to -2.3 mm. The Iba1+ cells were uniformly scattered in the hypothalamic parenchyma, and no-regional enrichment was observed across the three age groups (Figure 5.1, A – F).

In the Arc nucleus, young animals presented Iba1+ cells with ramified morphology, small, rounded cell body and slender, elongated processes (Figure 5.1, A', A'', D, D''). In the middle-aged, the ramified phenotype was also predominant, although some cells appeared to possess slightly shorter process compared to their young counterparts (Figure 5.1, B', B'', E', E''). Contrarily, old animals presented Iba1+ cells with dystrophic morphology, enlarged cell bodies and shorter and thickened processes (Figure 5.1, C',

C'', F', F'') . In addition to the Arc, microglial cells present in the ME suffered the same phenotypical changes with age (Figure 5.2). Microglia morphology has been widely used to identify activation state, with ramified morphology typically observed in the resting state and ameboid morphology present in the activated form. Therefore, results suggest that hypothalamic microglia undergo progressive morphological changes from resting to activated phenotype with age.

5.3.1.2. Microglia number and size is not affected by ageing in the mediobasal hypothalamus

Since microglial morphology adopted an activated phenotype with age, the quantity and size of microglia were measured to see the effects on inflammation with age. Immunohistochemistry for Iba1 was performed in brain sections from young adult (n = 4), middle-aged (n = 4) and old mice (n = 3). Quantifications were performed in a minimum of 12 sections per animal, ranging from bregma -1.22 to -2.06 mm (see *section 2.8.3* for more details). Results showed no statistical differences in the microglial number (Figure 5.3, A) and size (Figure 5.3, B) in the hypothalamus with age.

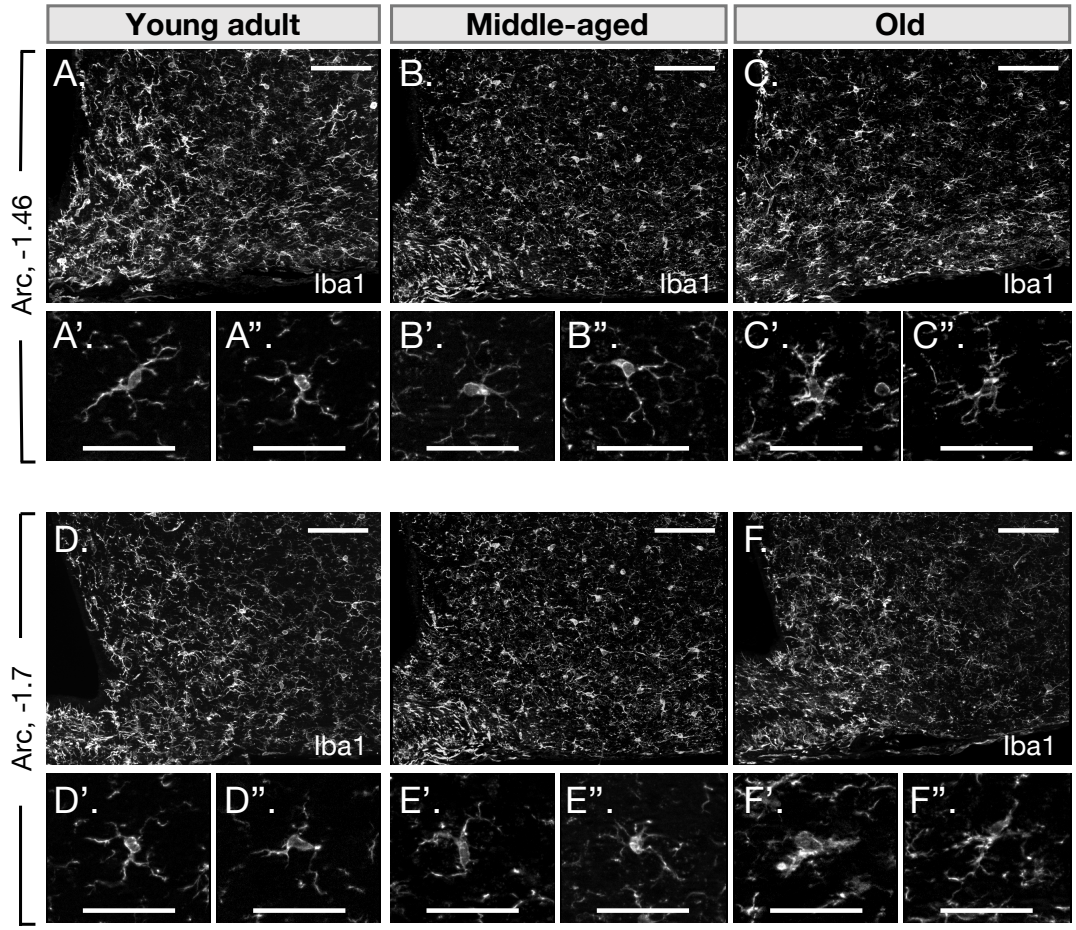


Figure 5.1. Iba1+ cells show an age-related switch from resting to activated phenotype in the MBH, featuring ameboid morphology and short and thickened processes. (A – C) Representative images of the Arc, bregma -1.46 mm, stained for Iba1, from young-adult (A), middle-aged (B) and old (C) animals. High power magnification of microglial cells for young (A', A''), middle-aged (B', B'') and old (C', C') showed age-related morphological features of microglial activation, such as ameboid cell body and short and thickened processes. (D, E, F) Representative images of the Arc, bregma -1.7 mm stained for Iba1, from young-adult (D), middle-aged (E) and old (F) animals. High power magnification of astrocytes for young (D', D''), middle-aged (E', E'') and old (F', F) showed an age-related change in microglial morphology. Bregma positions are approximated and indicated. Scale bar (A, B, C, D, E, F) 50 µm, (A'-A'', B'-B'', C'-C'', D', D'', E', E'', F' F'') 25 µm.

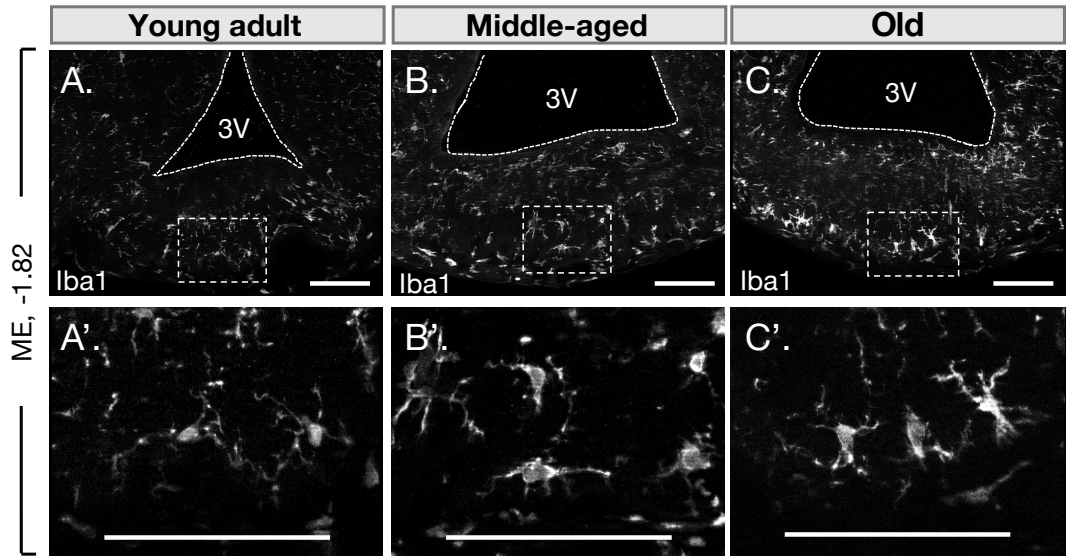


Figure 5.2. Iba1+ cells show an age-related switch from resting to reactive microglia morphology in the ME. (A, B, C) Representative images of the Arc, bregma -1.46 mm, stained for Iba1, from young adult (A), middle-aged (B) and old (C) animals. Dashed box shows a higher power of microglial cells for young (A', A''), middle-aged (B', B'') and old (C', C'). Bregma position is approximated. Dashed lines outline the 3V. Scale bar 50 µm. 3V – third ventricle. ME – median eminence. Scale bar 50 µm

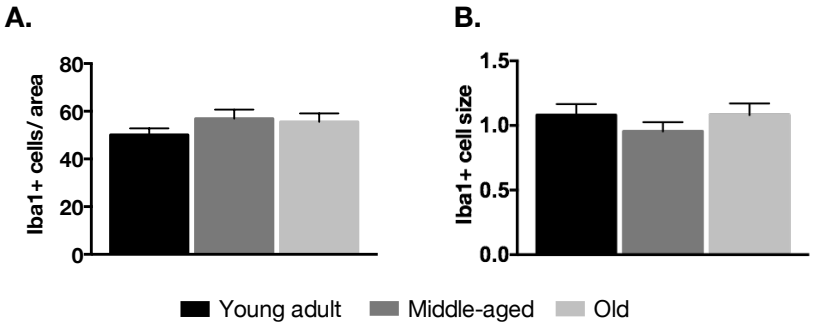


Figure 5.3. The number of Iba1+ microglia and size remain stable during ageing in the MBH. (A) Number of Iba1+ cells within the rectangle defined as Iba1+ per area in young adult (black column), middle-aged (dark grey) and old animals (light grey). (B) Iba1+ cell size in young adult (black column), middle-aged (dark grey) and old animals (light grey). Results show no changes in Iba1+ cell number and size with age. Data is represented as mean ±SEM, and a minimum of 4 animals per age group was quantified. All significance testing was performed using one-way ANOVA followed by Tukey post-hoc test (*p<0.05).

5.3.1.3. Expression of pro-inflammatory cytokine TNFα and the M1 microglia marker CD86 is increased with age in the hypothalamus

To further investigate and confirm the activation state in aged microglia, different markers of microglia activation were assessed. Microglial activation in the CNS is heterogeneous, which can broadly categorise in two main types: M1 and M2, with different roles in neuroinflammation. Some specific markers have been described for both subtypes: The M1 neurotoxic phenotype expresses specific markers such as CD86, and also produce inflammatory cytokines such as TNFα; the M2 neuroprotective phenotype typical markers include Arg1 and CD206. In this study, the protein levels of TNFα, CD86, CD206 and Arg1 were assessed via western blot in hypothalamic protein extracts from young adult, middle-aged and old mice (CD86, CD206, Arg1 $n = 3$ for each age group, $n = 6$ for TNFα).

Expression of TNFα and CD86 expression was detected in the hypothalamus. Anti-TNFα antibody detected two strong bands of 17 KDa and 27 KDa (**Figure 5.4, A**), which both increased with age as highlighted by densitometry analysis (**Figure 5.4, B**). In turn, anti-CD86 antibody detected multiple isoforms with a molecular weight between 52 KDa to 70 KDa (**Figure 5.4, C**). Densitometry analysis of the 70 KDa showed no differences with age (**Figure 5.4, D**). However, the 52 KDa isoform was highly expressed in aged animals, with the middle-aged group showing a bigger increased compared to young animals (**Figure 5.4, E**). Contrarily, expression of CD206 and Arg1 was not detected in the hypothalamus in any age group (results not shown). The data suggests that microglial cells in the hypothalamus may adopt a neurotoxic, pro-inflammatory (M1) activated phenotype with age, although M2 activation cannot be ruled out completely.

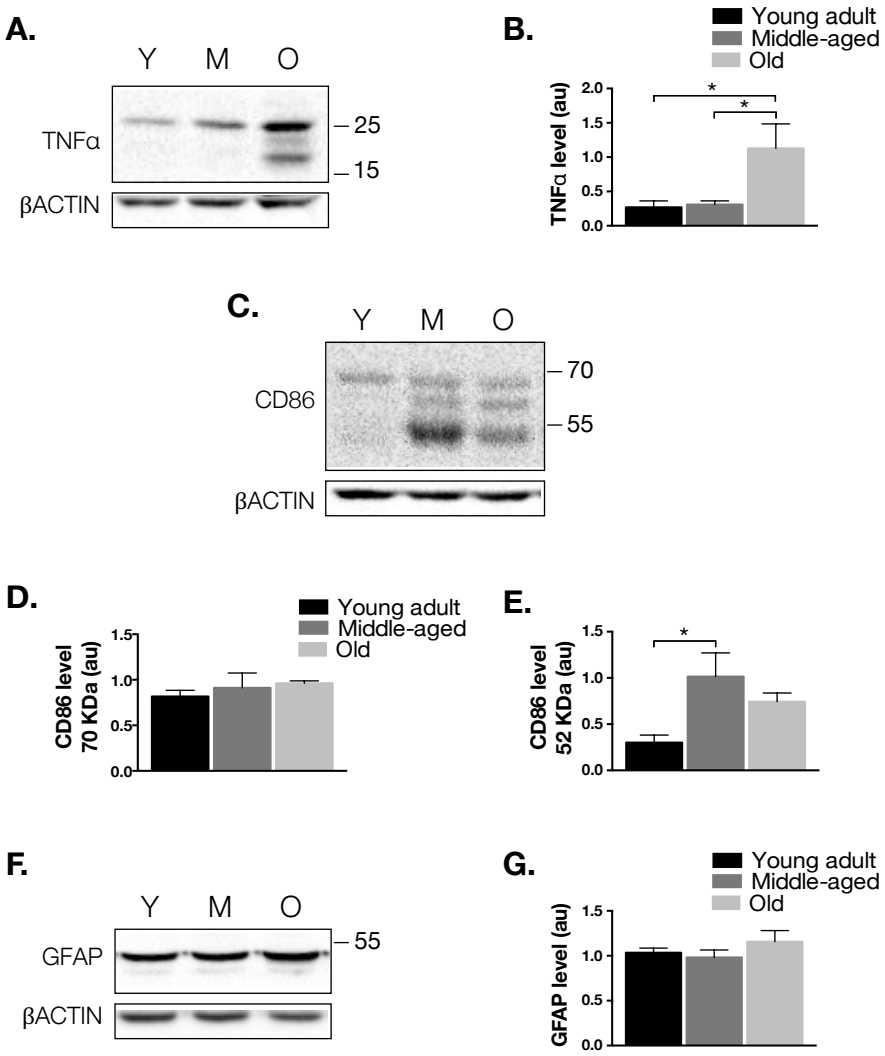


Figure 5.4. The protein levels of TNFα, CD86 and GFAP in the hypothalamus. (A) Representative image of immunoblot for TNFα and the loading control β-ACTIN. TNFα runs at an approximated MW of 27 KDa (transmembrane domain). Additional bands corresponding to the soluble form of 17 KDa can only be observed in the old animal lane. (B) Comparison of densitometric analysis of TNFα 27 KDa level relative to the loading control, in young adult (black), middle-aged (dark grey) and old (light grey) animals, show a significant increase with age. (C) Representative image of immunoblot for CD86 and the loading control β-ACTIN. CD86 antibody detects multiple bands with MW between 52 to 70 KDa, approximated. (D) Comparison of densitometric analysis of TNFα 70 KDa level relative to the loading control, in young adult (black), middle-aged (dark grey) and old (light grey) animals, show no age-related differences. (E) Comparison of densitometric analysis of TNFα 52 KDa level relative to the loading control, show a significant increase with age. (F) Representative image of immunoblot for GFAP and the loading control β-ACTIN. GFAP antibody detects a double band running at an approximated MW of 51 KDa. (G) Comparison of densitometric analysis of GFAP level relative to the loading control, in young adult (black), middle-aged (dark grey) and old (light grey) animals, show no age-related differences. Y – young. M – middle-aged. O – old. Numbers indicate bands of the molecular weight marker used as a size standard (KDa). Data is represented as mean ±SEM. A total of 6 animals per age group were quantified and for GFAP and TNFα and 3 animals per age group for CD86. All significance testing was performed using one-way ANOVA followed by Tukey post-hoc test (*$p<0.05$).

5.3.2. Hypothalamic astrocytes

5.3.2.1. Age-related effect on astrocytes distribution and morphology in the mediobasal hypothalamus

Given that astrocytes release pro-inflammatory cytokines in the hypothalamus with age, the effects of ageing in the hypothalamic astrocytes were investigated. The distribution and morphology of the cells were investigated in the hypothalamus using immunohistochemistry for GFAP on brain sections from young adult (n = 4), middle-aged (n = 4) and old mice (n = 3); between bregma positions -1.22 to -2.3 mm.

Results show GFAP+ cells in the parenchymal region flanking the 3V. Also, some GFAP+ cells were observed in the ependymal layer of the 3V but were not taking into consideration due to their tanycytic identity (**Figure 5.5**, **A – F**) (Goodman and Hajihosseini 2015). Therefore, the parenchymal GFAP+ astrocytes distribution was analysed in the three groups of age. Results showed that GFAP+ astrocytes were not homogeneously distributed in the hypothalamus, and an age-dependent regional enrichment was observed. In young animals, the Arc nucleus shows higher GFAP expression, compared to the rest of the hypothalamic nuclei (**Figure 5.5**, **A**, **D**). In addition to the Arc nucleus, high GFAP signal was also observed in the VMN of middle-aged animals (**Figure 5.5**, **B**, **E**). Finally, in old animals, high GFAP levels were additionally detected in the DMN (**Figure 5.5**, **C**, **F**). These results suggest that GFAP signal increase initially in the Arc-ME in middle-aged, which becomes more prevalent in the rest of nuclei of old animals.

Concomitantly to their distribution, astrocyte morphology also presented age-related changes. Results showed that GFAP+ astrocytes from young animals presented short and thin processes, representative of the resting morphology (**Figure 5.5**, **A'**, **A''**, **D'**, **D''**). The astrocytes in the middle-aged group presented longer processes than young animals. However, variability was observed between animals (**Figure 5.5**, **B'**, **B''**, **E'**, **E''**). Finally, in old mice, astrocytes appeared bigger with longer and thicker processes – features of the reactive phenotype (**Figure 5.5**, **C'**, **C''**, **F'**, **F''**). Moreover, in the aged hypothalamus, astrocytes seem to form an interconnected network thought their projections, termed syncytium (Scemes and Spray 2003). Both, the reactive morphology present in aged astrocytes and the syncytium formation, are signs of age-related

astrogliosis. In conclusion, hypothalamic astrocytes seem to undergo morphological changes towards a reactive phenotype with age, suggesting that the remodelling of the GFAP astrocytes is a dynamic process that develops gradually with age.

5.3.2.2. Astrocytes number and size is increased during ageing in the mediobasal hypothalamus but GFAP total hypothalamic levels remain stable

After exploring the astrocytes distribution and morphology, the next step was to investigate if the astrocyte number and/or size was also affected with age. To do so, immunohistochemistry for GFAP was performed in brain sections from young adult (n = 4), middle-aged (n = 4) and old mice (n = 3). Quantifications were performed in a minimum of 12 sections per animal, ranging from bregma -1.22 mm to -2.06 mm. Results showed that GFAP+ astrocytes show a significant increase in number (**Figure 5.6**, **A**) and size (**Figure 5.6**, **B**) in the hypothalamus with age.

The total levels of GFAP in the hypothalamus were assessed via western blot in protein extracts from young adult, middle-aged and old mice (n = 6 for each age group). Anti-GFAP antibody detected a strong band of 51 KDa, the predicted molecular weight of the full-length protein. Additionally, the antibody detected a weaker band of 45 KDa (**Figure 5.4**, **A**). Densitometry analysis of the full-length protein showed no significant differences in GFAP expression with age, although a trend towards increase can be observed (**Figure 5.4**, **B**).

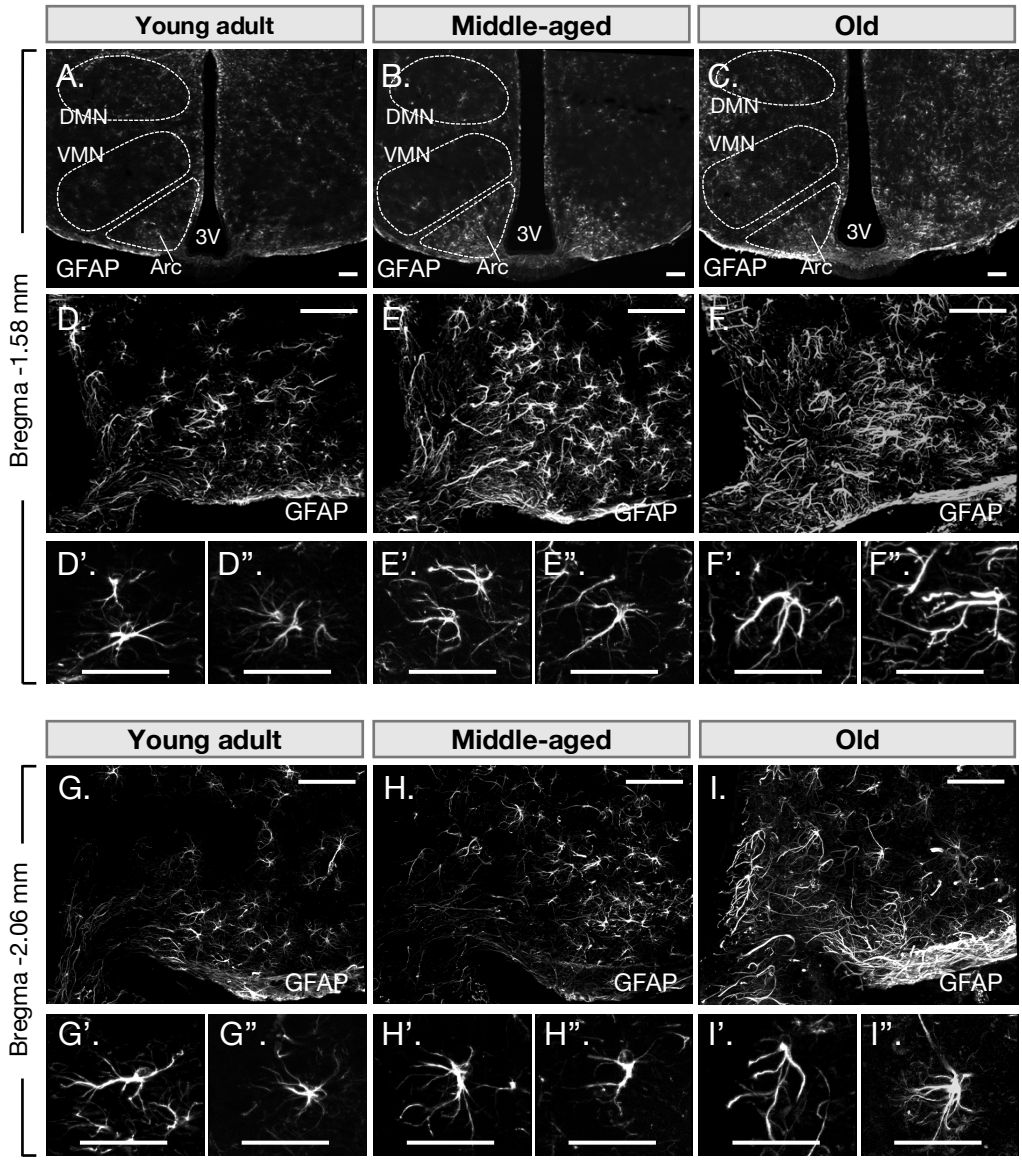


Figure 5.5. GFAP expression in the MBH increases with age and GFAP+ astrocytes display reactive morphology features. (A, B, C) Representative images of the MBH stained for GFAP (White), from young-adult (A), middle-aged (B) and old (C) animals. GFAP expression increase can be first observed in the Arc nucleus of middle-aged animals and later expands to the VMN and DMN in old animals. (D-F) Representative images of the Arc at bregma -1.58 mm stained for GFAP (White), from young-adult (D), middle-aged (E) and old (F) animals. High power magnification of astrocytes for young (D', D''), middle-aged (E', E'') and old (F', F'') show an age-related change in astrocyte morphology. (G-I) Representative images of the Arc at bregma -2.06 mm stained for GFAP (White), from young-adult (G), middle-aged (H) and old (I) animals. High power magnification of astrocytes for young (G', G''), middle-aged (H', H'') and old (I', I'') show an age-related change in astrocyte morphology. Bregma positions are approximated and indicated. Dashed lines outline different hypothalamic nuclei. DMN – dorsomedial nucleus. VMN – ventromedial nucleus. Arc – arcuate nucleus. Scale bar (A, B, C, D, E, F, G, H, I) 50 µm. Scale bar (D', D'', E', E'', F' F'', G', G'', H', H'', I, I'') 25 µm.

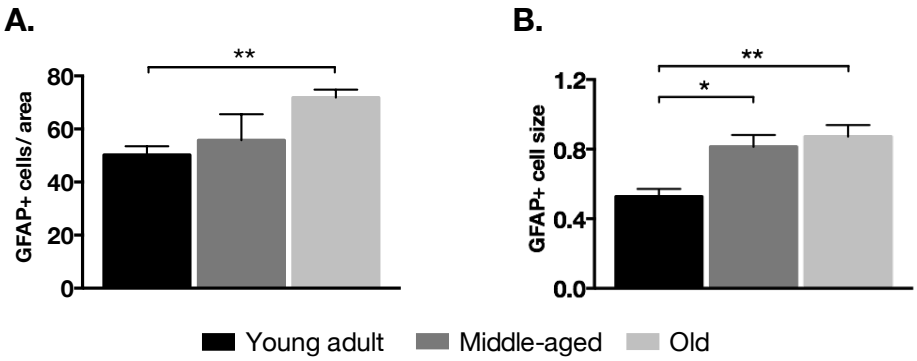


Figure 5.6. The number and size of hypothalamic GFAP+ astrocytes increase with age. (A) Number of GAFP+ cells in the Arc nucleus of young adult (black column), middle-aged (dark grey) and old animals (light grey). Data show a significant age-related increase in GFAP+ cells density. (B) GFAP+ cell size in young adult (black column), middle-aged (dark grey) and old animals (light grey). Data show a significant age-related increase in GFAP+ cell size. Data is represented as mean ±SEM, and a minimum of 3 animals per age group were quantified. All significance testing was performed using one-way ANOVA followed by Tukey post-hoc test (*$p<0.05$, **$p<0.01$).

