Neural Stem / Progenitor Cells in the Adult Mouse Hypothalamus

Thesis presented for the degree of PhD
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Abstract

Adult neural stem cells are now widely accepted to exist in the neurogenic regions of the subventricular zone and dentate gyrus; however there is increasing evidence to suggest that neurogenesis may also occur in other brain regions. It has been proposed that one such population of neural stem cells resides in the hypothalamus, more specifically in the ependymal lining of the third ventricle. In this thesis, I tested the hypothesis that stem cells exist in defined regions of the adult mouse hypothalamus.

My work confirms the presence of stem / progenitor cells in the adult mouse hypothalamus. Analysis of neural 'stem cell markers,' both in vivo and in vitro, suggests the presence of different populations of stem / progenitor cells occupying discrete territories of the ependymal zone. Some markers are common to those found in other adult neural stem cell niches, whilst others are unique to the hypothalamus. I have isolated hypothalamic stem / progenitor cells, and assayed their character and potential for both self-renewal and differentiation using the neurosphere assay. I show that hypothalamic neurospheres can be propagated in culture for extended periods of time, and that they can differentiate into cells of all three neural lineages. I have also determined the precise location of neurosphere-forming cells in the hypothalamus, showing that proliferative capacity is restricted to defined regions. Within these regions, I have also identified separate