5.4. Discussion

Growing evidence indicate that hypothalamic inflammation participates in the development of systemic ageing. Inflammation in the CNS is linked to glial remodelling and activation; however, the effects of ageing in the hypothalamic microglial and astrocytic populations are still in the early stages of characterisation. In this study, immunohistochemistry for the microglial marker Iba1 and astrocytic marker GFAP was employed. In aged microglia, Iba1+ cells acquired an enlarged cell body with ameboid morphology, typical of the activated phenotype. In turn, aged GFAP+ astrocytes increased in number and presented bigger and thicker ramifications, forming a syncytium of interconnected cells. The changes observed suggest that reactive gliosis involving both microglial and astrocyte populations develop gradually in the hypothalamus with age. In addition, western blot analysis suggest that aged hypothalamic microglia adopt a neurotoxic/pro-inflammatory (M1) activated phenotype accompanied by the release of the pro-inflammatory cytokine TNFα.

In summary, results in this chapter suggest that inflammation, mediated by activation of microglia and astrocytes, and release of pro-inflammatory factors is a feature of the aged hypothalamus. This detrimental environment may affect various neural cell types and disrupt key hypothalamic signalling pathways essential for energy balance, glucose homeostasis and blood pressure.

5.4.1. Age-related changes in microglia and astrocytes morphology, number and distribution in the hypothalamus

5.4.1.1. Age-related changes in hypothalamic microglia

To characterise the hypothalamic microglia and any age-related changes, immunohistochemistry for Iba1 was performed in brain sections of young, middle-aged and old animals. Iba1 is a calcium-binding protein expressed in the cytoplasm of microglia, but not present in other neural cell types (Ahmed et al. 2007). Microglial cells (Iba1+) were observed uniformly distributed in the hypothalamic parenchyma in non-overlapping territories, and their density was stable across the three age groups. The results presented in this chapter contradict with previous reports that observed

hypothalamic microglia increase during ageing (Yin et al. 2018; Zhang et al. 2013) and in diet-induced inflammation (Baufeld et al. 2016; Valdearcos et al. 2017). The differences observed between the present study and Zhang *et al*. could be due to the different counting methods employed, and the bregma positions analysed. Interestingly, in other regions of the brain, including the hippocampus, the total number of microglia in the brain is not increased during healthy ageing (Long et al. 1998; VanGuilder et al. 2011b).

In contrast, an age-related microglial phenotypic change towards the activated state is observed. Furthermore, the microglia activation seems to occur gradually as the animal ages, and the first signs of microglial activation are observed in middle-aged animals. Consistent with the data present here, studies in mammals observed microglia with activated morphology in different areas across the healthy aged CNS (von Bernhardi et al. 2015; Cerbai et al. 2012; Conde and Streit 2006; Lee et al. 2017). In the hypothalamus, size change in conjunction with altered morphology in microglia was observed after HFD feeding (Berkseth et al. 2014; Thaler et al. 2012); however, this is the first study providing evidence of morphological changes in hypothalamic microglia during ageing.

5.4.1.2. Age-related changes in hypothalamic astrocytes

To characterise age-related changes in hypothalamic astrocytes, immunohistochemistry for GFAP was performed in brain sections of young, middle-aged and old animals.

In agreement with previous reports, an age-related increase in hypothalamic astrocytes (GFAP+) density was detected (Santos, Bobermin, et al. 2018; Wang et al. 2006). The increase in GFAP+ number could be originated from different sources: (1) mature astrocytes that reactivate the cell cycle and proliferate (Bardehle et al. 2013; Gadea, Schinelli, and Gallo 2008); (2) parenchymal progenitors (Li et al. 2012); (3) ependymal/tanycytes cell progenitors (Chaker et al. 2016; Robins, Stewart, et al. 2013). Importantly, although GFAP is a widely used marker for astrocytes, presents some caveats: (1) is not expressed by all astrocytes (Sofroniew and Vinters 2010); (2) as an intermediate filament protein of the cytoskeleton, immunolabelling for GFAP only represents about 15% of the total astrocyte volume (Bushong et al. 2002).

The results presented in this chapter indicate that aged astrocytes acquire a reactive phenotype, with general hypertrophy of the cell body that increase in size with age. Reactive astrocytes lose the ability to carry out their normal functions and could induce the death of neurons and OLs (Clarke et al. 2018; Liddelow et al. 2017). In the hypothalamus, and consistent with their reactive phenotype, aged astrocytes exhibit functional changes (Santos, Roppa, et al. 2018) and up-regulate the expression of neuroinflammatory genes (Boisvert et al. 2018). Following the reactive phenotype, in the hypothalamus, aged GFAP+ astrocytes seem to overlap neighbouring astrocyte processes, ultimately forming a network or syncytium (Scemes and Spray 2003). These phenotypical changes are first observed in middle-aged animals and become more prevalent in the old group, suggesting that the activation and remodelling of the hypothalamic astrocytes is a dynamic process that develops with age.

Taken together, cellular hypertrophy, loss of individual astrocyte domains and increase in astrocyte number suggests that reactive astrogliosis occurs in the ageing hypothalamus (Sofroniew 2009; Sofroniew and Vinters 2010). Chronic astrogliosis can also impair the neuronal functions, limit axonal regeneration, and decrease neurogenesis in aged mice (Menet et al. 2003; Pekny and Nilsson 2005). In the rodent hypothalamus, reactive astrogliosis has been associated with a reduction in POMC neurons (Thaler et al. 2012), essential for energy balance and metabolism regulation. Therefore, the gradual and chronic inflammatory response observed in the hypothalamus during ageing may be detrimental for the correct functioning of neuronal and glial populations present in the area and may underlie some of the metabolic defects observed during ageing.

5.4.2. Pro-inflammatory markers

Increasing evidence indicates that microglial activation is heterogeneous, which can be categorised into two main opposite types: M1 and M2. The M1 phenotype exerts neurotoxic proprieties, and M2 has a phagocytic/neuroprotective role in the inflammatory response. (von Bernhardi et al. 2015; Gordon 2003; Tang and Le 2016). In addition to neurotoxicity, the M1 microglia is required for the activation of adjacent astrocytes by releasing factors such as TNFα and interleukins (Liddelow et al. 2017).

Protein levels of M1- and M2-type microglial markers were assessed via western blot in total hypothalamic extracts. Increased expression of the M1 marker CD86 and the pro-inflammatory cytokine TNFα in the hypothalamus was detected with age (**Figure 5.4**). Interestingly, in the hypothalamus, previous studies showed that upregulation of TNFα is restricted to the microglial cells during early ageing, but became prevalent across the neighbouring cells in 22-months-old mice (Zhang et al. 2013)

Contrarily, expression of the M2 markers CD206 and Arg1 was not detected in the hypothalamus. The failure to detect both M2 phenotype markers could be due to multiple reasons: (1) the antibodies employed are not suitable for western blot analysis; (2) CD206 and Arg1 are not expressed in the hypothalamus, or their expression falls below the detection threshold of the method. In support of (2), the transcriptomic experiment in *Chapter 3* failed to detect age-related changes in the classical M2 markers Cd206, FIZZ1 and Chil3l3 (results not shown).

In conclusion, results suggest that hypothalamic microglia acquire a neurotoxic phenotype (M1) during ageing, accompanied by the expression of pro-inflammatory cytokines such as TNFα, that may contribute to the age-related astrocyte activation in the hypothalamus.

5.4.3. Activators of hypothalamic age-related inflammation

This chapter showed that hypothalamic microglia and astrocytes undergo gradual changes towards a pro-inflammatory phenotype with age. The gradual changes observed in both populations indicate an escalation of inflammation during age progression, although it is still unclear how microglial and astrocyte activation is initially triggered. Different mechanisms could contribute, including mitochondrial dysfunction (Drougard et al. 2015; Tang et al. 2015), loss of autophagy (Kaushik et al. 2011, 2012) and intracellular RNA stress response (Yan et al. 2014). It is also possible that tissue changes during ageing development, such as adiposity and immune system dysfunction, secondarily contribute to hypothalamic inflammation (Deleidi, Jäggle, and Rubino 2015; Kuk et al. 2009).

Regardless of insufficient understanding as to its primary causes, age-related hypothalamic inflammation mediated by astrocytes and microglia disrupts key hypothalamic signalling pathways and mediate the development of the metabolic syndrome (Purkayastha and Cai 2013; Purkayastha et al. 2011; Tang et al. 2015; Zhang et al. 2008). Therefore, strategies that target age-related inflammation in the hypothalamus, like suppression of NF-κB reported by Zhang *et al*., may delay the development of age-related conditions and increase the lifespan.

6. Proliferation studies in the ageing hypothalamus

6.1. Introduction

Adult neural stem cell niches harbouring neural stem and progenitor cells able to differentiate through different glial and/or neuronal lineages have been identified in several species. Specifically, the canonical NSC niches include the SVZ, lining the walls of the lateral ventricles, and the SGZ of the hippocampus (Barnea and Nottebohm 1994; Eriksson et al. 1998; E. Gould et al. 1999; Gould et al. 1998; Kempermann, Kuhn, and Gage 1997). Besides the well-known neurogenic niches, the hypothalamus has emerged as a novel niche for postnatal and adult neurogenesis and gliogenesis.

In the hypothalamus, two main potential neurogenic sites have been identified, the ependymal cells lining the 3V and the parenchyma; although, the exact location and identity of the proliferative niche is object of strong controversy. The studies reporting neurogenesis in the postnatal and adult rodent hypothalamus, using both BrdU incorporation studies and cell lineage analysis using inducible Cre lines, are summarized in Table 6.1 and 6.2, respectively.

On the one hand, previous studies in rodents indicated that tanycytes lining the ependymal layer of the 3V harbours NSC that can give rise to both neurons (NeuN+, HuC/D+) and glial cells (GFAP+) and may self-renew by generating other tanycytes (Niels Haan et al. 2013; D. A. Lee et al. 2012; Robins, Stewart, et al. 2013). The new-born neurons originated from tanycytes integrate into the appetite and energy balance regulating nuclei, as lineage tracing studies revealed (Lee and Blackshaw 2012; Xu et al. 2005). Proliferation of tanycytes lining the 3V is frequently observed in postnatal ages, but it seems to decrease in adults (Niels Haan et al. 2013; D. A. Lee et al. 2012). In fact, young mice (2 – 3 months old) treated with the thymidine analogue BrdU, which effectively labels new-born cells in the hypothalamus, showed BrdU+ throughout the hypothalamic parenchyma but very few BrdU+ cells lining the 3V, (Djogo et al. 2016; Kokoeva et al. 2007; Robins, Trudel, et al. 2013; Zhang et al. 2017). In addition to decreased BrdU labelling, a recent study reported loss of NSC in the 3V with age, by visualizing proteins strongly expressed in NCS, such as Sox2, Nestin, Bmi1, and Msh1 (Goodman and Hajihosseini 2015; Zhang et al. 2017). Interestingly, one study using lineage tracing of Nestin expressing tanycytes showed that neurogenesis occurs even in mice as old as 9 months, and that deletion of IGF-1 receptors in Nestin expressing cells and their progeny enhances neurogenesis (Chaker et al. 2016).

Several studies have suggested that the neural stem/progenitor cells reside within the hypothalamic parenchyma. Parenchymal new-born cells have been identified throughout many hypothalamic regions, including the Arc, VMN, DMN nuclei and the ME (Niels Haan et al. 2013; Kokoeva et al. 2007; D. A. Lee et al. 2012; Matsuzaki et al. 2009; Robins, Trudel, et al. 2013). Besides, some studies identified a regional enrichment, with the ME containing 15-fold more BrdU+ cells than the rest of the hypothalamus (Djogo et al. 2016; Kokoeva et al. 2007; D. A. Lee et al. 2012). Analysis of the adult hypothalamus seven weeks post BrdU treatment revealed that parenchymal BrdU+ cells give rise to OLs (oligodendrocytes, CC1+) and, to a lesser extent, neurons (DCX+, HuC/D+, Tuj1+) (Kokoeva et al. 2007). Moreover, recent studies identified the majority of parenchymal dividing cells as NG2-glia in the adult mice (Robins, Villemain, et al. 2013). Hypothalamic NG2-glia is highly regenerative and can undergo multiple self-renewing divisions (Robins, Trudel, et al. 2013). Independent studies using the mitotic blocker arabinofuranosyl cytosine (AraC) reached the same conclusion and identified the majority of cycling cells in the adult hypothalamus as NG2-glia, being the microglia the second proliferative population in this region (Djogo et al. 2016).

The NG2-glia comprise an OL progenitor population that has an important role in myelination post-development (McTigue and Tripathi 2008), with a dense distribution throughout the CNS and a high potency to regenerate. In the hypothalamus, NG2-glia has been found enwrapping LepR processes from arcuate neurons, with the AraC NG2-glia induced ablation causing degeneration of LepR processes in the ME. Thus, proliferative NG2-glia has a critical role in the normal functioning of the hypothalamic neuronal circuits, and a short-term NG2 elimination can cause permanent neuronal processes impairment (Djogo et al. 2016).

In conclusion, many studies have reported cell proliferation and generation of new cells of neuronal and glial lineage in the postnatal and adult hypothalamus, with the majority of proliferative cells identified as NG2-glia; however, the characterisation of this niche is still in its early stages compared to the canonical SGZ and SVZ niches. Consequently, in this chapter, I decided to characterise the proliferating niche within the hypothalamus and define any changes during normal ageing. A decline in hypothalamic neurogenesis could disrupt key signalling pathways, contributing to functional changes in the hypothalamus with age.

6.2. Aims

Given that cycling cells with neuro- and gliogenic properties have been identified in the postnatal and adult hypothalamus, the next step was to investigate and characterise the dynamics of the hypothalamic stem cell niche during normal ageing:

- Defining the identity and spatial location of the proliferative cells in the adult hypothalamus.
- Characterisation of the number and distribution of the proliferative cells within the adult hypothalamus.
- Defining the changes in the number and distribution of the different proliferative subpopulations during ageing.

Table 6.1. Studies reporting neurogenesis in postnatal and adult hypothalamus of rodents using the mitotic marker BrdU

Location proliferative cells	Identity cycling cells and progeny	Species	Age BrdU treatment	BrdU labelling paradigm	Treatment	Treatment effect on proliferation	Reference
Periventricular zone Parenchyma	GFAP+, NeuN+	Rt	P20-40	IP 1x daily for 3 d (chased for 17 d)	Gonadal hormones	Up	Ahmed *et al.*, 2008
Parenchyma (Arc)	HuC/D+	Ms	P70-84	ICV osmotic pump for 9 d (acute harverst or chased for 34 d)	-	-	Bless *et al.*, 2014, 2016
Parenchyma (Arc-ME)	Iba1+, NG2+	Ms	2-3 m	ICV osmotic pump for 7 d (acute harvest) IP 6x within 30 h (acute harvest)	AraC ICV 6 d	First down, Up within following 14 d	Djogo *et al.*, 2016
Parencyma	NeuN+, POMC+	Ms	P50	ICV for 3 days (chased for 3 d, 7 d, 17 d or 21 d)	HFD 3-7 d	Up for 3 d, down after	Gouaze *et al.*, 2013
Ependymal layer Parenchyma (Most)	BGal+ ($Fgf10^{nLacZ}$), NeuN+	Ms	P28-32 P60-70	DW for 15 d (acute harvest)	-	-	Haan *et al.*, 2013
Ependymal layer Parenchyma	Prss56+ (Prss56-Cre), Sox2+, Vim+	Ms	3 m	ICV osmotic pump for 7d (chased for 42 d)	FGF ICV 7 d	Up	Jourdon *et al.*, 2016
Parenchyma (Arc-ME)	APC (CC1)+, DCX+,HuC/D+, NPY+, POMC+, Tuj1+	Ms	P60	ICV osmotic pump 7d (chased for 3 d, 7 d, 21 d, 35 d or 49 d)	CNTF ICV 7 d	Up	Kokoeva, Yin and Flier, 2005
Parenchyma	APC (CC1)+, DCX+, HuC/D+, Tuj1+	Ms	P60	ICV osmotic pump for 7 d (chased for 2 d, 9 d, 22 d or 42 d)	CNTF ICV 7 d	Up	Kokoeva, Yin and Flier, 2007
Ependymal layer (ME) Parenchyma	HuC/D+, Nestin+	Ms	P10	IP 2x daily for 9 d (chased for 26 d)	HFD 30 d	Up	Lee *et al.*, 2012
Parenchymal (Arc-ME)	HuC/D+, Nestin+	Ms	P42	IP 2x daily for 9 d (chased for 26 d)	HFD, High protein diet 30 d	Up (ME) Down (Arc)	Lee *et al.*, 2014
Parenchyma (Arc-ME)	NeuN+, NPY+, POMC+, RIP+ (OL), S100β+	Ms	4-8 m	ICV for 7 days (chased 10 d or 30 d)	Chronic HFD	Down	Li, Tang and Cai, 2013
Periventricular zone Parenchyma	APC+, DCX+, NeuN+, SYN+	Rt	P50	IP 1x daily for 5 d (chased for 6 d, 13 d, 23 d, 33 d, 43 d or 53 d)	Heat exposure	Up	Matsuzaki *et al.*, 2009

Table 6.1(cont). Studies reporting neurogenesis in postnatal and adult hypothalamus of rodents using the mitotic marker BrdU

Location proliferative cells	Identity cycling cells and progeny	Species	Age BrdU treatment	BrdU labelling paradigm	Treatment	Treatment effect on proliferation	Reference
Ependymal layer (P50) Parenchyma	APC+, DCX+, GFAP+, NeuN+	Rt	P50 10–11 m 22–25 m	IP 1x daily for 5 d (chased 40-50 d)	Heat exposure	Up	Matsuzaki *et al.*, 2015
Ependymal layer Parenchyma	GAD67+, Glut+, NeuN+	Rt	P45	IP 1x daily for 5 d (chased for 6 d or 40 d)	Heat exposure	Up	Matsuzaki *et al.*, 2017
Parenchyma	HuC/D+, NeuN+	Ms	P60	ICV osmotic pump for 7 d (chased for 28 d)	HFD 28 d	Down	McNay *et al.*, 2012
Ependymal layer Parenchyma	GFAP+, NeuN+	Rt	P28	ICV osmotic pump for 28 d (chased for 10 d)	-	-	Mohr, Don Carlos and Sisk, 2017
Ependymal layer Parenchyma	HuC/D+, GFAP+, Nestin+, NeuN+, Vim+	Rt	2.5-3 m	IP 1x daily for 5 d (chased for 2 d or 21 d)	Voluntary exercise	Up	Niwa *et al.*, 2016
Parenchyma	MAP2+, Tuj1+	Rt	Adult (not specified)	ICV osmotic pump for 7 d (chased for 28 d)	BDNF ICV 12 d	Up	Pencea *et al.*, 2001
Periventricular zone Parenchyma	GFAP+, Iba1+, NeuN+, S100β+	Rt	P60	IP 2x daily for 3 d (chased for 18 d)	IGF ICV 7 d	Up	Perez-Martin *et al.*, 2010
Parenchyma (Arc)	ACTH (POMC)+, AgRP+	Ms	3 m	ICV osmotic pump for 42 d (acute harvest)	Degeneration Agrp-neurons	Up	Pierce and Xu, 2010
Ependymal layer Parenchyma	GFAP+ (α-tanycytes), Vim+	Ms	P45-60	ICV osmotic pump for 7 d (acute harverst or chased for 42 d)	FGF2 ICV 7 d	Up	Robins, Stewart, *et al.*, 2013
Parenchyma (Arc-ME)	HuC/D+, NG2+, Sox2+	Ms	P60	ICV osmotic pump for 7 d (acute harverst or chased for 30 d) DW 28 d (acute harvest)	-	-	Robins, Trudel, *et al.*, 2013
Periventricular zone Parenchyma	GFAP+, HuC/D+, Orexin A+	Rt	P60	IP every 2 h for 48 h (chased for 3 d, 7 d, 28 d or 56 d)	bFGF ICV single dose	Up	Xu *et al.*, 2005
Ependymal layer (only 2-4 m)	-	Ms	2-4 m 11-16 m >22 m	IP 2x daily for 7 days (acute harvest)	-	-	Zhang *et al.*, 2017

Table 6.2. Studies reporting neurogenesis in postnatal and adult hypothalamus using lineage tracing mouse models

Identity and location progenitor cells	Identity progeny	Progeny location	Transgenic line	Reporter	Age of induction	Tamoxifen induction paradigm	Reference
Ependymal layer (ependymocytes and α/β-tanycytes)	GFAP+, GLAST+, Glut-R+, GABA-R+, NeuN+, NPY+, GHRH	Arc, VMN, DMN, LH, PH	Nestin-CreER^{T2}	R26^{tdTom}	3 m	IP 2x daily for 5 d (chased for 1 m, 6 m or 13 m)	Chaker *et al.,* 2016
Parenchymal OPC	BrdU+, Sox2+	Arc, ME,VMN	NG2-CreER^{T2}	R26^{tdTom}	2-3 m	IP 2x daily for 5 d (chased for 18 d)	Djogo *et al.,* 2016
Ependymal layer (β-tanycytes)	NeuN+	Arc, VMN	Fgf10-CreER^{T2}	R26^{LacZ}	P60	IP 1x daily for 7 d, then in diet for 10 d (chased for up to 84 d)	Haan *et al.,* 2013
Ependymal layer (α2-tanycytes)	BrdU+, Huc/D+, Sox2+	Arc, VMN, DMN	Prss56-Cre	R26^{tdTom}	3 m	-	Jourdon *et al.,* 2016
Ependymal layer (β2-tanycytes)	DCX+, HuC/D+	Arc, ME	Nestin-CreER^{T2}	R26^{YFP}	P4.5	IP single dose (chased for 30 d)	Lee *et al.,* 2012
Parenchymal progenitor cell	NeuN+, NPY+, POMC+, S100β+, RIP+	Arc, VMN	Sox2 promoter-Cre lentivirus	R26^{YFP}	3 m	Delivery not stated (chased for 80 d)	Li, Tang and Cai, 2013
Ependymal layer (α-tanycytes)	DCX+, GFAP+, NeuN+	Arc, VMN, DMN	Glast-CreER^{T2}	R26^{LacZ}	P56–P84	IP 1x daily for 10 d (chased for 42 d and 270 d)	Robins, Stewart, *et al.,* 2013
Parenchymal OPC	APC (CC1)+, BrdU+, CD13+ (pericytes), HuC/D+, NeuN+, Sox2+	Arc, ME, VMN	NG2-CreER^{T2}	R26^{tdTom}	P56–P84	IP 2x daily for 5 days (chased for 2 d, 14 d, 20 d and 60 d)	Robins, Trudel, *et al.,* 2013

Abbreviations used: AraC - arabinofuranosyl cytosine. Arc - Arcuate nucleus. d - day. DMN - dorsomedial nucleus. DW - drinking water. HFD - high fat diet. ICV - intracerebroventricular. IGF - insulin-like growth factor. IP - intraperitoneal. FGF - fibroblast growth factor. LH - lateral hypothalamus. m - month. ME - median eminence. PH - posterior hypothalamus. VMN - ventromedial hypothalamus.

6.3. Results

6.3.1. Characterisation of proliferating cells in the hypothalamus

Given that previous research suggested that the adult hypothalamus contains a proliferative stem cell/progenitor niche, the next step was to characterise the hypothalamic proliferative niche during ageing. To do so, immunohistochemical detection of BrdU was performed in hypothalamic brain sections from young adult (3 – 7 months old), middle-aged (10 – 14) and old mice (18 – 24; *n* = 4 animals per age group). As a result, BrdU+ cells were detected in the hypothalamic parenchyma between bregma positions -1.22 mm to -2.46 mm (Figure 6.1). The dividing cells were observed in the Arc-ME, with minor contributions to the VMN. The BrdU+ cells were present in the same hypothalamic regions across the three age groups (Figure 6.2). As a general observation, the BrdU+ cells were found in close proximity to each other, in some cases resembling to cell pairs, suggesting that they recently underwent cell division. As a control, the corpus callosum was immunolabelled for BrdU, and positive cells were observed in the region (Figure S 4).

Additionally, very few BrdU+ were observed in the ependymal layer of the 3V, where the neurogenic tanycytes are located. The low number of proliferating cells lining the 3V suggested that ependymal cells, including tanycytes, are a non-dividing or a very slow dividing population in the adult brain.

In summary, the BrdU+ cells spatial distribution is maintained during ageing, being specially concentrated in the Arc-ME region.

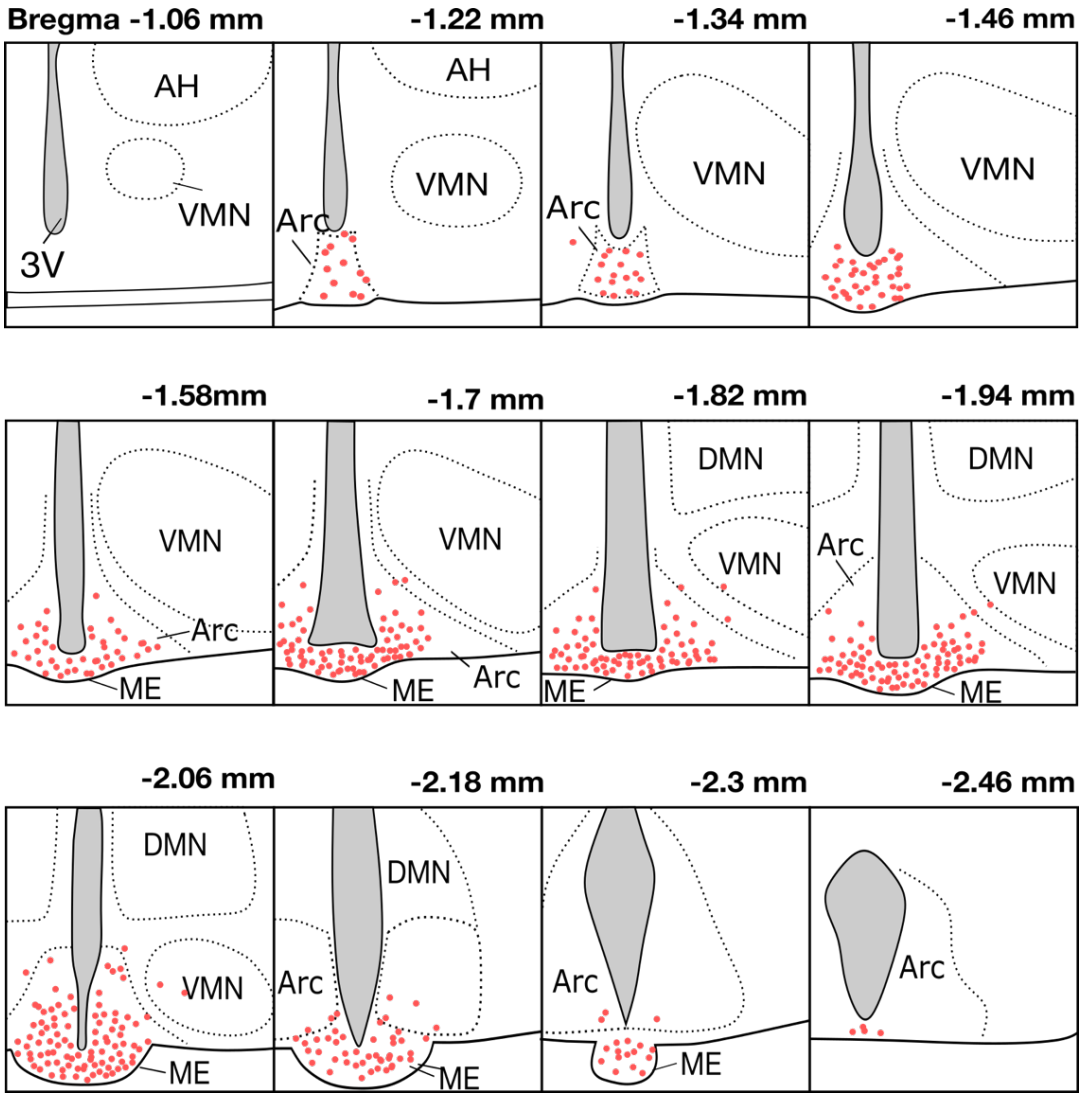


Figure 6.1. Representative distribution and density of BrdU+ cells in the hypothalamus. Approximated bregma positions are indicated. Red circles indicate position of the groups of BrdU+ cells. Results obtained from young, middle-aged and old animals, with a minimum of 3 animals analysed per age group, were employed for the diagram design. Dashed lines outline the 3V and the limits of the different hypothalamic nuclei. 3V – third ventricle. VMN – Ventromedial nucleus. AH – Anterior hypothalamus. Arc – Arcuate nucleus. ME – Median eminence.

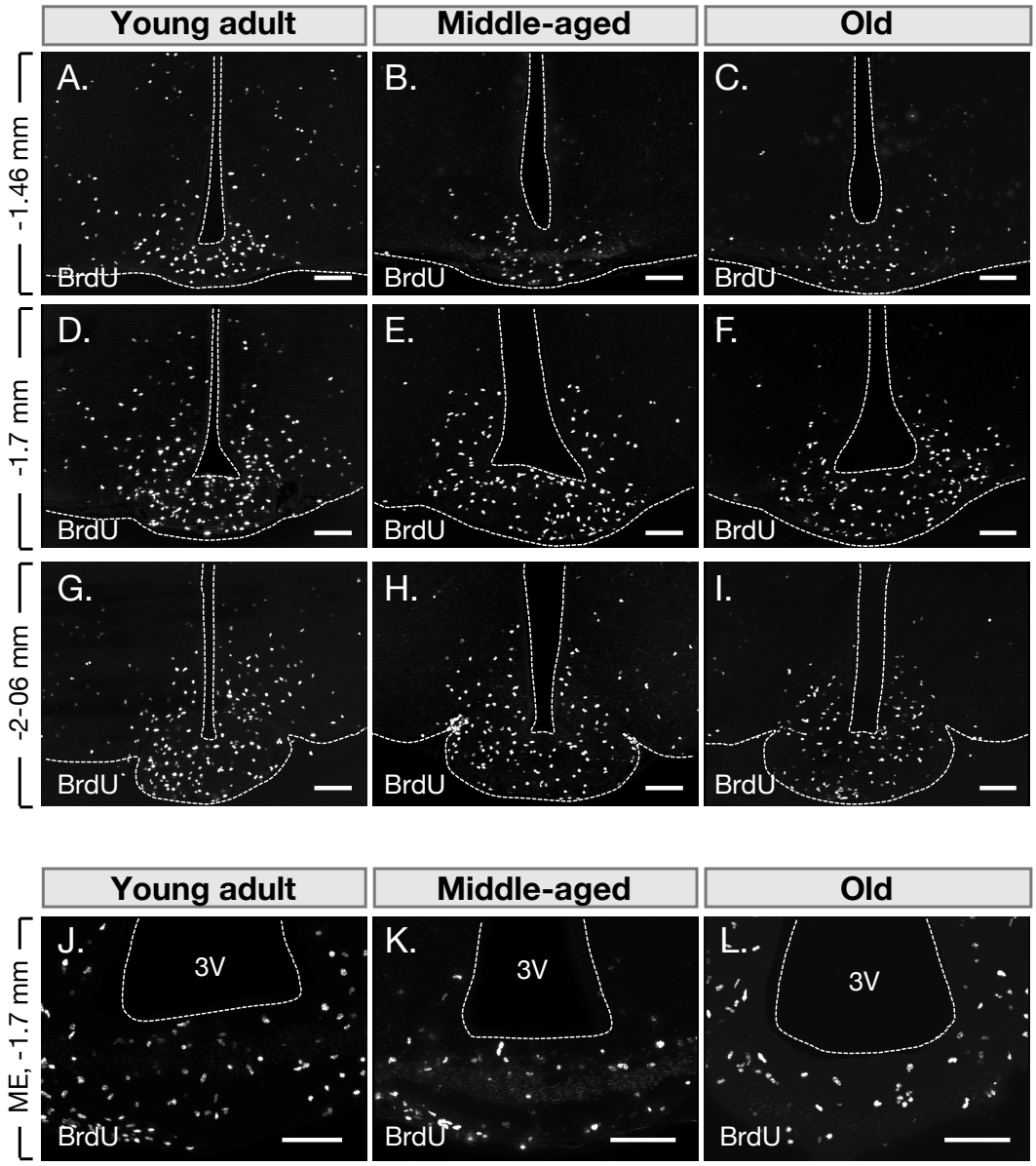


Figure 6.2. Proliferative cells within the hypothalamus were mainly observed in the Arc-ME in young, middle-aged and old animals. Mice were treated for 15 d with BrdU in drinking water to mark proliferative cells. (A, B, C) Representative images of the MBH at bregma -1.46 mm stained for BrdU (white), from young-adult (A), middle-aged (B) and old (C) animals. (D, E, F) Representative images of the MBH at bregma -1.7 mm stained for BrdU (white), from young-adult (D), middle-aged (E) and old (F) animals. (G, H, I) Representative images of the MBH at bregma -2.06 mm stained for BrdU (white), from young-adult (G), middle-aged (H) and old (I) animals. (J, K, L) Representative images of the ME at bregma -1.7 mm stained for BrdU (white), from young-adult (J), middle-aged (K) and old (L) animals. Bregma positions are approximated and indicated. Experiments performed in young, middle-aged and old animals with 4 animals analysed per age group. Dashed lines outline the 3V and the limits of the hypothalamus. Scale bar 50 µm. 3V – third ventricle.

6.3.2. Characterisation of the hypothalamic proliferative cells

Next, the identity of the proliferating cells identified in the Arc-ME regions was confirmed. To do so, BrdU co-localisation with different cell type makers was assessed in the hypothalamus via immunohistochemistry in young, middle-aged and old mice (minimum of *n* = 3 animals per age group). The specific cell type markers included Olig2 (OL lineage), Iba1 (microglia), GFAP (astrocytes, α-tanycytes), NeuN (neurons) and S100β (astrocytes, OLs and α-tanycytes). In addition, transgenic mice selectively expressing GFP in the Pomc and Npy neuronal populations (Pomc-GFP and Npy-GFP, respectively) were used to determine BrdU+ cells identity.

Results showed that BrdU+ cells co-localised with Olig2 and Iba1, specific makers of OLs and microglia respectively (**Figure 6.3** and **6.4**). Co-localisation of BrdU with S100β was also observed, but the BrdU+/S100β+ cells were always Olig2+, confirming that they belong to the OL lineage (**Figure 6.5**).

Moreover, in old animals but not in young adults, a very small number of GFAP+ astrocytes co-localised with BrdU staining (**Figure 6.6**). However, BrdU co-localisation with NeuN, Pomc and Npy (**Figure S 5**) was not observed, suggesting the neurogenic capacity of the proliferative cells is very low or totally absent in the adult hypothalamus. Also, co-localisation of BrdU with LacZ ($Fgf10^{nLacZ}$), that labels Fgf10-expressing tanycytes and derived cells, was not detected in any age group (**Figure S 6**).

In addition to co-localisation studies, immunohistochemistry for the neuronal new-born marker doublecortin (DCX), a transient marker for early postmitotic neurons, was assessed in the hypothalamus of young, middle-aged and old animals. This antibody was previously validated by former members of the group in postnatal tissue with positive staining. In the adult hypothalamus, however, staining for DCX was not detected.

In conclusion, results confirmed that the hypothalamic parenchymal stem cells are capable of gliogenesis and give rise to new-born microglial cells and OLs throughout the lifespan.

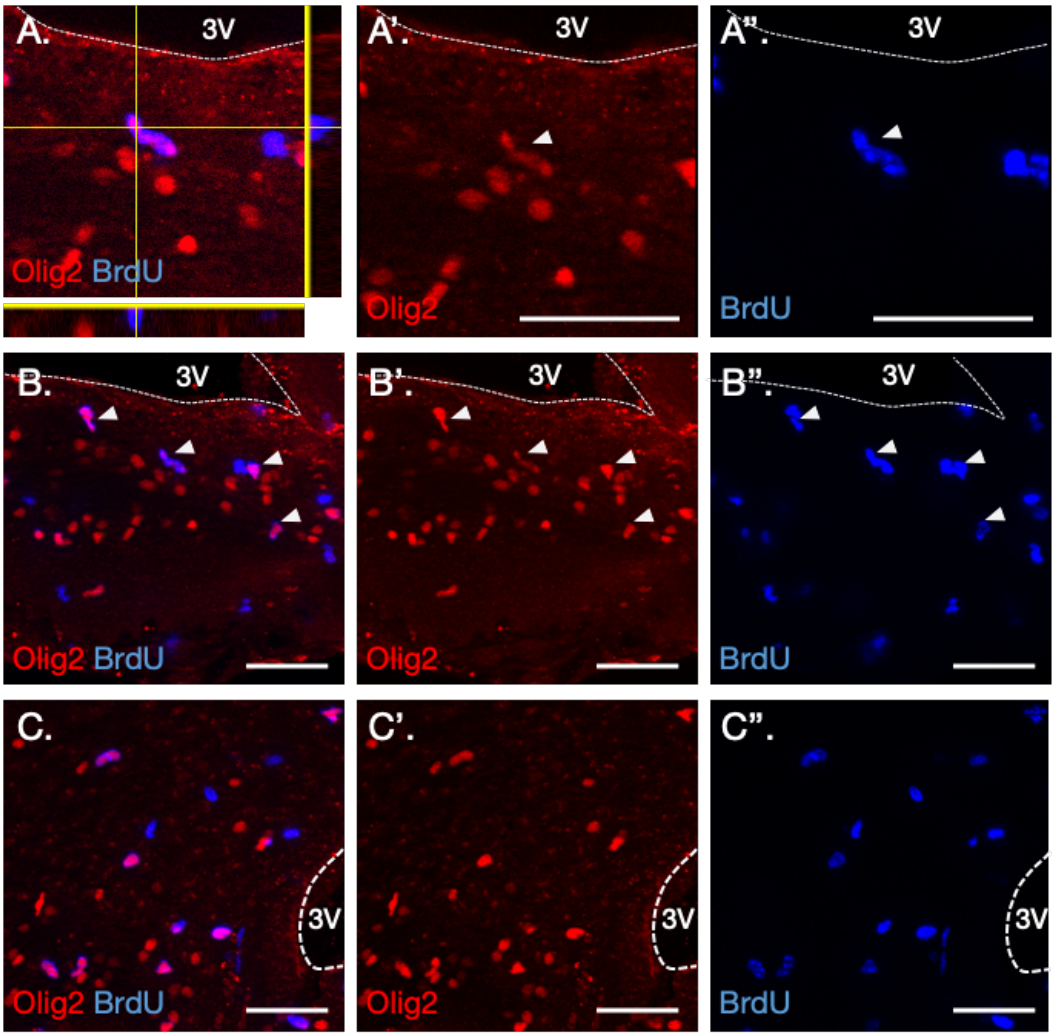


Figure 6.3. BrdU+ cells co-localise with the oligodendrocyte marker Olig2 in the Arc-ME. (A – A'') Orthogonal view z-stack images of BrdU (blue) and Olig2 (red) immunostaining show co-localisation (white arrow). Immunostaining for Olig2 (red) and BrdU (blue) show co-localisation in the ME (B – B'') and the Arc nucleus (C' – C''). The pictures shown were obtained from young animal tissue (n = 3). Dashed lines outline the borders of the 3V. Experiments performed in young, middle-aged and old animals with a minimum of 3 animals analysed per age group. Scale bar 25 µm. 3V – third ventricle.

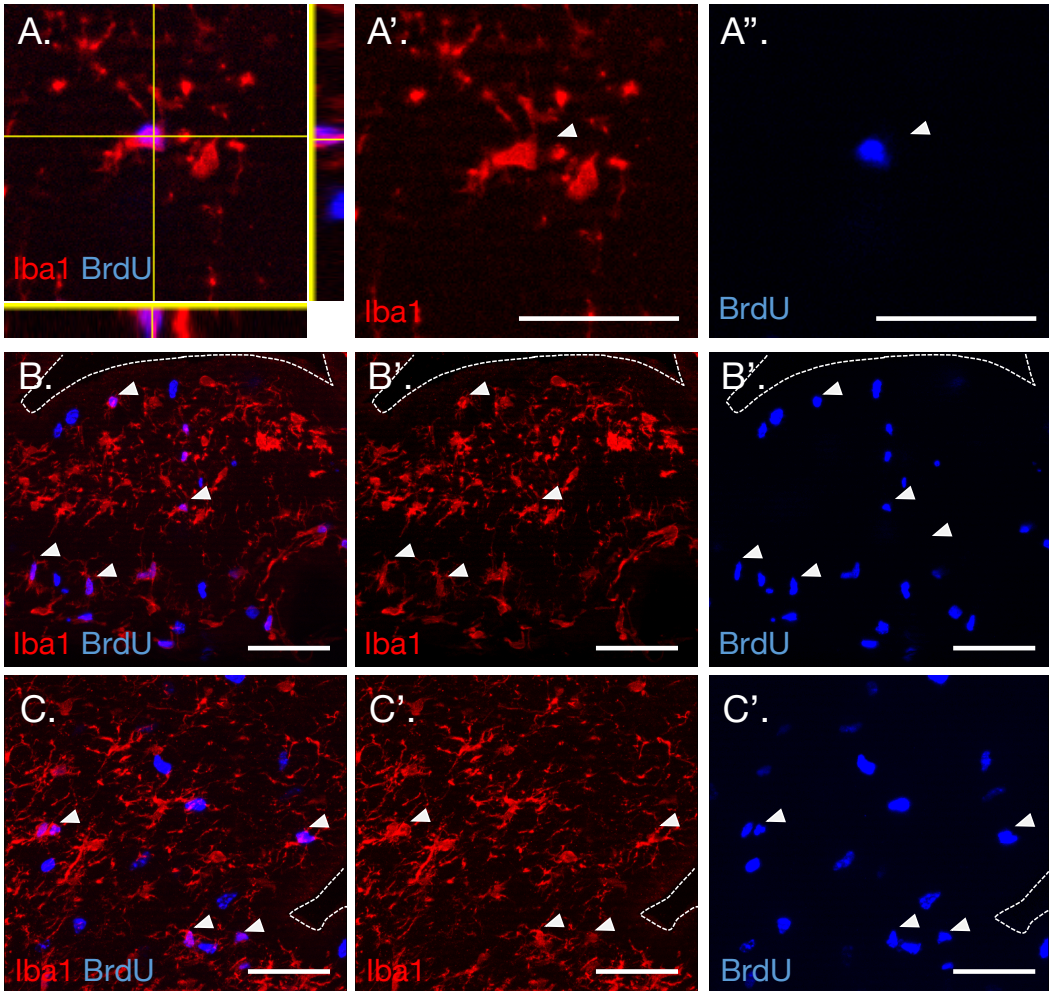


Figure 6.4. BrdU+ cells co-localise with the microglia marker Iba1 in the Arc-ME. (A – A'') Orthogonal view z-stack images of BrdU (blue) and Iba1 (red) immunostaining show co-localisation (white arrow) in the hypothalamus. Immunostaining for Iba1 (red) and BrdU (blue) show co-localisation in the ME (B – B''') and the Arc nucleus (C – C''). Experiments performed in young, middle-aged and old animals with a minimum of 3 animals analysed per age group. The pictures shown were obtained from young animal tissue. Dashed lines outline the borders of the 3V. Scale bar 25 µm.

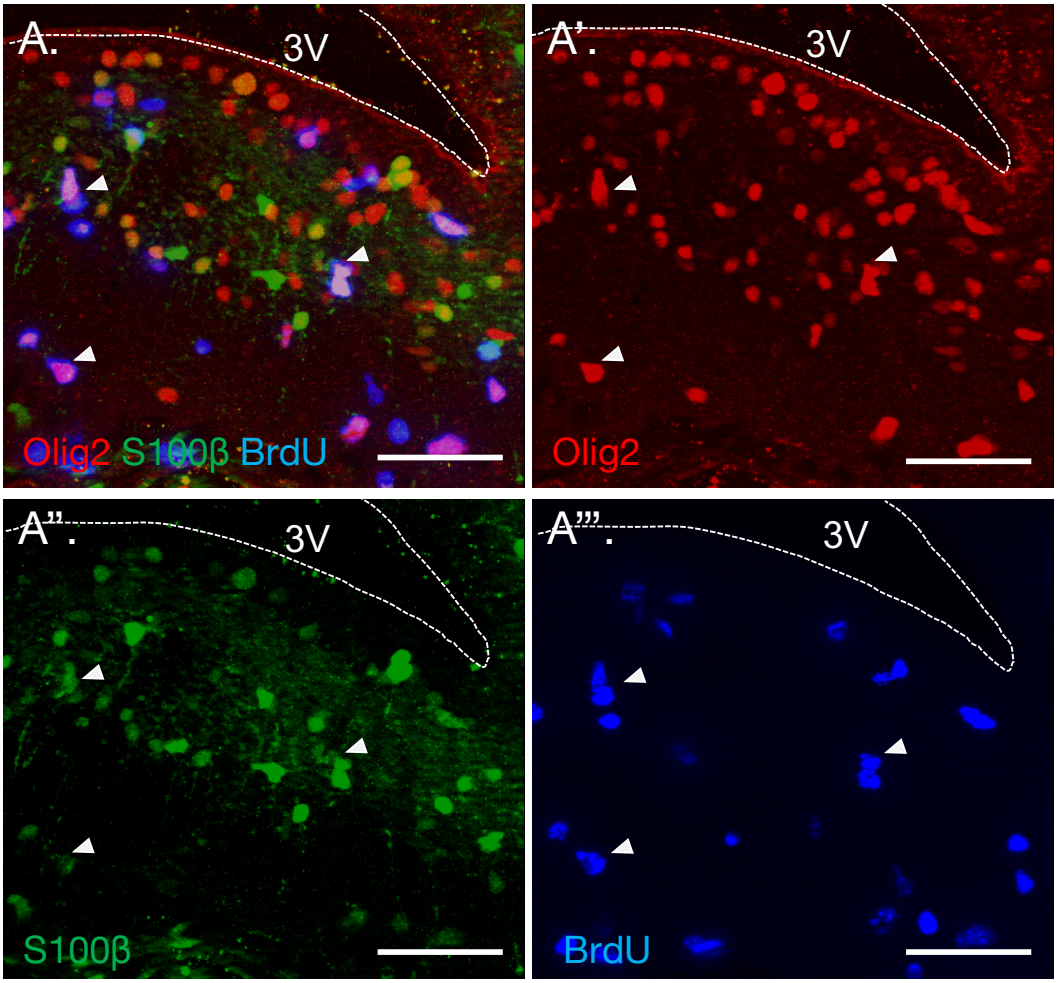


Figure 6.5. BrdU+ cells co-localise with S100β+/Olig2+ cells but not S100β+/Olig2- cells. (A – A''') Immunostaining for S100β (green), Olig2 (red) and BrdU (blue) in bregma -1.7 mm, approximated. Dashed lines outline the borders of the 3V. White arrows signal S100β, Olig2 and BrdU co-localisation. No S100β+/Olig2- cells were found to co-localise with BrdU staining. The pictures shown were obtained from young animal tissue. Experiments performed in young, middle-aged and old animals with a minimum of 3 animals analysed per age group. Scale bar 25μm. 3V – third ventricle.

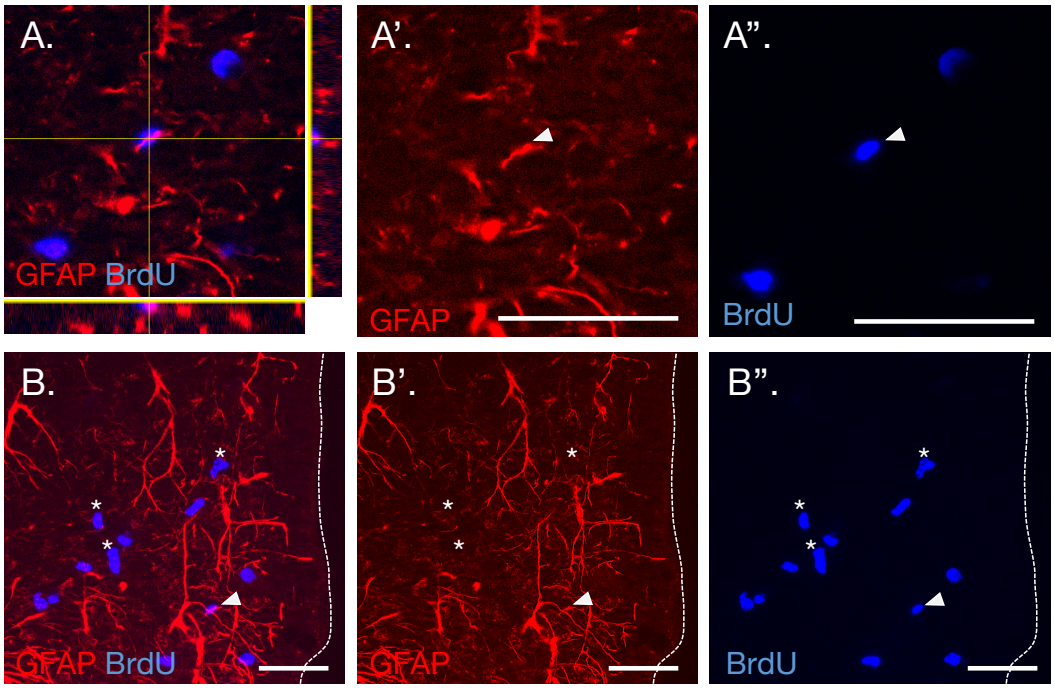


Figure 6.6. GFAP+ astrocytes that co-localise with BrdU+ cells are rare and only detected in aged animals. (A – A'') Orthogonal view z-stack images of GFAP (Red) and BrdU+ (Blue) immunostaining show co-localisation (white arrow) in the hypothalamus of old mice. (B – B'''). Immunostaining for GFAP (Red) and BrdU (Blue) show a BrdU+/GFAP+ cell in the Arc (white arrow) and BrdU+/GFAP- cells (asterisks) in the Arc nucleus. Experiments performed in young, middle-aged and old animals with a minimum of 3 animals analysed per age group. Dashed lines outline the borders of the 3V. Pictures were obtained from old animal tissue. Scale bar 25 µm.

6.3.3. Age-related effects in number and rostro-caudal distribution of hypothalamic proliferative cells

After confirming the identity of the new-born cells, I wished to investigate whether the proliferation rate of these parenchymal proliferative cells suffered any age-related changes. To do so, the total number BrdU+ cells in the Arc-ME were quantified in young adult, middle-aged, and old mice (n = 4 for each age group); between bregma positions -1.22 to -2.46 mm. Statistical analysis of young adult 151 ± 26.2 (n = 4), middle-aged 116 ± 25.2 (n = 4), and old mice 107 ± 30.4 (n = 3) results showed a significant age-related decrease in the total number of BrdU+ cells (**Figure 6.7 – A**).

The rostro-caudal distribution of BrdU+ cells was also investigated and BrdU+ cells were mainly observed between bregma -1.58 mm and -2.06 mm (**Figure 6.1 and Figure 6.7 – F**). BrdU+ distribution showed that the age-related decline in proliferation was homogenous throughout the Arc-ME region (**Figure 6.7 – F**).

6.3.4. Age-related effects in the identity of the new-born cells derived from hypothalamic proliferative cells

Given that the results showed an age-related decline in the number of dividing cells, I wanted to investigate the effect on the production of new-born OLs (Olig2+) and microglia (Iba1+). To do so, the number (**Figure 6.7 – B** and **C**) and percentage (**Figure 6.7 D** and **E**) of BrdU cells that co-localised with Olig2 or Iba1 was obtained for each age group (n = 4). Results showed that 45 – 50% BrdU+ co-localised with Olig2+ and 30 – 35% with Iba1+, making the Olig2+ population the most proliferating in the hypothalamus. Importantly, these percentages were not affected with age, indicating that the age-related decline in proliferation affects both populations equally. Additionally, rostro-caudal distribution of BrdU+/Olig2+ and BrdU+/Iba1+ cells was also investigated, with no changes observed with age (**Figure 6.7 – G** and **– H**).

In conclusion, proliferation in the hypothalamus suffers an age-related decline, affecting both the microglia and OLs. However, Olig2+ and Iba1+ are not the only proliferating populations, as they together accounted only for the 80% of the proliferating cells. Thus, the identity of the remaining proliferating cells remains unidentified, although their identity

could be inferred due to their localisation. As an example, some BrdU+ could be pericytes and/or endothelial cells as they are found in close contact with blood vessels, requiring confirmation by the use of specific markers for both populations.

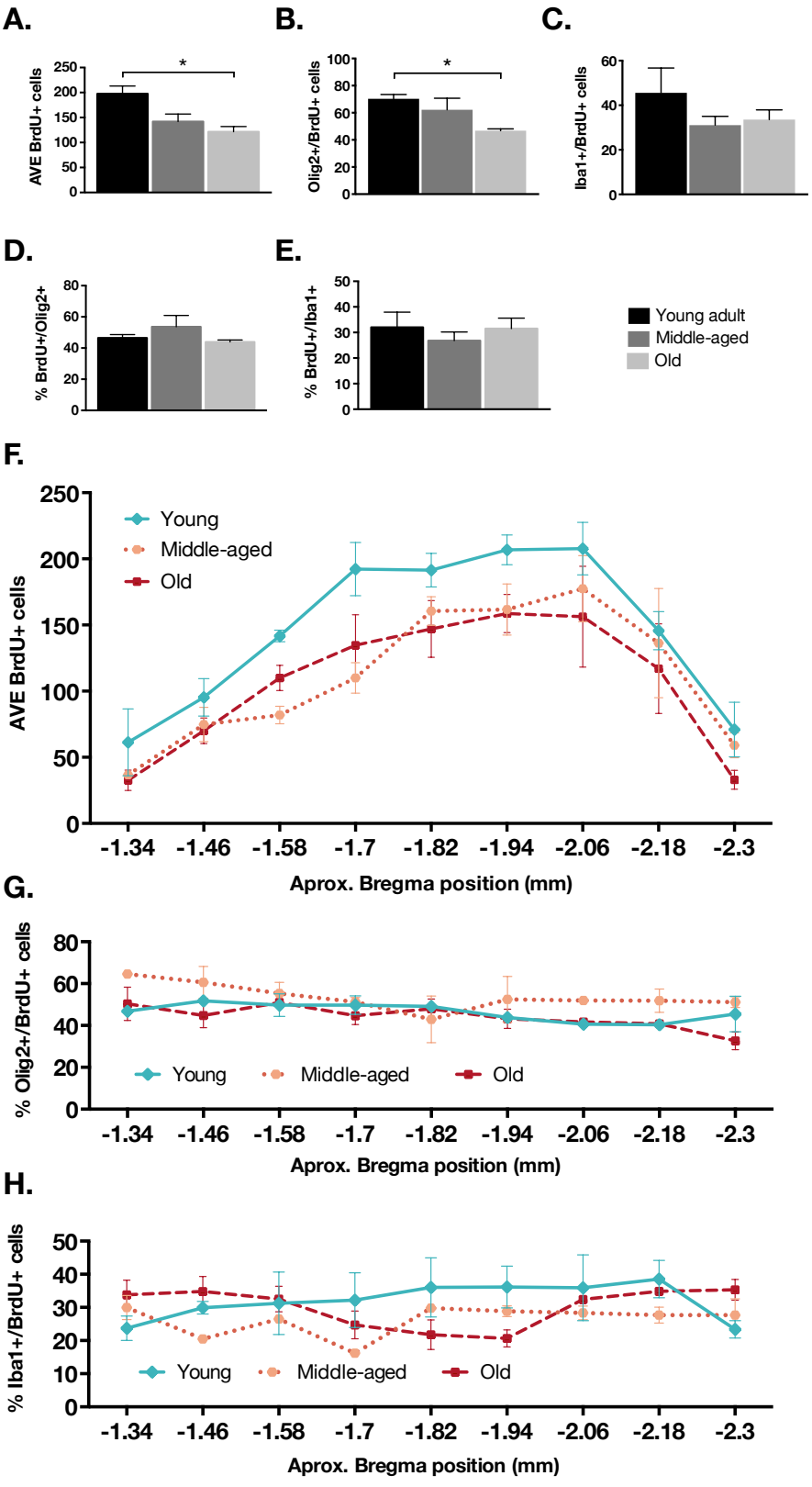


Figure 6.7. BrdU+ cells decline in the hypothalamus with age, affecting the two main proliferative populations (Olig2+ and Iba1+) equally. (A) Number of BrdU+ cells in young adult (black), middle-aged (dark grey) and old animals (light grey). Data show a significant age-related decline in the BrdU+ cell number. (B) Number of Olig2+/BrdU+ cells in young adult, middle-aged, and old animals. Data show a significant age-related decline. (C) Number of Olig2+/BrdU+ cells in young adult, middle-aged and old animals. (D) Percentage of Olig2+/BrdU+ show no age-related differences. (E) Percentage of Iba1+/BrdU+ show no age-related differences. (F) Distribution of BrdU+ cells in the hypothalamus, represented as the average number of BrdU+ cells in sections ranging from bregma -1.34 mm to -2.3 mm. Different colours represent the three age groups, young adult (blue), middle-aged (orange) and Old (red) (G) Distribution of proliferative Olig2+ cells in the MBH, represented as the percentage Olig2+/BrdU+ cells respect to the total BrdU+ cell count, between bregma -1.34 mm to -2.3 mm. (H) Distribution of proliferative Iba1+ cells in the MBH, represented as the percentage Iba1+/BrdU+ cells respect to the total BrdU+ cell count. Data is represented as mean ±SEM and a minimum of 4 animals per age group were quantified. The significance testing for A, B and C was performed using one-way ANOVA followed by Tukey post-hoc test (*$p<0.05$).

6.4. Discussion

Previous studies have suggested that a population of neural stem/progenitor cells reside within the adult hypothalamus, although the anatomic location of the proliferative niche is the object of strong controversy. In this study, using the mitotic marker BrdU, cycling cells have been identified in the hypothalamic parenchyma of the Arc nucleus and the ME; thus, suggesting that the putative progenitor population is located in the parenchyma. The proliferative cells were identified as Olig2+ (oligodendrocyte) and Iba1+ (microglia), accounting for 80% of the BrdU+ cells. However, co-localisation with mature neuronal markers failed to give positive results, thus suggesting that the parenchymal progenitor cells predominantly give rise to glial fate cells.

Characterisation of the parenchymal progenitor population in three age groups (young adult, middle-aged and old) indicated that the number of BrdU+ cells in the Arc-ME decline gradually with age, affecting both Olig2+ and Iba1+ populations. These results suggest that either the progenitor cells lose self-renewing capacity with age, or they became senescent as the animals age.

6.4.1. BrdU incorporation assays label new-born cells in the hypothalamus

Over the past two decades, the hypothalamus has emerged as a putative novel region for adult neurogenesis and gliogenesis, in addition to the well-documented SGZ and SVZ neurogenic niches. The development of new techniques to track cycling cells facilitated the detection of additional proliferative regions outside the canonical stem cells niches. BrdU is the most common method for directly tracking DNA replication (Cavanagh et al. 2011; Zeng et al. 2010), and is especially useful for labelling slowly dividing cells as incorporation can be tracked *in vivo* over the course of several days.

Therefore, BrdU was applied for 15 days via drinking water (1 mg/ml solution containing 0.25 mg/ml glucose), in preference to other labelling routes (intraperitoneal and intracerebroventricular injections) that require surgery and/or can be stressful for the animals (Kokoeva et al. 2007; Pérez-Martín et al. 2010). The BrdU concentration within this paradigm was sufficient to be detectably incorporated into the DNA of dividing cells in the hypothalamus, the SVZ and the SGZ (Niels Haan et al. 2013). Also, given that

prolonged BrdU treatment can induce toxicity, the analysis was limited to 15 days and animals showed no overt phenotype at this time point (Niels Haan et al. 2013).

Alternative methods based upon detecting endogenously expressed markers associated with cell proliferation, such as Ki67 or PCNA, were not considered in this study given that they provide a snapshot of a cell population at the time of assay (Kee et al. 2002; Miller et al. 2018), and label too few cells to allow a satisfactory evaluation of cell proliferation in regions with lower mitotic activity than the SVZ or SGZ.

Caveats of the BrdU assays include that the thymidine analogue can be incorporated into DNA repair or during cell death (Kuan et al. 2004; Yang et al. 2012). Consequently, alternative methods were assessed to confirm cell proliferation via BrdU incorporation. First, in this study, BrdU+ cells are often observed in pairs, in close proximity, indicative of recently divided cells (Rakic 2002). Also, cell death studies in *Chapter 1* reported that apoptotic and necroptotic events are rarely observed in the hypothalamus of adult mice, suggesting that cell death cannot explain the full magnitude of the hypothalamic proliferation and supporting the proliferative identity of the hypothalamic BrdU+ cells. In agreement with these results, very few cells were detected by CC3 immunohistochemistry and TUNEL assay in the hypothalamus of young rats (Guyenet et al. 2013).

6.4.2. The spatial distribution of the proliferative cells suggested that the adult neural stem cell/progenitor niche resides within the hypothalamic parenchyma

The existence of a neuronal stem cell/progenitor niche within the adult hypothalamus has been the object of strong controversy and the precise identity of the neurogenic cells, and their dynamics with age remained elusive, particularly during adulthood. Several studies have suggested that proliferative cells with neurogenic capacity reside in the adult hypothalamic parenchyma (Djogo et al. 2016; Kokoeva et al. 2007; Pencea et al. 2001; Pierce and Xu 2010). Contrarily, additional studies have speculated that tanycytes located in the ependymal layer lining of the 3V are the neurogenic cells (Lee *et al.*, 2012; Haan *et al.*, 2013; Robins *et al.*, 2013).

The lack of agreement in the identification and location of the hypothalamic progenitor/NSC niche could be caused by a combination of multiple factors. First, the different methodology employed, including the BrdU labelling routes (IP, ICV or DW), the length of treatments and the different transgenic models used. Second, the fact that there is no specific marker for neural stem cells could underlie the discrepancy concerning the precise anatomic origin of the adult hypothalamic new-born cells (Doetsch et al. 1999; Johansson et al. 1999). Third, the majority of the studies used animals of postnatal stages (Table 6.1 and 6.2), thus encompassing significant developmental effects. To date, only a handful of studies included rodents older than 3 months (Pierce and Xu, 2010; Matsuzaki *et al*., 2015; Djogo *et al*., 2016; Jourdon *et al*., 2016; Niwa *et al*., 2016; Zhang *et al*., 2017). Rodents are considered fully mature adults when they reach 3 months of age (Flurkey et al. 2007), and in the hypothalamus, the organisation and final maturation of the glia and neuronal circuits continue for, at least, 1 month after birth (Bitsch and Schiebler 1979; Padilla, Carmody, and Zeltser 2010).

Therefore, in order to identify the location and of the hypothalamic adult neural stem cell/progenitor niche, the present study focuses on fully adult animals, older than 3 months of age. This study demonstrates that the parenchymal region surrounding the 3V wall harbours adult proliferative cells, and their proliferative capacity continues in the adult and ageing brain (Figure 6.3). These proliferative cells appear restricted to the Arc nucleus and the ME, with scarce BrdU+ staining lining the 3V. The relative and spatial distribution of BrdU+ cells in the hypothalamic compartments was comparable to previous reports despite the different labelling routes and length of treatments employed (Bless et al. 2014; Djogo et al. 2016; Kokoeva, Yin, and Flier 2005; Lee et al. 2014; Li et al. 2012; Robins, Trudel, et al. 2013; Robins, Villemain, et al. 2013).

Besides, the results showed rare proliferating cells in the ependymal layer of the 3V of young, middle-aged and old animals, similarly to results obtained in rodents older than 2 months (Niels Haan et al. 2013; Kokoeva et al. 2007; Matsuzaki et al. 2015; Pierce and Xu 2010; Robins, Villemain, et al. 2013). The scarce amount of cycling cells observed lining 3V wall suggests that the ependymal layer contains non-proliferative and/or slow-cycling cells, in agreement with previous reports that classified tanycytes as a slow-cycling cell population, containing non-dividing as well as proliferative subtypes with long cell cycles ranging 9 to 15 days (Haan *et al.*, 2013; Robins *et al.*, 2013).

Interestingly, lineage tracing studies in adult mice using Prss56$^{Cre/+}$ Rosa26$^{tdTom/+}$ found that α2 tanycytes can migrate into the parenchyma in physiological conditions and upon mitogenic stimulation (Jourdon et al. 2016). Also, it has been suggested that tanycytes proliferation is not restricted to the ependymal compartment, and upon departure of the ependymal layer, they can remain undifferentiated and continue to divide within the neighbouring parenchyma. In support of this notion, Haan *et al*., found pairs of BrdU+/βGal+ cells in close contact within the hypothalamic parenchyma of Fgf10^{nLacZ} brains of postnatal mice, suggesting that a subset of parenchymal cycling cells might be tanycytes in origin. In the hypothalamus, Fgf10 expression is restricted to β1 and β2 tanycytes (Hajihosseini et al. 2008), and the use of Fgf10^{nLacZ} transgenic mice enables their transient lineage tracing (Niels Haan et al. 2013). However, experiments presented in this chapter using the Fgf10^{nLacZ} animal model and the same BrdU labelling paradigm than Haan *et al*. failed to show BrdU/βGal co-localisation, either lining the 3V or in the parenchymal region, with the only difference that this study was performed in adult animals. Therefore, the results suggested that the adult parenchymal cycling cells are not derived from tanycytes, at least not Fgf10+, supporting the notion that adult tanycytes are either non-dividing or slow-dividing cells. The use of additional lineage tracing transgenic mice for other tanycytes subtypes would help to assess this possibility.

The regional enrichment of the BrdU+ cells is particularly important given the unique anatomical relationship of the hypothalamus with the BBB, where the ME and the mediobasal region of the Arc nucleus are located outside the BBB (Yulyaningsih et al. 2017), allowing the cells contained in these regions to sense and respond to a variety to blood-borne molecules. Remarkably, in the hypothalamus, BrdU incorporation can be modulated by dietary changes (Gouazé et al. 2013b; D. A. Lee et al. 2012; Lee et al. 2014; Li et al. 2012; McNay et al. 2012), hormonal levels (Ahmed et al. 2008), and voluntary exercise (Niwa et al. 2016). Finally, a similar connection between the NSC and the vasculature has been observed in the hippocampal niche, where studies highlighted a role of the vascular context in the regulation of adult neurogenesis (Kokovay et al. 2010; Palmer, Willhoite, and Gage 2000; Shen et al. 2004). Thus, the anatomical location of the proliferative niche in the hypothalamus suggested that these cells are able to sense and respond to different environmental and peripheral cues.

Environmental conditions may therefore have a crucial role in the modulation of hypothalamic proliferation, neurogenesis and gliogenesis.

6.4.3. The co-localisation studies suggested that the identity and fate of the parenchymal proliferating is predominantly glia (oligodendrocytes and microglia)

BrdU incorporation assays can be used in combination with immunohistochemistry (Houck and Loken 1985), making it feasible to determine the identity of the proliferative cells. Co-localisation studies with different neuronal and glial cells markers, including Olig2 (OL lineage marker), Iba1 (microglia), S100β (astrocytes), GFAP (astrocytes) and NeuN (mature neurons) were performed. Also, different transgenic models were employed to assess BrdU co-localisation with Fgf10+ tanycytes and derived cells (Fgf10-nLacZ), and Npy- and Pomc-expressing neurons (Npy-GFP and Pomc-GFP, respectively).

According to the results presented in this chapter, the majority of the parenchymal proliferative cells have glial Identity: 45 – 50% BrdU+ cells expressed the transcriptional factor Olig2, and 30 – 35% expressed the microglial marker Iba1. However, Olig2+ and Iba1+ are not the only proliferating populations, as they together accounted only for the 80% of the proliferating cells. Thus, the identity of the remaining proliferating cells remains unidentified, although their identity could be inferred due to their location. As an example, some BrdU+ could be pericytes and/or endothelial cells from blood vessels, as previous reports indicated (Robins, Trudel, et al. 2013; Robins, Villemain, et al. 2013). Confirmation by the use of specific markers such as CD13 for pericytes, or CD31 for endothelial cells, may help to answer this question.

Olig2 is a universal oligodendrocyte lineage marker (Valério-Gomes et al. 2018), expressed by progenitors (OPC) and maturing OLs. In line with the findings presented in this chapter, previous BrdU labelling studies identified that more than two-thirds of the BrdU+ cells in the adult hypothalamus co-express NG2, a specific OPC marker (Robins, Trudel, et al. 2013). More than 90% of NG2 cells co-express Olig2 in the brain, in contrast, only 18% of the Olig2+ cell population expressed NG2 (Ligon et al. 2006); however, when Olig2 expression is examined in proliferating cells, the ratio of Olig2+

proliferating cells was almost the same as the ratio of NG2+ proliferating cells (Mori et al. 2009). Thus, the different proportion of cycling OPCs observed in this study and Robins *et al*. could be due that NG2 expression, but not Olig2, is detected in pericytes associated with blood vessels (Ligon et al. 2006).

In addition, NG2-glia is highly regenerative and can undergo multiple self-renewal divisions and, in the hypothalamus is able to differentiate into mature OL (CC1+, RIP1+), pericytes (CD13+) and a small subset of neurons (DCX+, HuC/D+, NeuN+, NPY+, POMC+, Tuj1+; Kokoeva, Yin and Flier, 2007; Li, Tang and Cai, 2012; Robins, Trudel, *et al.*, 2013).

The present study also identified the Iba1+ microglia, as the second proliferative population in the adult hypothalamus. In addition to this study, another two reports identified proliferating microglia in the hypothalamus (Djogo et al. 2016; Pérez-Martín et al. 2010). Interestingly, microglia proliferation has been reported in different regions of the mammalian CNS following its experimental depletion (Bruttger, Karram, Wörtge, et al. 2015; Lloyd et al. 2019; Renee et al. 2015). Genetic ablation of microglia in adult mice revealed that these cells are able to self-renew, proliferate and rapidly repopulate the region of the CNS inspected (Bruttger, Karram, Prinz, et al. 2015). Thus, the results suggest that the hypothalamic parenchyma hosts a microglial progenitor pool, capable of self-renewal and, possibly, able to generate new microglia across the mouse lifespan.

Regarding the neuronal identity of the proliferating cells, this study failed to identify co-localisation of BrdU+ cells with the neuronal markers NeuN, Npy and Pomc; however, the possibility that new neurons are generated in the adult hypothalamus cannot be rejected. First, although NeuN is a widely employed marker to identify mature neurons, not all differentiated neurons express NeuN (Mullen, Buck, and Smith 1992; Weyer and Schilling 2003). Also, the hypothalamus contains multiple neuronal types that are distinct from NPY neurons and POMC neurons (Campbell et al. 2017). Thus, the absence of NeuN, Npy and Pomc expression in new-born hypothalamic cells does not preclude that these cycling cells lack neurogenic potential, as they may generate a different cohort of neurons. The use of additional neuronal markers should help resolve this question. Second, the length of the BrdU treatment (15 days) may not be long enough for progenitors to differentiate into neurons, given that evidence following experimental ablation suggested that the time needed to terminal neuronal differentiation in the

hypothalamus seems to be longer than 10 days (Yulyaningsih et al. 2017). Indeed, adult-born cells in the hypothalamus failed to express the mature neuronal markers Tuj1, NeuN and HuC/D two weeks after BrdU ICV infusion; however, BrdU+ co-localisation with Tuj1+ and HuC/D+, but not NeuN, was observed 7 weeks post-ICV BrdU infusion (Kokoeva et al. 2007). Earlier studies from the same group also identified several BrdU+ cells expressing HuC/D+, Tuj1+ and the neuropeptides NPY or POMC characteristic of hypothalamic neurons in CNTF-treated animals 42 days after BrdU ICV infusion (Kokoeva et al. 2005). In agreement with this findings, the number of adult-born cells with neuronal fate in the hypothalamus (NeuN, POMC, NPY) increased when the BrdU-labelled cells were chased for longer periods (greater than 20 days after treatment; (Gouazé et al. 2013b; Li et al. 2012; Matsuzaki et al. 2009; Xu et al. 2005). Therefore, longer treatments and/or longer tracing time should help resolved if progenitor cells residing within the hypothalamic parenchyma could differentiate into neurons that integrate the hypothalamic circuitry. Third, some of the previous evidence indicates that the neurogenic potential of the hypothalamic progenitors may be lower in adult rodents, given that new-born cells with neuronal fate were hardly observed in rats older than 3 months (Matsuzaki et al. 2015). Also, lineage-tracing studies with NG2-CreER^{T2}:R26^{tdTom} found that over 60 days following induction, a minority (0.54%) of NeuN+ neurons in the adult hypothalamus have been derived from NG2-glia (Robins, Trudel, et al. 2013). Together, this evidence suggested that new-born neurons may represent a very small fraction of the hypothalamic adult-born cells, making them mostly undetectable with the methods used in this study.

As an alternative indicator for adult neurogenesis to BrdU incorporation, the expression of the immature neuronal marker DCX in the adult hypothalamus was assessed. In contrast to NeuN, DCX is transiently expressed in early postmitotic neurons, but not in mature neurons (Brown et al. 2003). Indeed, some BrdU+/DCX+ cells in the adult hypothalamus can be observed around 11 – 15 days post-BrdU infusion; early before than co-localisation with NeuN is detected (Kokoeva et al. 2007; Matsuzaki et al. 2009). However, the results present in this chapter failed to detect convincing DCX expression in the adult hypothalamus, suggesting that either very little new-born neurons are being produced or that new-born hypothalamic neurons express DCX at relatively low levels and therefore might have escaped detection.

Finally, the results suggested that adult hypothalamic astrocytes are either quiescent or have very long cycles, as adult born GFAP+ astrocytes were scarce and only observed in the aged group. Also, co-localisation of S100β with BrdU was observed in the three age groups; however, BrdU+/S100β+ cells also expressed Olig2+, thus suggesting they belong to the OL linage. Although S100β is an alleged astrocytic marker, its expression is upregulated in OPC, as well as immature and mature OLs (Hachem et al. 2005).

6.4.4. The proliferation in the hypothalamus declines with increasing age

Although new-born cells are continuously produced throughout life in discrete regions of the CNS, a significant decline in neurogenesis been reported with advancing age (Kuhn, Dickinson-Anson, and Gage 1996; Lazarov and Marr 2013; Tang et al. 2009). In the hypothalamus, a decline in parenchymal progenitor proliferation has been reported in aged rats. The same study also found that not only progenitor cell proliferation was decreased, but survival and maturation of the new-born cells were also impaired in the aged hypothalamus (Matsuzaki *et al.*, 2015).

The hypothalamic proliferation, measured by the number of BrdU+ cells, declines significantly and gradually with increasing age (**Figure 6.7 – A**). Also, the decline affected both progenitor populations, Olig2+ and Iba1+ **Figure 6.7 – B, C**). The decreased proliferation rate observed leaded to speculate that (1) hypothalamic parenchymal progenitors may be lost gradually during ageing; (2) progenitor cell cycle times increase significantly with advancing age or a subpopulation of the parenchymal progenitors became senescent; (3) hypothalamic progenitor responsiveness to stimulating environmental factors decreases with age and/or (4) these environmental factors decrease or disappear with age and/or inhibitory factors appear or accumulate with age. In support of (1, 2), previous reports associated the age-related accumulation of damaged proteins resulting in a reduction in the NSC proliferation rate (Vilchez, Saez, and Dillin 2014). Also, studies in the mouse demonstrate that OPC cycle times increase significantly in cortex and corpus callosum with age (Psachoulia et al. 2009). Finally, DNA damage, oxidative stress and telomerase shortening have been proposed as a mechanism for the ageing-induced deterioration of stem cell functions, including mitotic potential (Back et al. 2001; Balaban et al. 2005; Schultz and Sinclair 2016; Sharpless and DePinho 2007).

In support of (3, 4), previous studies have shown that hypothalamic proliferation can be modulated by a range of trophic factors, such as BDNF, IGF, CNTF, and FGFs (Jourdon et al. 2016; Kokoeva et al. 2005; Pencea et al. 2001; Pérez-Martín et al. 2010; Robins, Stewart, et al. 2013; Xu et al. 2005). Consistent with these observations, inhibition of the IGF-1 pathway significantly delayed the age-related decline of hypothalamic neurogenesis (Chaker et al. 2016). Also, hypothalamic neurogenesis is known to be highly sensitive to nutrition and metabolic status of the animal nutrition (Gouazé et al. 2013b; D. A. Lee et al. 2012; Lee et al. 2014; Li et al. 2012; McNay et al. 2006; Niwa et al. 2016). Therefore, it can be speculated that age-related environmental and nutrient factors can influence and/or underlie the decline in proliferation observed with aged hypothalamus.

6.4.5. The age-related decline in the number of proliferative cells affects the Olig2+ and Iba1+ populations equally

This study reported an age-related decrease in hypothalamic proliferation, that could be associated with reductions in the number of new-born OLs (Olig2+) and microglia (Iba1+) cells, and possibly, other neural cell types.

OPC proliferation and differentiation is essential for maintaining CNS myelination throughout life (Franklin and Ffrench-Constant 2008). However, studies in aged mice have observed that the OPC have a diminished ability to self-renew and to differentiate, causing a reduction in oligodendrogenesis (Neumann et al. 2019; Psachoulia et al. 2009). In the hypothalamus, OPCs seem to hold a critical role in preserving the axonal tracts, as permanent neuronal processes impairment has been observed after short-term OPC elimination (Djogo et al. 2016). However, only one study reported this, and further investigation will be required to confirm the NG2-glia role in neuronal processes maintenance. Taking all together, the decline in OPC proliferation could lead to a reduced number of new-born OLs, myelin defects and axonal damage in the hypothalamus with age.

Together with OPC decline, microglia proliferation was also reduced in the aged hypothalamus. As mentioned in the introduction, microglia are the resident macrophages in the CNS and mediate immunity and inflammation by producing and

releasing a range of inflammatory mediators (Clarke et al. 2018; Colombo and Farina 2016; Valdearcos et al. 2017). In humans and mice, the microglia population homeostasis is maintained during the lifetime by the spatial and temporal coupling of proliferation and cell death (Askew et al. 2017; Renee et al. 2015). Therefore, according to the results presented here, turnover dynamics and homeostasis of the microglia population could be affected in the aged hypothalamus.

7. Myelin dynamics in the ageing hypothalamus

7.1. Introduction

Myelin is a special type of membrane composed mainly of lipids and a few protein components. Myelin primarily function consists in enwrapping axons, insulating them and allowing an energy-efficient saltatory transmission of the action potentials. In addition, myelin is involved in axonal maintenance by covering and protecting target axons from any damage and degeneration (Aggarwal et al. 2011).

Biochemical analysis of myelin composition identified a high lipidic content (73 – 83%), with enrichment of glycosphingolipids (galactosylceramide and sulfatide) and plasmalogens. The major protein constituents are the myelin basic protein (MBP; 30% total protein fraction), and the proteolipid protein (PLP; 50%), that hold structural function by stabilising and compacting myelin membranes (Baron and Hoekstra 2010). Additional myelin proteins include the myelin oligodendrocyte glycoprotein (MOG), myelin-associated glycoprotein (MAG), myelin-associated oligodendrocyte basic protein (MOBP), and 2′,3′-cyclic nucleotide phosphodiesterase (CNP). Although MOG, MAG, MOBP and CNP are associated with myelin, they lack a primarily structural function (Morell and Quarles 1999; Nakamura et al. 1996).

The MBP gene encodes different isoforms, with molecular weights ranging from 14 to 21.5 KDa in rodents (Akiyama et al. 2002), which appear to localise to different microdomains in the myelin membrane (Boggs 2006; DeBruin et al. 2006). Likewise, the PLP gene also encodes different isoforms, PLP with structural function, and DM20, that seems to assist the trafficking of PLP (Sinoway et al. 1994). Strikingly, although both MBP and PLP are structural proteins, MBP is the only protein essential for myelin formation, with myelination still occurring in the PLP-null mice, although the myelin stability decreases over time (Boggs 2006; Klugmann et al. 1997).

In the CNS, the different myelin proteins are produced by the OLs, a specialised type of glial cells. These glial cells suffer a terminal differentiation staged process, from progenitors (OPC) to post-mitotic pre-myelinating OLs (pre-OL), and then into myelinating OLs (Emery 2010). The terminal differentiation of OPCs is essential for the synthesis of the different myelin components and, the different stages are controlled by the expression of certain proteins that can be used as markers to study the process. However, in the CNS the master regulator of OPC differentiation has not been yet

identified and, the axonal signals identified are primarily inhibitory of OPC differentiation (Piaton, Gould, and Lubetzki 2010; Taveggia, Feltri, and Wrabetz 2010).

With the onset of myelin synthesis, terminally differentiated OLs must organise the delivery of different components to the growing myelin sheath. The myelin components are transported to the OL projections terminals where they assemble and organise the myelin sheath surrounding target axons. The myelin components are expressed in a timely fashion and transported to the growing myelin sheath by different mechanisms. After its biosynthesis, the myelin protein PLP is sorted and transported in vesicles to the OL projections, before reaching the myelin sheath (Sinoway et al. 1994). In marked contrast, the Mbp mRNA is trafficked away from the cell body to the oligodendrocyte projections, where it is translated 'on-site' and inserted within the myelin membrane (Barbarese et al. 1999).

The Mbp mRNA transport relies on microtubules, and different RNA-binding motor proteins are involved: the vast kinesin family and single cytosolic dynein that binds the dynactin activator (Barbarese et al. 1999; Baron and Hoekstra 2010). Previous studies in zebrafish identified the kinesin Kif1b as an essential member for the anterograde Mbp transport. Kif1b mutants showed a disrupted Mbp transport, causing Mbp to accumulate in the OL perikaryon, where it is locally translated (Lyons et al. 2009). At the same time, the Kif1B mutant presented ectopic myelin-like membranes in processes that did not ensheath axonal processes (Lyons et al. 2009). Similar to Kif1b mutants, mutations in the retrograde motor complex dynein/dynactin resulted in arrested Mbp transport and defective myelination in zebrafish and mammalian OL primary cultures (Herbert et al. 2017). In humans, mutations in the kinesin Kif1b and dynein genes have been linked with susceptibility to MS (Aulchenko et al. 2008), and are known to cause Charcot-Marie-Tooth (CMT), both demyelinating diseases (Gentil and Cooper 2012).

Importantly, loss of myelin is a hallmark of the ageing brain, although differences in the magnitude of decline have been observed across different brain regions (Bowley et al. 2010; Peters 2002; Peters et al. 2000; Sandell and Peters 2001; Wang et al. 2020; Xing et al. 2012). Also, impairment of OPC proliferation, recruitment and differentiation into myelinating OLs have been reported with age (Psachoulia et al. 2009; Sim et al. 2002). Consequently, given that myelin enwraps axons to facilitate neuronal transmission and protect them from damage, the loss of myelin could lead to defective neuronal

communication and, ultimately, axonal degeneration with increasing age. Therefore, following the changes in myelin gene expression in the ageing hypothalamus reported in *Chapter 4*, the next step was to investigate if myelination defects occur in the hypothalamus with age.

7.2. Aims

- Characterise the myelination pattern in the hypothalamus during ageing and identify any age-related changes with age.
- Investigate the myelin-axon relationship in the ageing hypothalamus and identify any age-related changes in axonal integrity.
- Study the expression levels of the proteins involved in the transport of myelin components to target axons in the ageing hypothalamus.
- Study the OL number and the process of terminal differentiation from OPC to mature-myelinating OLs in the ageing hypothalamus.

7.3. Results

7.3.1. The myelination pattern in the hypothalamus is maintained with age, although differences in the myelin microstructure are observed with advancing age

Given that transcriptomic data presented in *Chapter 4* showed an age-related upregulation of myelin-related genes in the hypothalamus, the next step was to investigate the effects on the myelination in the hypothalamus. To do so, the myelination pattern in the hypothalamus was characterised using immunohistochemistry for MBP in brain sections of young adult (2 – 6 months old), middle-aged (10 – 12 months old) and old (18 – 25 months) mice, with a minimum of 5 animals per age group; between bregma positions -1.22 to -2.46 mm.

Results showed a region-specific myelination pattern in the hypothalamus, as evidenced by detection of dense MBP+ fibers in the ME and the ventral region of the Arc nucleus. In contrast, the dorsal part of the Arc nucleus and the periventricular regions were practically devoid of myelinated fibres (**Figure 7.1**, **A – C** and **D – F**). This region-specific pattern is maintained during ageing, with high MBP density observed in the Arc-ME of young, middle-aged and old mice.

Then, given that our results indicated that the Arc-ME are highly myelinated areas, the next step was to investigate the microstructure of the myelin in those areas. To do so, high power magnifications of MBP immunostaining in the MBH were obtained. Results for young animals showed a continuous MBP staining resembling to fibres with multidirectional orientation. In contrast with the continuous meshwork of the MBP+ fibres observed in the young animals, MBP staining showed a disrupted appearance in middle-aged and old animals (Figure 7.2). In addition to the disrupted pattern, myelin circumferences were observed in the Arc-ME of old mice.

To assess if the aberrant pattern also was observed in extra-hypothalamic regions, the corpus callous was analysed for MBP expression. Results failed to show the abnormal MBP labelling observed in the MBH, thus suggesting that the age-related aberrant pattern is specific to the MBH (Figure S 7).

In conclusion, although the localisation of the myelinated areas in the hypothalamus is maintained during ageing, defects in the myelin microstructure are associated with age progression.

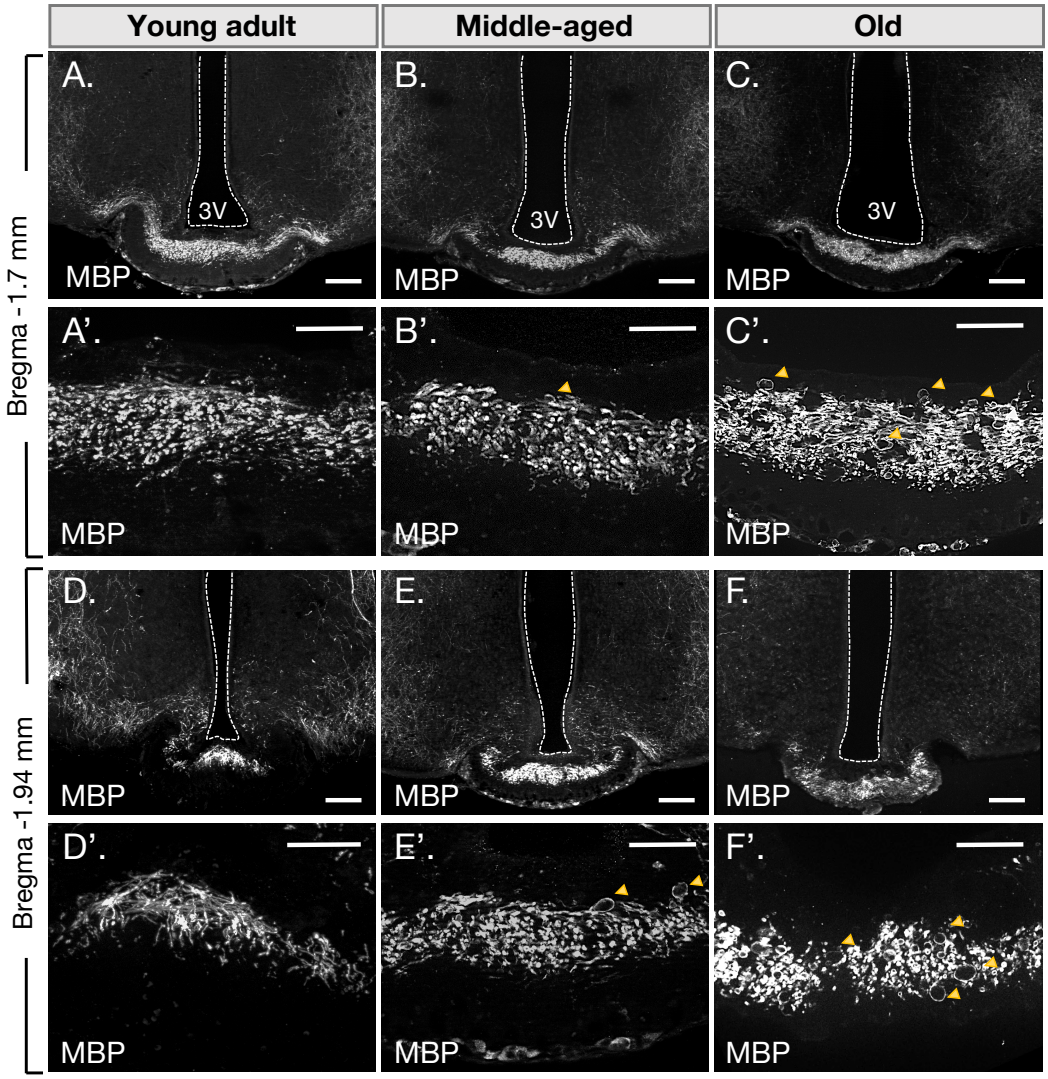


Figure 7.1. MBP expression pattern in the hypothalamus during ageing. (A, B, C) Representative images of MBH brain sections immunolabelled for MBP (white) at bregma -1.7 mm, from young-adult (D), middle-aged (E) and old (F) animals. Images show a high expression of MBP in the ME and the ventral part of the Arc nucleus. (A', B', C') High power images of ME show age-related changes in MBP staining observed as circumferences (yellow arrows). The circular staining is only present in middle-aged and old animals, the later showing a higher number. (D, E, F) Representative images of MBH brain sections stained for MBP (White) at bregma -1.94 mm, from young-adult (D), middle-aged (E) and old (F) mice. Images show a high expression of MBP in the ME and the ventral part of the Arc nucleus. (D', E', F') High power images of ME show age-related changes in MBP staining (white) observed as circumferences (yellow arrows). Bregma positions are approximated. Scale bar (A – C, D – F) 50 µm. Scale bar (A' – C', D' – F') 25 µm.

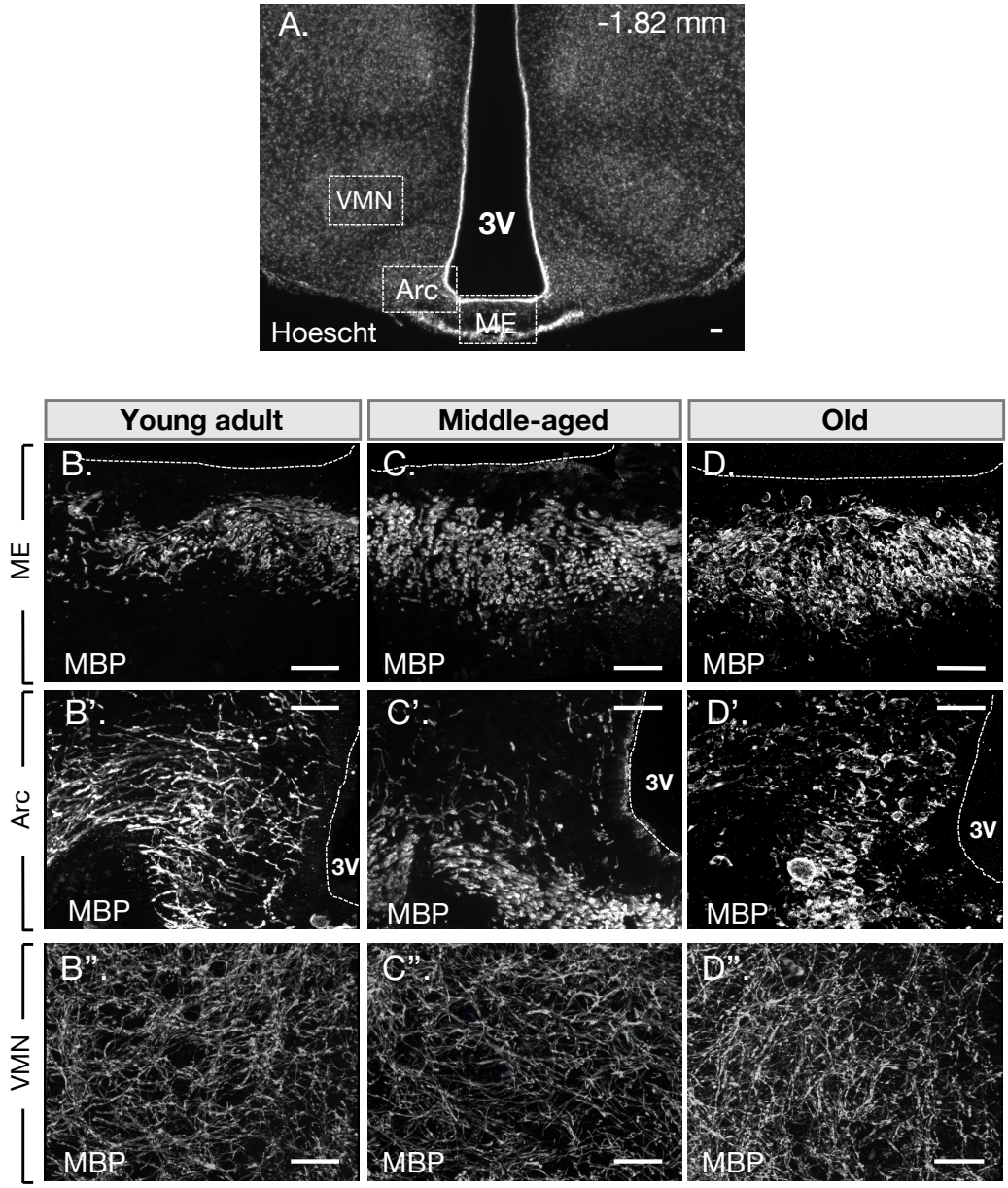


Figure 7.2. Age-related morphological changes in MBP expression in the ME and the ventral part of the Arc. (A) Representative image of MBH section with nuclear staining Hoechst (white). Dashed boxes outline the location ME, Arc and VMN regions at bregma -1.82 mm referred in the following images. (B, C, D) Representative images of ME stained for MBP (white) in young (B), middle-aged (C) and old (D) animals showed an age-related MBP+ circumferences. (B', C', D') Representative images of Arc stained for MBP (white) in young (B'), middle-aged (C') and old (D') animals showed an age-related MBP disrupted and circular staining. (D'', C'', D'') Representative images of VMN stained for MBP (white) in young (B''), middle-aged (C'') and old (D'') animals showed no age-related MBP differential staining and the aberrant myelin figures in the Arc-ME are not detected. Bregma position is approximated. Dashed lines outline 3V. Representative images of a minimum of 4 animals analysed per age-group. Scale bar 25 µm. 3V – third ventricle. Arc – Arcuate nucleus. ME – median eminence. VMN – Ventromedial nucleus.

7.3.2. MBP and Olig2 co-labelling indicates ectopic expression in the oligodendrocytes perikaryon of aged animals

Following the striking observations with MBP labelling, the next step was to investigate the relationship of MBP fibres with OLs in the Arc-ME. To do so, immunostaining for Olig2 and MBP was performed in was performed in 6 – 8 brain sections of young (*n* = 4), middle-aged (*n* = 3) and old (*n* = 3) animals, between bregma -1.58 mm to -2.3 mm. Results for young animals showed Olig2+ nuclei close to MBP+ fibres; however, co-localisation of both markers was hardly detected (**Figure 7.3**, **A**, **A'**). The same pattern was observed in middle-aged animals (**Figure 7.3**, **B**, **B'**). However, in the aged group, Olig2+ nuclei are often surrounded by MBP+ circumferences structures (**Figure 7.3**, **C**, **C'**), suggesting that MBP accumulates in the perikaryon of OL of aged animals.

In conclusion, the MBP staining observed in the perinuclear region could be a signal of ectopic deposition and aberrant myelination process that occurs in the MBH with age.

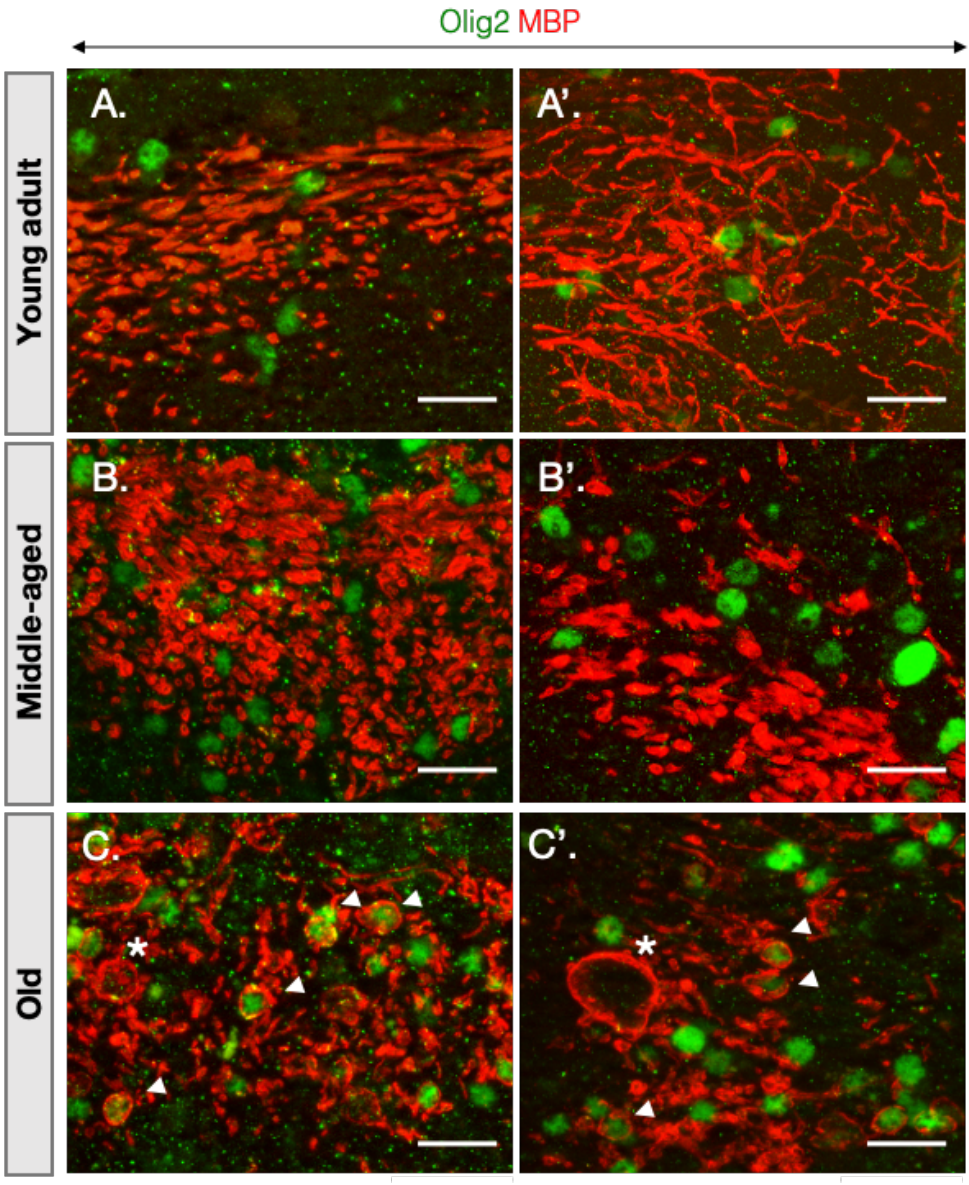


Figure 7.3. MBP expression is detected in the perinuclear region of Olig2+ cells in old animals. (A, B, C) Representative images of ME stained for MBP (red) and the nuclear marker Olig2 (green) in young (A), middle-aged (B) and old (C) animals. (B', C', D') Representative images of Arc nucleus stained for MBP (red) and Olig2 (green) in young (A), middle-aged (B) and old (C) animals. In the ME and Arc of old animals, some MBP staining enwrap Olig2+ nucleus (white arrows) while others do not (asterisks). Representative images of a minimum of 3 animals analysed per age group. Scale bar 12 µm.

7.3.3. Myelin-axon relationship in the Arc-ME is affected with age

Given that myelin is involved in axonal maintenance by covering and protecting axons from damage and degeneration, the next step was to investigate the relationship between myelin and axonal tracts in the MBH. To do so, co-immunostaining for MBP and the axonal neurofilaments (NFs) was performed in 6 – 8 brain sections of young, middle-aged and old animals (n = 3 per age group), between bregma -1.58 mm to -2.3 mm. Results showed NF+ axons orientated in different directions and co-localisation with MBP was observed in the Arc-ME (**Figure 7.4**).

Then, to investigate microstructural changes in the myelin-axon relationship with age, high power magnifications of MBP and NF immunostaining in the Arc-ME were obtained. Results for young adults showed NF+ axonal tracts in cross-section surrounded by MBP staining (**Figure 7.5**, **A – A''**). The middle-aged group showed the same pattern observed in young animals; however, MBP+ circumferences filled or partially filled by NF signal were observed in the ME (**Figure 7.5**, **B – B''**). In old animals, the MBP+ structures were detected widespread in the Arc and ME and were mainly devoid of NF labelling (**Figure 5.12**, **C – C''**), thus suggesting that ectopic myelin-like membranes are not enwrapping axonal tracts.

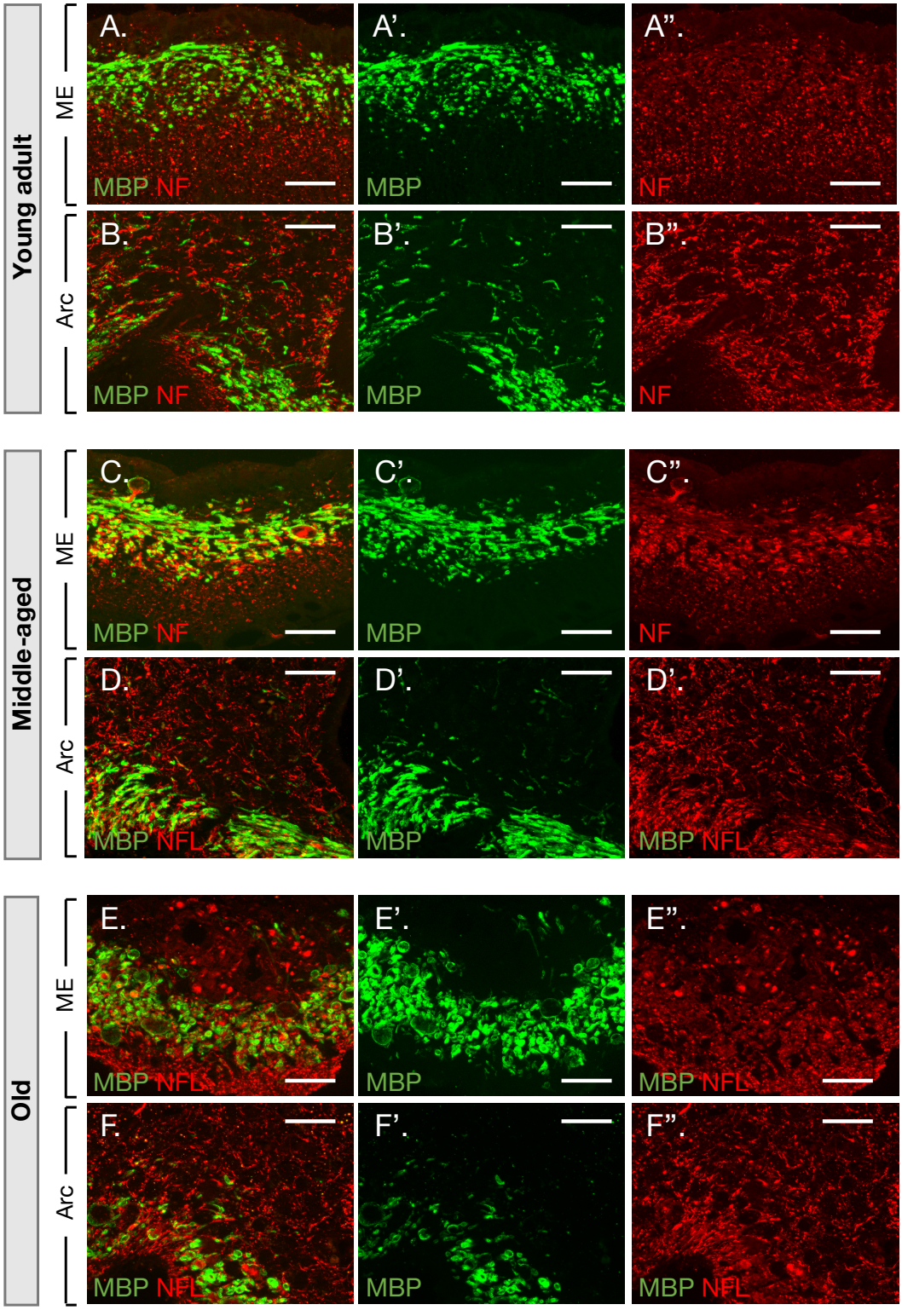


Figure 7.4. Co-immunostaining for MBP and NF in the Arc nucleus and the ME. (A – A", C – C", E – E") Representative images of ME stained for MBP (red) and the NF (green) in young, middle-aged and old animals. (B – B", D – D", F – F") Representative images of Arc nucleus stained for MBP (red) and NF (green) in young, middle-aged and old animals. Representative images of 3 animals analysed per age-group. Scale bar 25 µm.

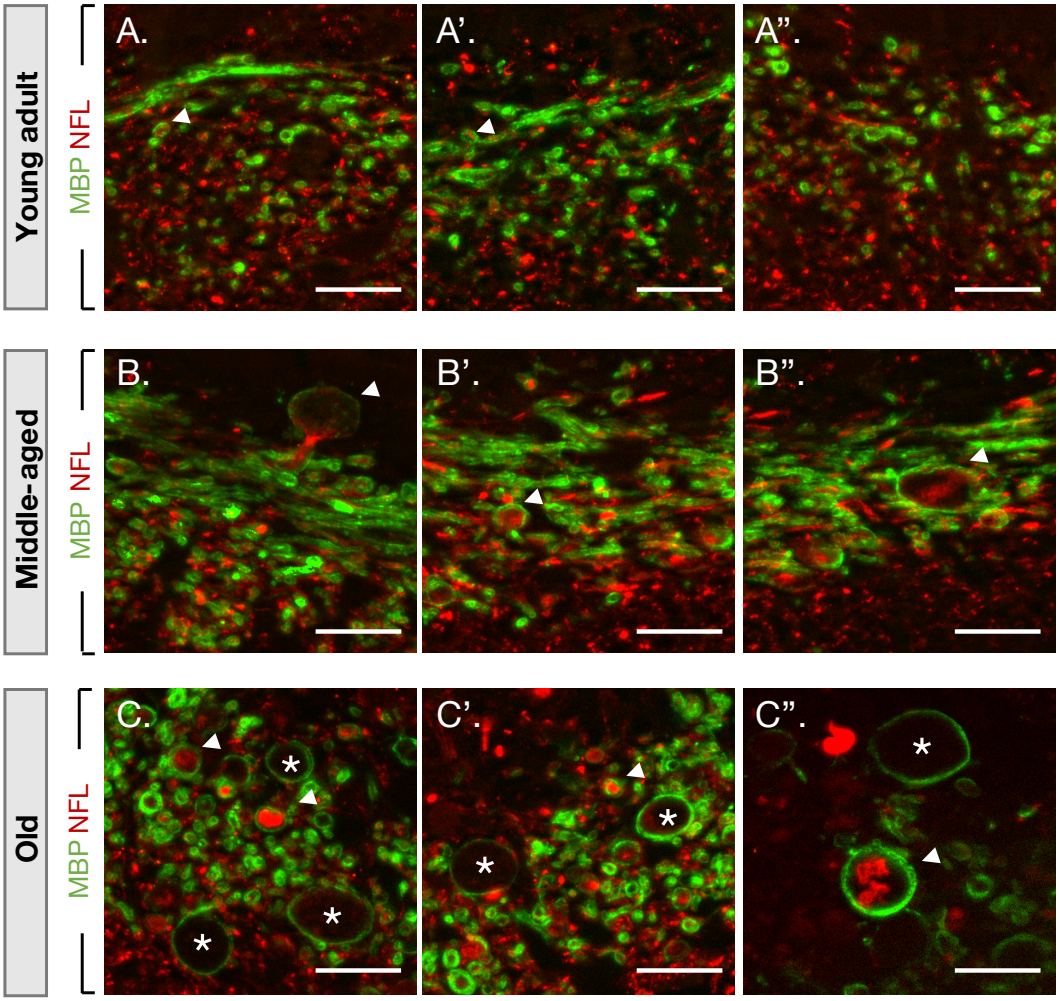


Figure 7.5. Axonal tracts immunolabeled for MBP and NF in the ME revealed age-related defects. (A, B, C) Representative images of ME stained for MBP (red) and the NF (green) in young (A – A"), middle-aged (B – B") and old (C – C") animals. In old animals, some MBP+ circumferences are filled by NF signal (white arrows) while others are devoid of NF staining (asterisks). Representative images of a minimum of 3 animals analysed per age group. Scale bar 12 µm

7.3.4. Expression of proteins involved in axonal stability in the aged hypothalamus

Lastly, given that myelination pattern changes with age in the hypothalamus, the axonal integrity was investigated in this region. Since visualisation of full-length axonal tracts was unachievable with the available methods, the expression of proteins involved in axonal stability and maintenance was investigated instead. To do so, the protein levels of βIII-tubulin (TUBB3) and NFM were assessed via western blot in hypothalamic extracts of young, middle-aged and old animals (n = 6 per age group).

Results for TUBB3 showed that the antibody detected a band of 55 KDa (**Figure 7.6**, **A**), and densitometric analysis of the 55 KDa showed no differences with age. (**Figure 7.6**, **B**). For NF, the antibody detected a multi banded pattern corresponding to the neurofilament medium isoform (NFM, 145 – 160 KDa) and additional lower and higher molecular weight weaker bands (**Figure 7.6**, **C**). Densitometric analysis of the NFM band revealed a significant decrease in the expression in old animals (**Figure 7.6**, **D**). In summary, the NFM protein levels showed decreased expression with age, while TUBB3 expression is maintained during ageing.

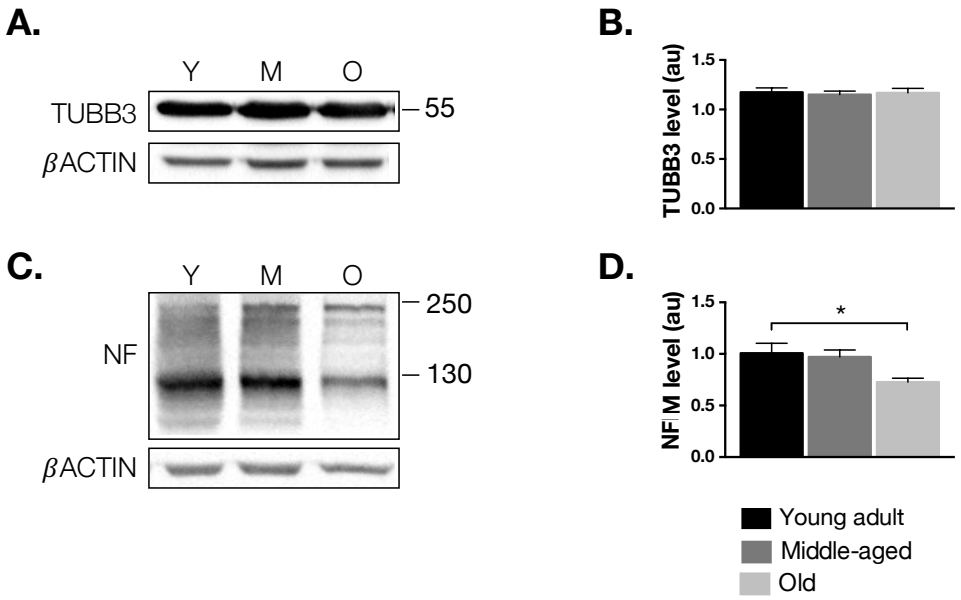


Figure 7.6. Expression of the neuronal cytoskeleton markers TUBB3 and NF in the ageing hypothalamus. (A) Representative image of immunoblot for TUBB3 and the loading control β-ACTIN. TUBB3 runs at an approximated MW of 55 KDa. (B) Comparison of densitometric analysis of TUBB3 relative to the loading control showed no significant changes with age. (C) Representative image of immunoblot for NF and the loading control β-ACTIN. NFM runs at MW of 145 – 160 KDa, approximated. (C) Comparison of densitometric analysis of NFM relative to the loading control showed a significant decline in the old group compared to the young animals. The letters above the immunoblot lanes refer to the different age groups, young adult as Y, middle-aged as M and old as O. Numbers indicate bands of the molecular weight marker used as a size standard (KDa). Data is represented as mean ±SEM and a minimum of 6 animals per age group were analysed. All significance testing was performed using one-way ANOVA followed by Tukey post-hoc test (*$p<0.05$).

7.3.5. The expression of motor proteins involved in the transport of Mbp is decreased in the aged hypothalamus

As described in *Chapter 4*, transcriptomic analysis showed an age-related decrease in RNA levels of the kinesin Kif1b and dynein Dync1h1, involved in Mbp transport from OL cell body to target axons. However, since mRNA levels do not always correlate with functional protein level, an alternative approach was used to confirm the transcriptomic results. To do so, the protein levels of KIF1B and DYNC1H1 were analysed via western blot in hypothalamic extracts of young, middle-aged and old animals (n = 6 per age group).

Results for KIF1B showed that the antibody detected a band of 204 KDa corresponding to the predicted size of the protein and, an additional band with lower molecular weight (**Figure 7.7**, **A**). Densitometric analysis of the 204 KDa showed lower KIF1B expression in the old group, compared to young and middle-aged animals (**Figure 7.7**, **B**). For DYNC1H1, the antibody detected a high molecular weight band of 520 KDa, approximated (**Figure 7.7**, **C**). Densitometric analysis of showed lower DYNC1H1 expression in the old group, compared to middle-aged (**Figure 7.7**, **D**).

In summary, the expression of RNA-binding motor proteins KIF1B and DYNC1H1 is decreased in aged animals.

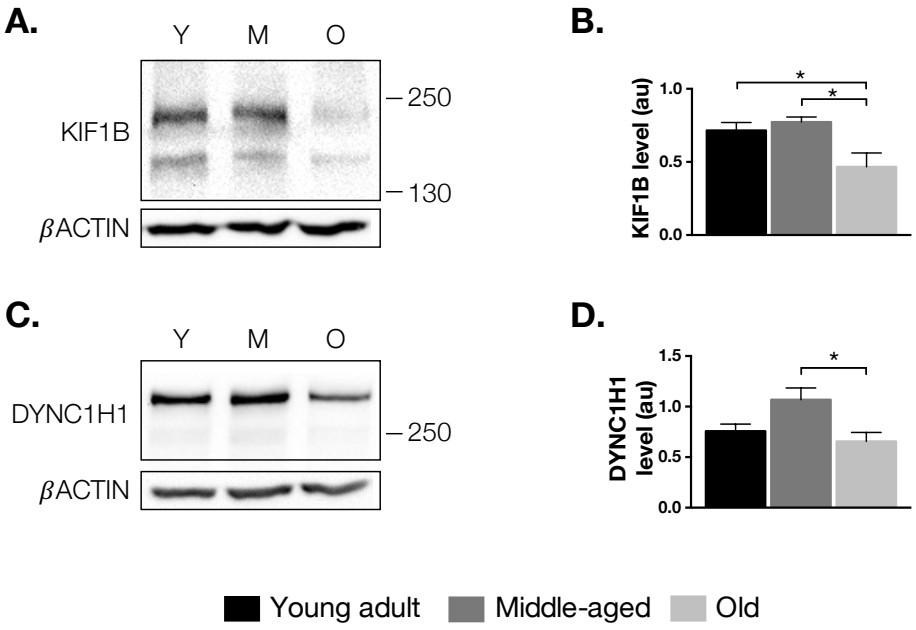


Figure 7.7. Age-related changes in the levels of KIF1B and DYNC1H1 in the hypothalamus. (A) Representative image of immunoblot for KIF1B and the loading control β-ACTIN. KIF1B runs at an approximated MW of 204 KDa. (B) Comparison of densitometric analysis of KIF1B relative to the loading control showed a significant age-related decline with age. (C) Representative image of immunoblot for DYNC1H1 and the loading control β-ACTIN. DYNC1H1 runs at an approximated MW of 530 KDa. (C) Comparison of densitometric analysis of DYNC1H1 relative to the loading control showed a significant decline in the old group compared to the middle-aged animals. The letters above the immunoblot lanes refer to the different age groups, young adult (Y), middle-aged (M) and old (O). Numbers indicate bands of the molecular weight marker used as standard (KDa). The column colours in the graph represent the three age groups: young adult (black), middle-age (dark grey) and old (light grey). Data is represented as mean ±SEM and a minimum of 6 animals per age group were analysed. All significance testing was performed using one-way ANOVA followed by Tukey post-hoc test (*$p<0.05$).

7.3.6. Oligodendrocyte number increases with age in the ME region

Next, given that the myelination pattern is affected in the hypothalamus with age, the next step was to investigate differences in the OL population during ageing. To do so, the distribution and density of OL was mapped in the MBH, using immunohistochemistry for Olig2 or APC (clone CC1) in 10 – 12 brain sections from young adult, middle-aged and old mice (n = 4 animals per age group). The analysis was performed between bregma -1.58 mm to -2.3 mm. The nuclear marker Olig2 is expressed at all stages of OL differentiation. To better characterise the myelinating OL subtype, CC1 was used as an additional marker to label specifically mature OL cell bodies. Thus, the use of the two markers allowed the characterisation OL at different differentiation stages (Figure 7.8).

Immunostaining for Olig2 revealed positive nuclei evenly distributed throughout the hypothalamic parenchyma, with greater concentration in the basal region of the Arc and the ME (Figure 7.9). The distribution was maintained during ageing, as observed in young, middle-aged and old animals. Coinciding with Olig2, CC1+ cells were also scattered in the hypothalamus with a higher concentration observed in the basal region of the Arc and the ME. Likewise, CC1 distribution was maintained with age (Figure 7.10). As a control, the CC was immunolabelled for CC1, and positive cells were observed in the region (Figure S 8).

In addition to defining the OL distribution, Olig2+ and CC1+ cell number was also quantified in the ME. Figure 7.11 shows that both Olig2+ and CC1+ cells increase in number with age. Also, given that Olig2 is a universal OL marker while CC1 is specific to the mature OL subtype, Olig2+ cells outnumbered CC1+ cells in the hypothalamus of young adults (17 ± 0.86 Olig2+ cells *vs* 8.33 ± 1.20 CC1+ cells), middle-aged (23.98 ± 0.60 Olig2+ cells *vs* 12.00 ± 1.73 CC1+ cells) and old mice (26.07 ± 0.68 Olig2+ cells *vs* 16.00 ± 0.58 CC1+ cells) (Figure 7.11, C).

In conclusion, OLs (Olig2+ and CC1+) are evenly distributed throughout the hypothalamus with a higher density in the ME, where OL accumulate with age.

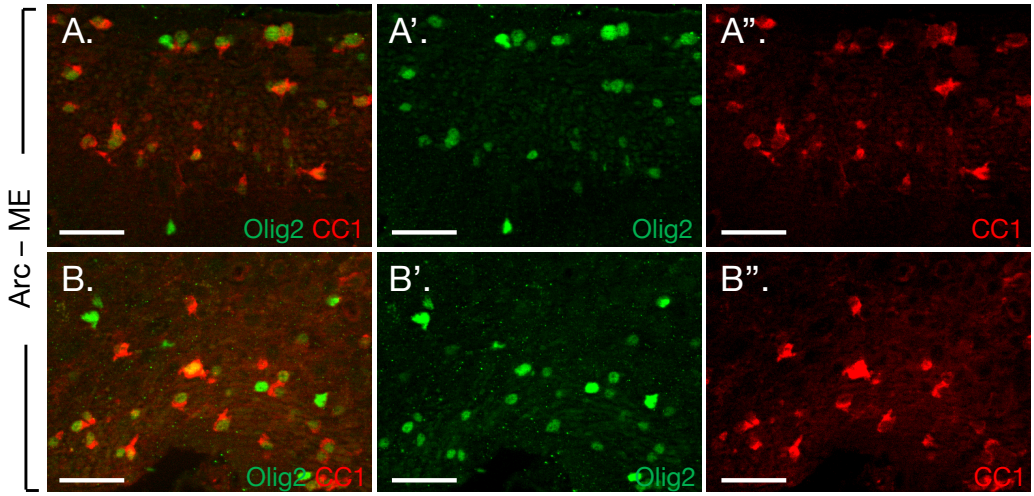


Figure 7.8. Immunostaining for the oligodendrocyte markers Olig2 and CC1 in the hypothalamus. (A-A'', B-B''). Representative images of the ME immunolabelled for Olig2 (green) and CC1 (red). The nuclear expression of Olig2 can be detected in all stages of OL differentiation, while CC1 labels specifically mature OL cell bodies. Images are representative of the results obtained for 4 animals analysed per age group. Scale bar 25 µm.

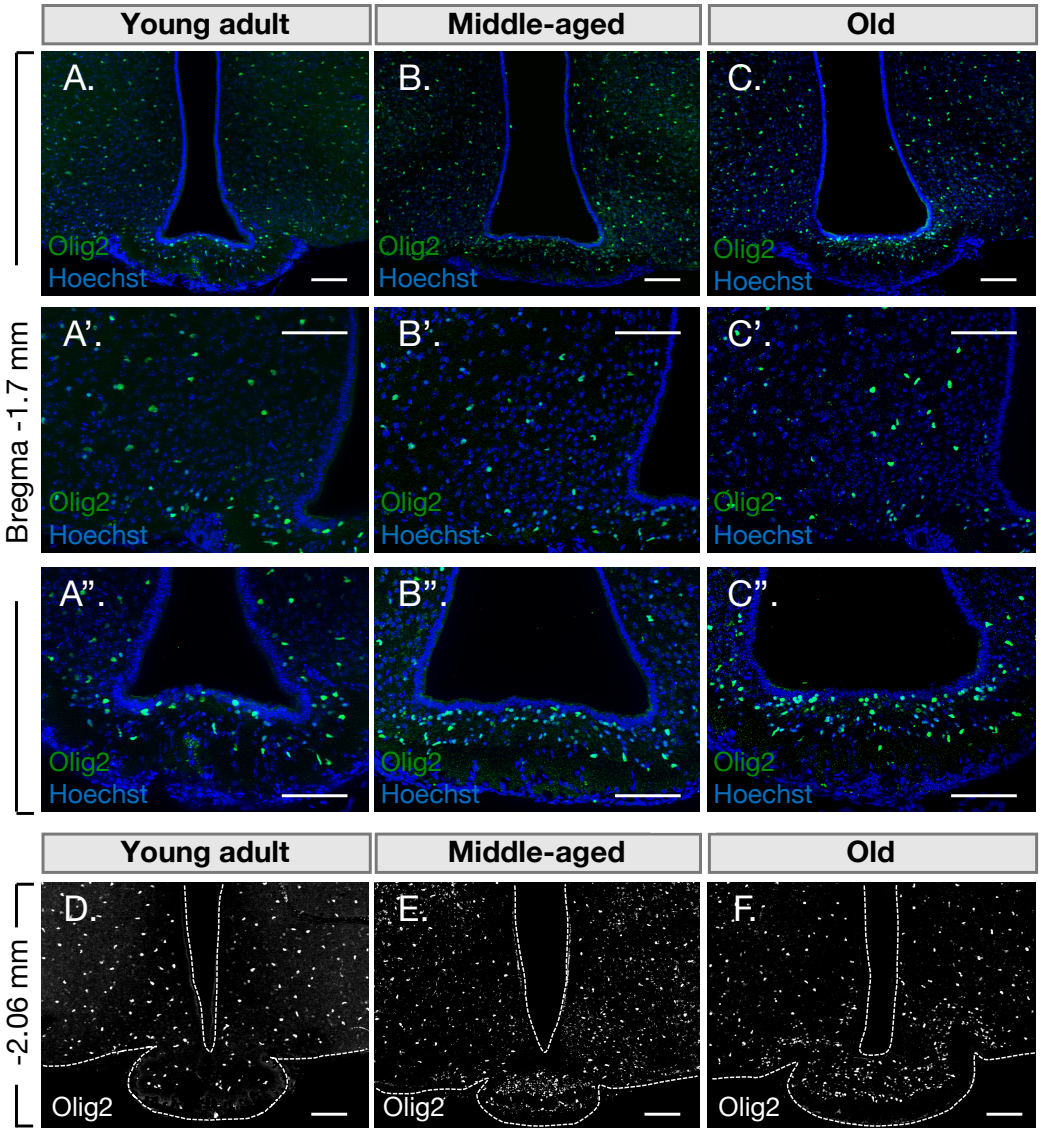


Figure 7.9. Oligodendrocytes showed a scattered pattern in the hypothalamus parenchyma with a higher density in the ME. (A-C) Representative images of the MBH for Olig2 (green) and Hoechst (blue) immunochemistry of young adult (A) middle-aged (B) and old (C) mice at bregma -1.7 mm. High power of the Arc nucleus (A', B', C') and ME (A", B", C") in young (A', A''), middle-aged (B', B'') and old animals (C', C''). (D-F) Representative images of the MBH for Olig2 (white) staining of young adult (D) middle-aged (E) and old mice (F) at bregma -2.06 mm. Dashed lines outline the borders of the hypothalamus and the 3V. Scale bar 50 µm.

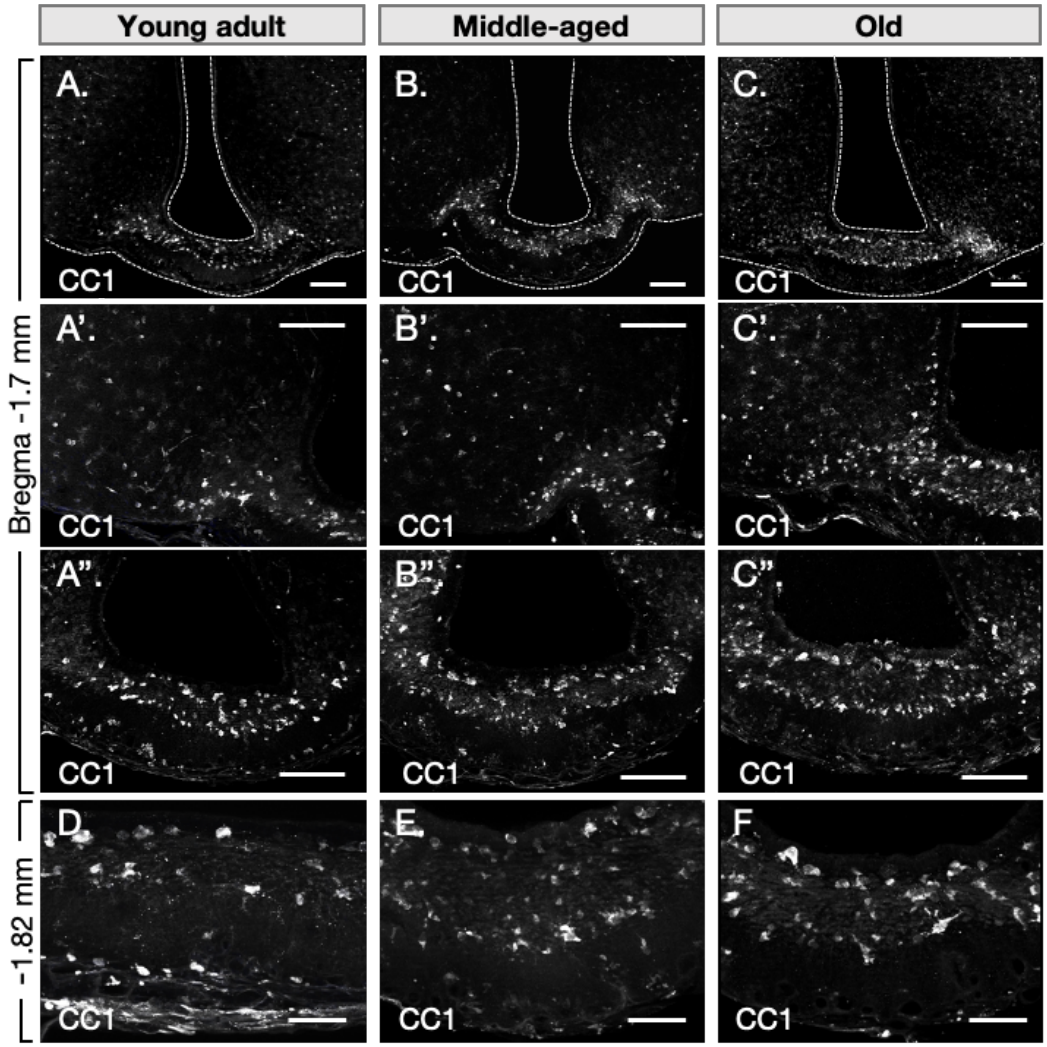


Figure 7.10. Mature oligodendrocytes showed higher density in the ME. (A-C) Representative images of the MBH for CC1 (white) immunochemistry of young adult (A) middle-aged (B) and old (C) mice at bregma -1.7 mm. High power of the Arc nucleus (A', B', C') and ME (A", B", C") in young (A', A''), middle-aged (B', B'') and old animals (C', C''), showed an age-related increase of CC1+ cells in the ME. (D-F) Representative images of the ME for CC1 (white) staining of young adult (D) middle-aged (E) and old (F) mice at bregma -1.82 mm. Dashed lines outline the borders of the hypothalamus and the 3V. Scale bar (A – A", B – B", C – C'') 50 µm. Scale bar (D' – F'), 25 µm.

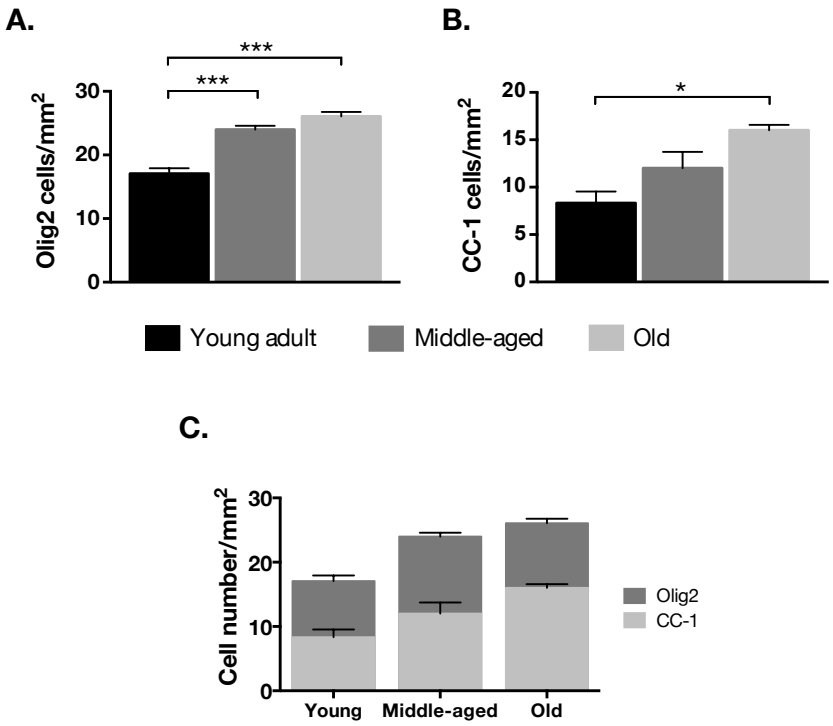


Figure 7.11. Age-related increase of Olig2+ and CC1+ cells in the ME. (A) Number of Olig2+ cells in the ME per square of area defined as Olig2+ cells per mm^2 in young-adults (black column), middle-aged (dark grey) and old animals (light grey). (B) Number of CC1+ cells in the ME per square of area defined as CC1+ cells per mm^2 in young-adults (black column), middle-aged (dark grey) and old animals (light grey). Data is represented as mean ±SEM and 4 animals per age group were quantified for both markers. All significance testing was performed using one-way ANOVA followed by Tukey post-hoc (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

7.3.7. Mature oligodendrocyte markers are normally expressed in the hypothalamus during ageing, suggesting the oligodendrocyte differentiation process is not impaired

Given that the terminal OPC differentiation to mature myelinating OLs is essential for myelin production, the OL differentiation process was investigated in the ageing hypothalamus. The OL differentiation is a multi-step process divided into three main stages: the OPC, the pre-myelinating and the mature myelinating OLs, which is defined by the expression of different markers. To gain a better understanding of the OL differentiation process, Olig2 was used as a pan-marker for OLs (**Figure 7.8**), while NG2 for OPC and CNP for pre-myelinating OL stages. To cover the final stage of the differentiation process, the transcriptional factor MYRF and the myelin proteins PLP and MBP as mature markers were chosen (**Figure 7.12**). The level of all markers was analysed via western blot in hypothalamic extracts of young, middle-aged and old animals (n = 6 per age group).

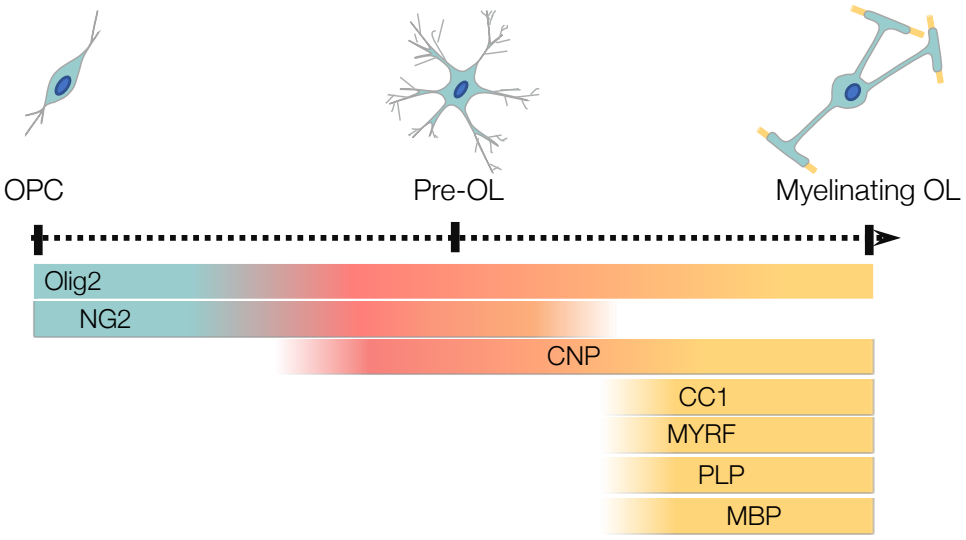


Figure 7.12. Markers of oligodendrocyte lineage differentiation and maturation. The schematic shows progression from OPC to pre-OL and, then to myelinating OL. The expression of different OLs markers is indicated by coloured gradients. The marker Olig2 is expressed in all the stages. The expression of the chondroitin sulphate proteoglycan NG2 is detected in the OPC and the intermediate pre-OL stages. CNP (2′,3′-cyclic nucleotide phosphodiesterase) is expressed in the intermediate pre-OL and in myelinating OL. Finally, CC1 (anti-adenomatous polyposis coli clone CC1), MYRF (myelin regulatory factor), PLP (myelin proteolipid protein) and MBP (myelin basic protein) expression is restricted to the mature myelinating type. OPC – oligodendrocyte progenitor cells. Pre-OL – pre-myelinating oligodendrocyte. OL – oligodendrocyte.

Western blot results for Olig2 showed that the antibody detected a distinct band of 32 KDa (Figure 7.13, A), and densitometric quantification showed no significant differences with age (Figure 7.13, B). As an early-stage marker, NG2 antibody recognised a high molecular weight band of 300 KDa, approximated (Figure 7.13, C). Densitometric analysis of the 300 KDa band revealed a significant decrease with age (Figure 7.13, D). For the pre-OL stage, CNP antibody detected a double band of 46 and 48 KDa (Figure 7.13, E) which expression remained unaffected with age (Figure 7.13, F).

For the mature stage, three makers were analysed, including the transcriptional factor MYRF and the structural myelin proteins PLP and MBP. For MYRF, densitometric quantification of the 123 KDa band (Figure 7.13, G) revealed a trend toward increase with age; however, the statistical analysis failed to show significant differences (Figure 7.13, H). Next, immunoblot for the PLP antibody detected two bands of 26 and 30 KDa, likely corresponding the DM20 and PLP isoforms (Figure 7.13, I), whose expression was not affected with age (Figure 7.13, J). Finally, the MBP antibody detected five bands with approximated molecular weights of 13, 14, 17, 18.5 and 21.5 KDa (Figure 7.13, K). Densitometric analysis revealed differential expression of some of the MBP isoforms with age. Results showed a decrease expression of the 21 KDa isoform (Figure 7.13, L), and an increase in the 13/14 KDa isoforms with age (Figure 7.13, N). However, the expression of the 17/18.5 KDa isoforms remained unaffected (Figure 7.13, M).

In summary, the expression of all the markers, including terminal differentiation markers, was observed in the hypothalamus, suggesting that OPC progression to mature OL is not disrupted in the hypothalamus with age. However, significant differences were observed for the OPC marker NG2 and, the terminal markers MYRF and MBP. Results showed that NG2 is decreased with age, MYRF expression is increased in aged animals and, the expression of the different MBP isoforms changes across the murine lifespan.

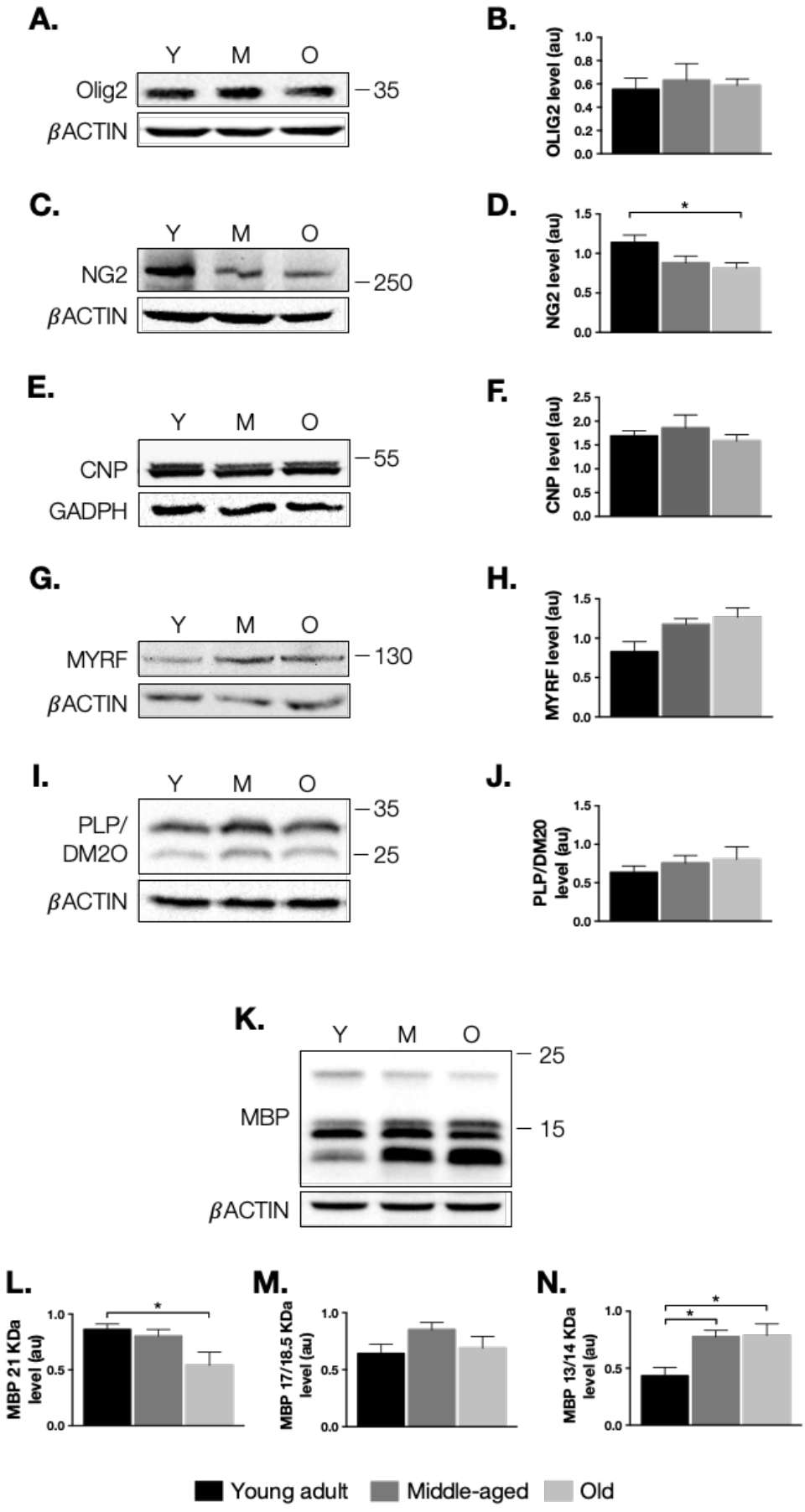

A.
Y M O
Olig2
βACTIN
35
B.
OLIG2 level (au)
C.
NG2
βACTIN
250
D.
NG2 level (au)
*
E.
CNP
GADPH
55
F.
CNP level (au)
G.
MYRF
βACTIN
130
H.
MYRF level (au)
I.
PLP/
DM2O
βACTIN
35
25
J.
PLP/DM20 level (au)
K.
MBP
βACTIN
25
15
L.
MBP 21 KDa level (au)
M.
MBP 17/18.5 KDa level (au)
N.
MBP 13/14 KDa level (au)
Young adult
Middle-aged
Old

Figure 7.13. Age-related changes in the levels of different oligodendrocyte differentiation markers. (A) Representative image of immunoblot for Olig2 and the loading control β-ACTIN. OlgG2 runs at an approximated MW of 32 KDa. (B) Comparison of densitometric analysis of Olig2 relative to the loading control showed no age-related differences. (C) Representative image of immunoblot for NG2 and the loading control β-ACTIN. NG2 runs at an approximated MW of 300 KDa. (D) Comparison of densitometric analysis of NG2 relative to the loading control showed an age-related decrease in the protein level. (E) Representative image of immunoblot for CNP and the loading control GADPH. CNP is detected as a double band at 46 and 48 KDa. (F) Comparison of densitometric analysis of CNP relative to the loading control showed no age-related differences. (G) Representative image of immunoblot for MYRF and the loading control β-ACTIN. MYRF runs at an approximated MW of 123 KDa. (H) Comparison of densitometric analysis of MYRF relative to the loading control showed tendency to increase with age. (I) Representative image of immunoblot for PLP and the loading control β-ACTIN. PLP and its spliced isoform DM20 can be detected at 30 KDa and 26 KDa, respectively. (J) Comparison of densitometric analysis of PLP/DM20 relative to the loading control showed a tendency to increase with age. (K) Representative image of immunoblot for MBP and the loading control β-ACTIN. A total of five MBP spliced isoforms are detected with different MW: 13, 14, 17, 18.5 and 21.5 KDa. (L) Comparison of densitometric analysis of 21 KDa MBP isoform relative to the loading control showed a significant decline with age. (L) Comparison of densitometric analysis of 17/18.5 KDa MBP isoforms relative to the loading control showed no age-related differences. (L) Comparison of densitometric analysis of 17/18.5 KDa MBP isoforms relative to the loading control showed a significant age-related increase. The letters above the immunoblot lanes refer to the different age groups, young adult (Y), middle-aged (M) and old (O). Numbers indicate bands of the molecular weight marker used as a size standard (KDa). The column colours in the graph represent the three age groups: young adult (black), middle-age (dark grey) and old (light grey). Data is represented as mean ±SEM and a minimum of 6 animals per age group were analysed. All significance testing was performed using one-way ANOVA followed by Tukey post-hoc test (*$p<0.05$).

7.4. Discussion

In healthy ageing, decline in neuronal functions has been linked to myelination defects, including myelin disruption, decreased expression in myelin proteins and changes in oligodendrocyte number (Psachoulia et al. 2009; Sim et al. 2002). However, the myelination pattern and the myelin dynamics in the hypothalamus throughout life remain unexplored. In this study, characterisation of the myelin pattern in young adult, middle-aged and old animals revealed that myelin microstructure in the MBH is impaired with age. Investigation of the myelin-producing cells showed an age-related increase in total and mature OL numbers. In addition to increase, hypothalamic OLs seem to retain their capacity to differentiate and are able to produce the major myelin components PLP and MBP throughout life. In contrast, the expression of the motor proteins KIF1B kinesin and DYNC1H1 dynein, involved in Mbp trafficking, are reduced in the aged hypothalamus. The loss of myelin could lead to defective neuronal connectivity and, ultimately, axonal degeneration with increasing age.

7.4.1. Age-related defects in the myelin microstructure in the mediobasal hypothalamus are associated to ectopic expression of MBP and inhibition of intracellular transport in oligodendrocytes

To characterise the myelin pattern in the hypothalamus and any age-related changes, in this study immunohistochemistry for MBP was performed in brain sections of young, middle-aged and old mice. MBP immunostaining allows for the rapid and efficient evaluation of age-related changes in myelin structure and is an excellent alternative approach to electron microscopy (Xing et al. 2012).

In the hypothalamus, MBP immunohistochemistry revealed relatively high densities of myelinated fibres in the basal Arc nucleus and the ME. The localisation of the myelinated axons in the hypothalamus was maintained across the lifespan, however, the integrity of myelin microstructure in the Arc-ME was affected with age. Myelin sheath disorganisation is first observed in middle-aged animals, although their frequency of occurrence increased with age. In line with these findings, the progressive loss of myelin and degeneration of myelinated nerve fibres has been extensively described in healthy ageing and in neurodegenerative diseases (Bartzokis 2011; Bowley et al. 2010; Braak

and Del Tredici 2004; Peters 2002; Peters et al. 2000; Sandell and Peters 2001; Wang et al. 2020; Xing et al. 2012). Also, the capacity to replace lost or damaged myelin progressively declines as animals grow older (Ibanez et al. 2003).

In the current study, myelin microstructure defects included myelin circumferences in the Arc-ME of aged animals that were never present in the young group. The aberrant myelin pattern could be a consequence of ectopic expression of myelin proteins and/or redundant myelin production, that lead to the formation of myelin-like membranes that do not ensheath axons. In support of this hypothesis, MBP expression was observed surrounding Olig2+ nuclei, in contrast to previous reports where this protein is restricted to myelinating processes (R. M. Gould, Freund, and Barbarese 1999; Lyons et al. 2009; O'Connor et al. 2000). Ectopic accumulation of MBP may exert deleterious effects for the OL function and affect the formation and maintenance of the myelin sheath. Indeed, altered intracellular localisation of myelin proteins has been reported in the *taeip* rat, a myelin mutant that presents characteristics of hypomyelination (O'Connor et al. 2000). Also, the results support the production of redundant myelin in the aged hypothalamus, a common age-related myelin defect (Peters 2002; Sandell and Peters 2001). Co-localisation studies of MBP with the axonal marker NF identified MBP+ circumferences with different diameter, among normal myelinated structures filled by NF labelling. In other cases, the myelin sheath in cross-sections appeared too large for the size of the enclosed axon. It is, therefore, possible that the presence of redundant myelin may be the result of continued formation of myelin. However, failure to assemble and elaborate the correct amount of myelin around axons may underlie the aberrant pattern observed in aged animals.

The process of myelin sheath formation requires a timely and spatially controlled transport of the different myelin components from the OLs to the expanding myelin sheath. The Mbp mRNA trafficking occurs through a multi-step pathway, including assembly into granules, transport along the processes, and localization within the myelin compartment (Ainger et al. 1993). The Mbp mRNA transport relies on microtubules, and the RNA-binding motor proteins KIF1B and dynein are required (Barbarese et al. 1999; Herbert et al. 2017; Lyons et al. 2009). The RNA-seq results in *Chapter 4* showed downregulation of Kif1b and the three subunits of the dynein Dync1H1, Dync1LI and Dync2H1, in the aged hypothalamus. Further validation via western blot analysis confirmed the decreased expression of KIF1B and DYNC1H1 at protein level. Hence, it

is possible that altered expression of molecules that regulate and coordinate the intracellular trafficking of Mbp could affect the myelin biogenesis. Indeed, studies in zebrafish identified that KIF1B and Dynein mutants showed a arrested Mbp transport OL perikaryon that resulted in myelination defects (Herbert et al. 2017; Lyons et al. 2009).

Myelin wraps around axons, thereby facilitating rapid saltatory conduction while protecting axons form degeneration. In the MBH, terminals from VSP and OXT neurons are myelinated (Yin and Gore 2010). The age-related aberrant myelination could reduce the conduction velocity, affect axonal connectivity, and ultimately, result in degeneration of myelinated nerves crossing the hypothalamus. Indeed, attenuation of POMC electrical responses has been reported in aged rats (Newton et al. 2013). Importantly, NFs are major determinants of axonal calibre, and decreased NFs expression has been associated with axonal atrophy (Parhad et al. 1995; Uchida et al. 2001). In line with this, results presented in this study showed that NFM protein levels in the hypothalamus are reduced with age.

7.4.2. Oligodendrocyte number, but not the differentiation along the oligodendrocyte lineage, seems to be altered in the aged hypothalamus

To address if the aberrant myelin pattern observed in the aged hypothalamus involved the dysfunction or loss of the myelin-producing cells of the CNS, the OL number and differentiation along the OL lineage was investigated across the mouse lifespan. In addition, the proliferative capacity of hypothalamic OPC was investigated in the *Chapter 6*, identifying an age-related decrease. In the hypothalamus, OPCs hold a critical role in preserving neuronal processes, and short-term OPC elimination leaded to permanent neuronal damage in the ME (Djogo et al. 2016). However, how the decreasing rate of OPC cell division affects directly the myelin production in the hypothalamus is still unknown. Life-long generation of OLs is required for myelination of new neuronal connections and repair of myelin lost through normal 'wear and tear' (Peters et al. 2000; Rivers et al. 2008). It is, therefore, possible that decline in the OPC proliferation contributes to reduced generation of new OLs, in turn promoting deficient myelination in the aged hypothalamus.

First, to address if the aberrant myelin pattern observed in aged animals was due to changes in OL number, immunohistochemistry for Olig2 (pan OL marker) and CC1 (mature-myelinating OL marker) was performed in brain sections of young, middle-aged and old animals. Coincidentally with the myelin distribution, Olig2+ and CC1+ showed a higher density in the Arc-ME region, and quantification of Olig2+ and CC1+ cells in the ME showed an increase in the number with both markers. This observation indicates that hypothalamic OLs are probably long-lived cells, as previously reported in the corpus callosum from mouse and humans (Tripathi et al. 2017; Young et al. 2013). Thus, the increase in number could be due to accumulation of adult-born OLs, that may augment the pre-existing population rather than replacing lost cells (Tripathi et al. 2017). Also, the increase in OL number has been linked to myelination defects. Previous reports demonstrated that dysregulation of OL number relative to target axons can lead to aberrant myelination patterns and ectopic myelin deposition (Almeida et al. 2018), similar to the defects reported in the present study.

Second, to address if myelin defects could be attributed to the inefficient or interrupted differentiation of OPCs, the expression of multiple stage-specific markers was investigated. Arrest in a pre-myelinogenic stage will translate in altered levels of OL lineage markers. In regard to this possibility, the RNA-seq data in *Chapter 4* showed an upregulated expression of the mRNA encoding myelin-related proteins expressed by OLs, including Cnp, Myrf, Plp1 and Mbp (Aggarwal et al. 2011; Bujalka et al. 2013). However, although transcriptomic studies provide valuable insights, most transcripts require translation to protein to deliver biological function of the expressed gene (Liu, Beyer, and Aebersold 2016). Thus, in the present chapter, the expression of OL stage-specific proteins was inspected via western blot in hypothalamic extracts of young, middle aged and old animals. Given that the whole hypothalamus was dissected out for protein extraction, western blot results will represent the total hypothalamic levels of the proteins analysed, and regional differences in the Arc-ME may be diluted or not represented.

Western blot results for the OPC maker NG2 showed lower protein levels with age concurring with to the decline in the number of actively proliferating OPCs described earlier in *Chapter 6*. However, no differences were detected for the pan OL marker Olig2, and the myelin-associated enzyme CNP expressed by both, pre- and myelinating OL (Goldman and Kuypers 2015). Interestingly, the mature myelinating protein MYRF

showed an increase in expression with age. MYRF is essential for the myelination process and, evidence suggest that is an important factor in the terminal OL differentiation (Emery et al. 2009; Koenning et al. 2012). Also, as a transcriptional factor MYRF regulates the expression of myelin genes such as, Plp1 and Mbp (Bujalka et al. 2013; Koenning et al. 2012). In turn, the Plp1 gene encodes the two isoforms PLP and DM20, which represent the predominant protein portion of myelin in the CNS (McLaughlin et al. 2002) and showed no age-related differences.

In regard to MBP, the relative amounts of MBP isoforms are affected with age. In rodents, MBP protein has 5 different isoforms consequence of alternative splicing of the same mRNA that translate in proteins with different molecular weights: 21.5, 18.5, 17, 14 and 13 KDa (Akiyama et al. 2002). Reduction in MBP expression has been reported across multiple brain regions with age, in both rodents and humans (Ansari *et al.*, 1985; Xing *et al.*, 2012; Xie *et al.*, 2013, 2014; Ahn *et al.*, 2017; Wang *et al.*, 2020); however, most of these studies have not examined the expression of the different MBP isoforms individually. In contrast, the present study identified age-related changes in the expression in the MBP isoforms, with decreased expression of the 21 KDa isoform and increase of the 13/14 KDa isoforms, that become the most abundant in the ageing hypothalamus. MBP acts as an important spatial and temporal regulator of myelination, by triggering disassembly of the actin cytoskeleton to promote initiation of myelin membrane wrapping (Zuchero et al. 2015). The exact role of each isoform during the formation and compaction of the myelin sheath is still unknown, although it has been reported that the 18.5 KDa and 14.0 KDa are predominant during active myelination in the rat brain (Akiyama et al. 2002). Interestingly, differences in both size and relative amounts of MBP isoforms have been reported between normal and myelin-deficient *jimpy* mouse (Fannon and Moscarello 1990). Thus, the age-related increased expression of the 14 KDa isoform may play a role in the aberrant myelination observed.

In summary, the number of OL, but not the differentiation along the OL lineage, seems to be altered in the aged hypothalamus. Potentially, an increase in OL number may lead to mistargeting of myelin to cell bodies, including those of neurons (Almeida et al. 2018). In addition, the relative amounts of MBP isoforms suffer an age-related change, although the functional consequences of this switch will require further investigation.

8. General discussion

Ageing is characterised by a progressive loss of physiological functions and constitutes the leading risk factor for the development of neurodegenerative diseases (López-Otín *et al*., 2013). Ageing represents a major health issue worldwide that is only expected to escalate due to the remarkable increase in life expectancy of the population. According to the World Health Organisation (WHO), the world's population over 60 years will nearly double from 12% to 22% between 2015 and 2050.

Even healthy people experience a decline in several physiological functions with age, including energy homeostasis, hormonal regulation, circadian rhythm, reproduction, and cognition. Interestingly, the majority of the physiological functions that deteriorate with age are controlled by the hypothalamus (Kim and Choe 2018). Therefore, in the recent years, the role of the hypothalamus as a central orchestrator of the systemic ageing has gained interest (Chen et al. 2015; Zhang et al. 2013, 2017).

The present study aimed to uncover the underlying cellular and molecular mechanisms involved in the age-related (Tang et al. 2015; Zhang et al. 2013, 2017)(Tang et al. 2015; Zhang et al. 2013, 2017)(Tang et al. 2015; Zhang et al. 2013, 2017)(Tang et al. 2015; Zhang et al. 2013, 2017)alterations occurring in the hypothalamic circuits that control energy balance. Main findings included age-related changes in appetite-regulating neuron number, inflammation, loss of stem cells and myelin defects in the hypothalamus.

8.1. Age-mediated inflammation mediated by astrocyte and microglia activation may damage the brain circuits that regulate energy balance

The hypothalamus plays a critical role in the control of energy homeostasis via the opposing action of two defined neuronal types, POMC and NPY/AgRP, that can directly respond to peripheral hormonal and nutritional status (Blouet and Schwartz 2010; Dietrich and Horvath 2013). Studies in rodents have shown that dietary excess and central administration of glucose or lipids lead to the activation of the NF-κB pathway

and the expression of pro-inflammatory genes (Berkseth et al. 2014; Posey et al. 2009; Valdearcos et al. 2017; Zhang et al. 2008). Hypothalamic inflammation disrupts key signalling pathways involved in the regulation of energy balance, glucose homeostasis and blood pressure, and participates in the development of metabolic syndrome components such as obesity, glucose intolerance, and hypertension (Cai and Khor 2019; Calegari et al. 2011; Cazettes et al. 2011; Purkayastha and Cai 2013).

Recent evidence indicates that hypothalamic inflammation is also crucial for the development of whole-body ageing (Tang et al. 2015; Zhang et al. 2013, 2017). In mice, systemic inflammation associated with age leads to NF-κB activation in microglia of different brain areas, including the hypothalamus (Korhonen, Helenius, and Salminen 1997; Zhang et al. 2013). Notably, ageing delay and lifespan extension have been achieved in mice through prevention of hypothalamic NF-κB activation (Zhang et al. 2013). Also, ageing correlates with a decline in the hypothalamic GnRH expression in mice, given that activated NF-κB inhibits the expression of GnRH. In turn, GnRH therapy promotes adult neurogenesis and deaccelerates age-related phenotypes (Zhang et al. 2013). However, future studies will be required to uncover the effect of NF-κB activation/inhibition on other neurotransmitters and in the neuronal populations present in the hypothalamus.

In the present study, results indicated that reactive gliosis, involving both microglia and astrocytes, develop gradually in the hypothalamus with age. Also, a neuroinflammatory response represented by the upregulation of inflammatory genes is observed in the aged hypothalamus, including upregulation of the complement pathway, MHC genes and inflammatory cytokines (see *Chapter 4*). In general, the inflammatory response is a result of NF-κB activation, given that the majority of the immune signalling and inflammatory responses genes are under NF-κB transcriptional control (Tilstra et al. 2011). In the hypothalamus, the age-related NF-κB activation is initially restricted to astrocytes and microglia cells (Zhang et al. 2013), known to be the primary source of a wide range of inflammatory mediators (Clarke et al. 2018; Colombo and Farina 2016; Pekny and Nilsson 2005). It is possible, therefore, that microglia and astrocyte activation is underlying the exacerbated neuroinflammatory response observed with age.

Inflammatory responses mediated by microglia and astrocytes can impair neuronal functions, limit axonal regeneration, decrease neurogenesis, and induce myelin defects

(Menet et al. 2003; Pekny and Nilsson 2005). Interestingly, within the hypothalamus, the physiological effects of neuronal NF-κB activation appear to be cell-type dependent (Purkayastha et al. 2011). TNFα has two known receptors: TNFα receptor 1 (TNFR1) and TNFα receptor 2 (TNFR2) (Wajant, Pfizenmaier, and Scheurich 2003). TNFR1 is barely detected in hypothalamic neurons; in turn, TNFR2 expression is detected in POMC neurons but hardly observed in NPY/AgRP neurons (Purkayastha et al. 2011). The differential expression pattern may suggest that POMC neurons are more sensitive to neuroinflammation mediated by NF-κB activation. In line with this, previous studies in rodents linked diet-induced inflammation with a reduction of hypothalamic POMC neurons but not NPY/AgRP (Li et al. 2012; Thaler et al. 2012), mimicking the results presented in *Chapter 3*. Diet-induced hypothalamic inflammation negatively impacts neurogenesis (Li et al. 2012), and recently, it has also been associated with myelin disruption (Huang et al. 2019). In turn, the age-related myelin breakdown contributes significantly to the wear and tear of microglia, which may exacerbate microglia senescence and immune dysfunction in the ageing brain (Safaiyan et al. 2016). Similarly, the present study showed that age-related inflammation in the hypothalamus is accompanied by a decreased proliferation capacity of stem/progenitor cell niche (*Chapter 6*) and myelination defects (*Chapter 7*).

Age-related activation of inflammatory responses can damage cellular functions and be detrimental for the correct functioning of the hypothalamic circuits; however, it is still unclear how microglial and astrocyte activation is initially triggered in the ageing hypothalamus. Different mechanisms are reported to contribute to hypothalamic inflammation, including mitochondrial dysfunction (Drougard et al. 2015; Tang et al. 2015), defective autophagy (Kaushik et al. 2012; Meng and Cai 2011) and intracellular RNA stress response (Yan et al. 2014). It is also possible that dietary stressors and tissue changes during ageing, such as adiposity and immune system dysfunction, also contribute to hypothalamic inflammation (Burfeind, Michaelis, and Marks 2016; Deleidi et al. 2015; Kuk et al. 2009). One of the most popular hypothesis suggests that accumulation of oxidative damage over time mediates microglia and astrocyte activation (Norden and Godbout 2013). In support of this, the hypothalamus from aged mice showed increased expression of enzymes involved in mitochondria respiratory chain compared to young animals (Jiang et al. 2001), suggesting an increased hypothalamic ROS production with age. Also, elevated oxidative stress (increased generation of free

radicals and lipid peroxidation levels), along with impaired antioxidant defences has been reported in the hypothalamus of aged rats (Rodrigues Siqueira et al. 2005).

Regardless of insufficient understanding as to its primary causes, age-related hypothalamic inflammation mediated by astrocytes and microglia may contribute to the decline in energy homeostasis with age and mediate the development of the metabolic syndrome (Purkayastha and Cai 2013; Purkayastha et al. 2011; Tang et al. 2015; Zhang et al. 2008). Therefore, strategies that target hypothalamic microglia/astrocyte activation may delay the development of age-related conditions and increase the lifespan.

8.2. Age-dependent changes in hypothalamic POMC neurons may contribute to the altered energy balance observed with age

In this study, an age-dependent remodelling of the hypothalamic neuronal circuits reported, accounting for one potential mechanism to explain the age-dependent decline in energy homeostasis. Main findings include: (1) The number of POMC neurons, but not NPY, decline with age; (2) POMC neuronal decline is not associated to cell death, however, ageing could be influencing silencing of Pomc expression in a specific subset of anorexigenic neurons.

The specific reasons underlying POMC neuronal decline are uncertain, although age-associated inflammation mediated via NF-κB may play a role (Zhang et al. 2013). In fact, *in vitro* studies revealed that Pomc gene is a downstream target of NF-κB (Shi et al. 2013). In addition to inflammation, the elevation of mTOR activity has also been associated with the deterioration of POMC neurons during ageing (Yang et al. 2012). Elevation of mTOR signalling by targeted deletion of the negative regulator Tsc1 in POM*C* neurons caused cell hypertrophy, silenced neuronal activity and reduced neurite projection to the PVN, contributing to age-dependent obesity (Mori et al. 2009; Yang et al. 2012). However, targeted Tsc1 deletion in NPY/AgRP neurons did not affect their size or firing rate (Yang et al. 2012). In old mice, the central administration of rapamycin, an inhibitor of mTOR pathway, enhanced the firing and neurite projections of POMC neurons, causing a reduction of food intake and body weight (Yang et al. 2012). Reportedly, rapamycin significantly extends the median and maximal lifespan in mice when fed at 20 months of age (Harrison *et al*., 2009).

Recent studies also revealed that POMC neurons exhibit increased sensitivity to age-associated reduced autophagy, leading to decreased lipolysis and α-MSH levels (Kaushik et al. 2012). In mice, selective deletion of essential autophagy genes in POMC neurons affected axonal growth and caused metabolic defects often associated with ageing, including obesity, adiposity, and glucose intolerance (Coupé et al. 2012; Kaushik et al. 2012; Quan et al. 2012). Interestingly, hypothalamic inflammation has been linked to autophagic dysfunction, at least partly, due to the effect of deficient hypothalamic autophagy in promoting obesity (Meng and Cai 2011).

In summary, given that POMC neurons are critical regulators of energy homeostasis, dysfunction of these cells likely represents the cause of the metabolic deregulation observed with age. Thus, it is possible that the decline in POMC neuronal number reported in this study, coupled with decreased autophagy, increased mTOR signalling and higher sensitivity of these cells to neuroinflammation (described in the previous section) underlies the ageing-related shift in energy homeostasis.

8.3. Ageing exacerbates myelin disruption and axon injury in the mediobasal hypothalamus

Although the loss of myelinated nerve fibres has been reported in multiple brain regions during normal ageing and in neurodegenerative diseases (Bartzokis 2011; Braak and Del Tredici 2004; Peters 2002; Peters et al. 2000; Sandell and Peters 2001; Xing et al. 2012), this study represents the first attempt to characterise the myelin dynamics in the ageing hypothalamus.

The present study identified an aberrant myelination pattern in the hypothalamus with age together with an age-related increase in the number of total and mature myelinating OLs in the MBH. Moreover, hypothalamic OLs seem to retain their capacity to differentiate and are able to produce the major myelin proteins PLP and MBP throughout life. Interestingly, the expression of the motor proteins KIF1B kinesin and Dynein, essential for Mbp trafficking and myelination *in vivo* (Herbert et al. 2017; Lyons et al. 2009), are reduced in the aged hypothalamus (*see Chapter 7*). These results suggest that inhibition of the transport, rather than the reduced expression of myelin proteins,

may contribute to the aberrant myelination reported with age. Defects in the transport of myelin membrane components have been linked to myelin dysfunction in demyelinating diseases, including MS (Aulchenko et al. 2008).

The remyelination capacity has been shown progressively decline as animals grow older (Ibanez et al. 2003) and is associated with changes in the inflammatory response (Graf et al. 2016; Hinks and Franklin 2000). The proliferative capacity of hypothalamic OPC was also investigated in *Chapter 6*, identifying an age-related decrease. In the hypothalamus, OPCs also hold a critical role in preserving neuronal processes, and short-term OPC elimination led to permanent neuronal damage in the ME (Djogo et al. 2016). Interestingly, in diet-induced mouse models, hypothalamic NF-κB activation led to reduced OPC proliferation and reduced production of new OLs in the hypothalamus (Li et al. 2012). Also, impaired myelin microstructure in the hypothalamus has been reported in diet-induced obesity models, due to pro-inflammatory cytokine expression and microglia activation (Huang et al. 2019).

Regardless of its causes, defective myelination may lead to interrupted neuronal communication and, ultimately, cause axonal degeneration (Stassart et al. 2018). In line with this, results in *Chapter 4* revealed reduced expression of genes involved in neuronal cytoskeleton organisation, axonal guidance and transport in the aged hypothalamus. It is possible, therefore, that the loss of myelin and the neuronal processes in the hypothalamus could underlie the decline in metabolism and energy homeostasis observed during ageing. Thus, strategies that preserve OPC proliferation and reduce gliosis may help maintain the normal hypothalamic functioning during ageing.

8.4. Concluding remarks

The present project provides novel information on the age-related alterations which occur in the hypothalamic circuits that control energy homeostasis. The detailed characterisation of both appetite-regulating populations showed that the anorexigenic POMC, but not the orexigenic NPY, suffers a significant decline with age. Also, increased inflammation, mediated by activation of microglia and astrocytes, and release of pro-inflammatory factors was reported. Finally, defective myelin in the aged hypothalamus,

possibly due to the decline in OPC proliferation and oligodendrogenesis, may lead to interrupted neuronal communication and axonal degeneration.

Combined, the findings presented here may help improve the understanding of the different roles that hypothalamic neurons and glial cells may play during the ageing development. These results open the possibility that potential anti-ageing and ageing-control technologies to target the cellular and molecular mechanisms affected in the ageing hypothalamus.

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Annex

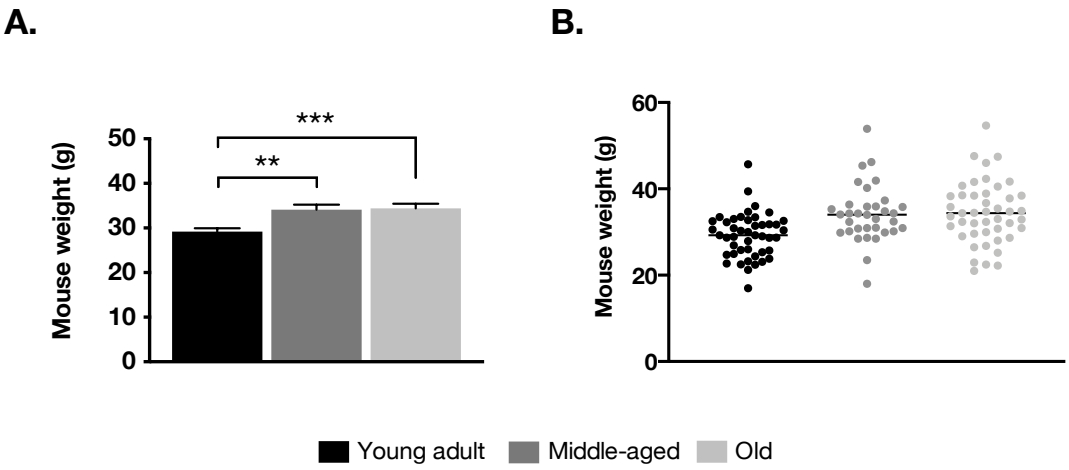

Figure S 1. Changes in mice weight with age. Graph (A) and Scatter dot plot (B) display body weight in young adult (black), middle-aged (dark grey) and old animals (light grey). Data in (A) is represented as mean ±SEM and a minimum of 35 animals per age group were analysed. Data in (B) is represented as the median and the range of mice body weight. All significance testing was performed using one-way ANOVA followed by Tukey post-hoc test (*p<0.05, **p<0.005, *p<0.0005). g – grams.

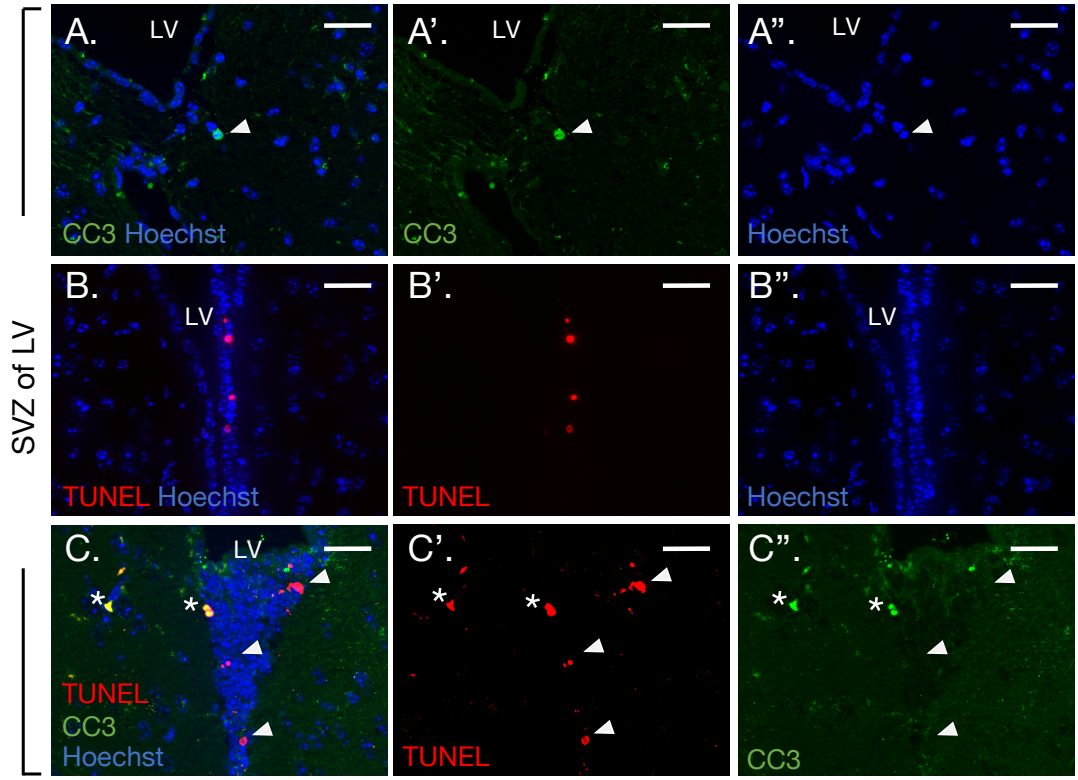

Figure S 2. Cleaved Caspase 3 (CC3) and TUNEL assay staining in the SVZ of lateral ventricles, a neural stem cell niche of the brain known to undergo cell division and apoptosis in adult mice. (A – A'') CC3 immunostaining (green) and Hoechst (blue) in the LV and surrounding parenchyma. White arrow signals a CC3+ cell and its apoptotic nucleus. (A – A'') CC3 immunostaining (green) and Hoechst (Blue) in the LV surrounding parenchyma. White arrow signals a CC3+ cell and its apoptotic nucleus. (B – B'') TUNEL staining (red) and Hoechst (blue) in the LV and surrounding parenchyma. TUNEL+ cells can be detected in the LV wall. (B – B'') CC3 immunostaining (green), TUNEL (red) and Hoechst (blue) in the LV and surrounding parenchyma. White arrows signal TUNEL+/CC3- cells and asterisks indicate TUNEL+ cells/CC3+. Scale bar 25 µm. LV – lateral ventricle. SVZ – subventricular zone

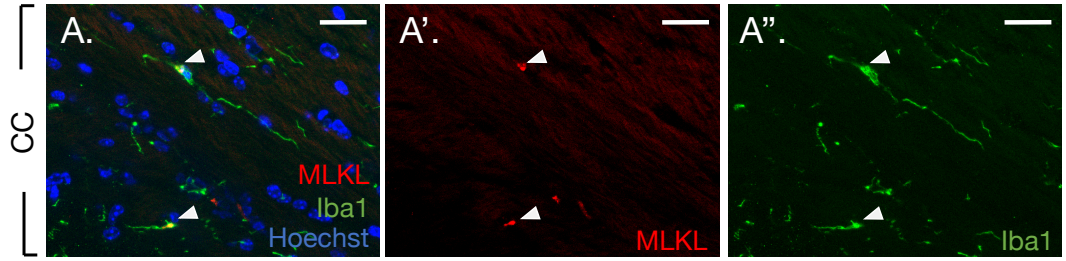

Figure S 3. Co-localization of the necrosis marker MLKL and the microglia marker Iba1 in the corpus callosum. (A – A'') MLKL immunostaining (red), Iba1 and Hoechst (blue) in the Corpus callosum area. White arrows signal MLKL+/Iba1+ cells. Experiments were performed in young (*n* = 3), middle-aged (*n* = 3) and old animals (*n* = 3). Scale bar 25 µm. CC – corpus callosum.

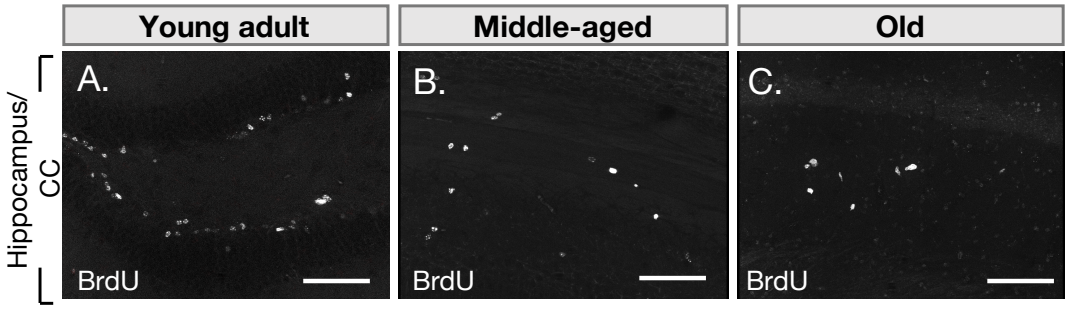

Figure S 4. Proliferative cells were observed in the hippocampus and corpus callosum of young, middle-aged and old animals. As a general observation, fewer BrdU+ cells were detected in the old animals. (A, B, C) v images of the from young-adult (A), middle-aged (B) and old (C) animals, a minimum of 3 animals were analysed per age group. Scale bar 50 µm. CC – Corpus callosum.

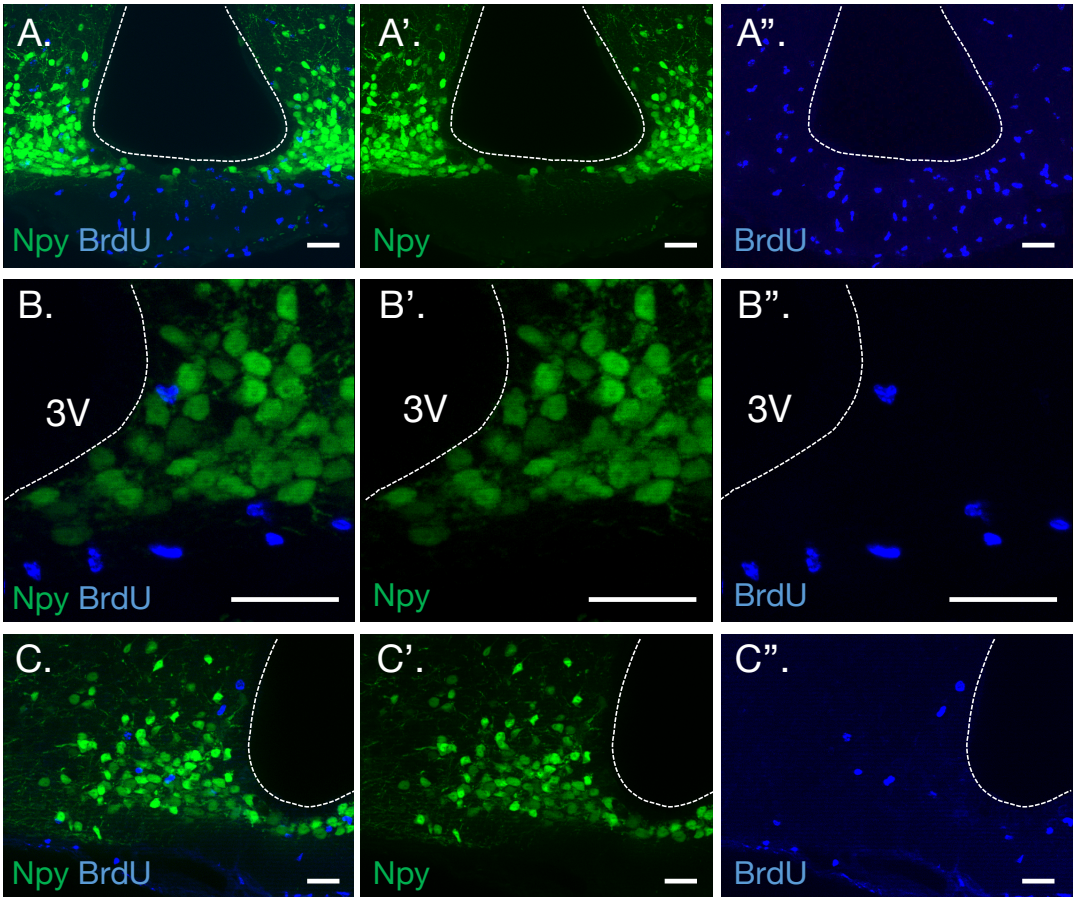

Figure S 5. BrdU+ cells do not co-localise with Npy-GFP+ cells. (A – A'') Npy-GFP+ (green) and BrdU+ (blue) cells do not show co-localization in Arc-ME in bregma -1.7 mm, approximated. (B – B''') High power of (A-A'') confirm the lack of co-localization in the Arc nucleus. (C – C'') Npy-GFP+ (green) and BrdU+ (blue) do not show co-localization in hypothalamus in bregma -1.46 mm, approximated. Dashed lines outline the borders of the 3V. The pictures shown were obtained from young animal tissue. Experiments performed in young, middle-aged and old animals with a minimum of 3 animals analysed per age group. Scale bar 50 µm. 3V – third ventricle.

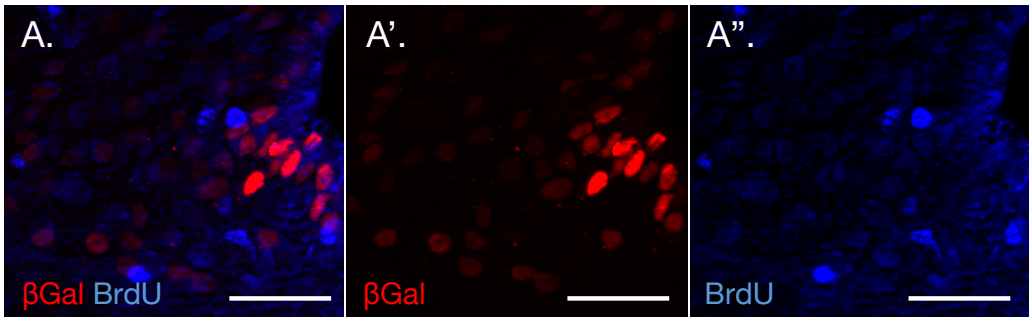

Figure S 6. BrdU+ cells do not co-localise with βGal cells. (A – A'') βGal (red) and BrdU+ (blue) cells do not show co-localisation in Arc in bregma -1.58 mm, approximated. (B – B''') Experiments performed in young, middle-aged and old animals with a minimum of 3 animals analysed per age group. Scale bar 50 µm.

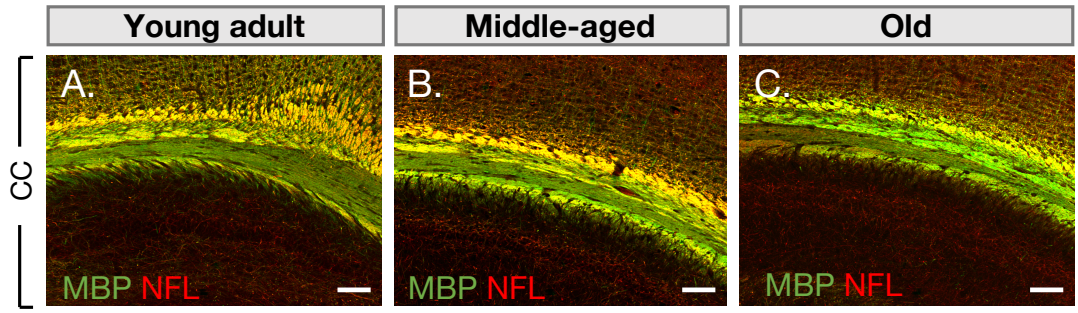

Figure S 7. CC1+ cells were observed in the h corpus callosum of young, middle-aged and old animals. Representative images of the from young-adult (A), middle-aged (B) and old (C) animals. CC1 immunostaining (red) and Hoechst (blue) in the Corpus callosum area. Experiments performed in young, middle-aged and old animals with a minimum of 3 animals analysed per age group. Scale bar 50 µm. CC - corpus callosum.

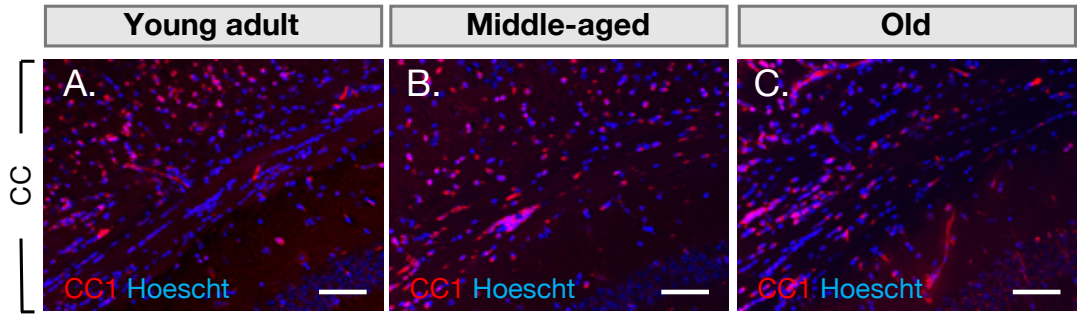

Figure S 8. CC1+ cells were observed in the corpus callosum of young, middle-aged and old animals. Representative images of the from young-adult (A), middle-aged (B) and old (C) animals for CC1 immunostaining (red) and Hoechst (blue) in the Corpus callosum area. Experiments performed in young, middle-aged and old animals with a minimum of 3 animals analysed per age group. Scale bar 50 µm. CC – Corpus callosum.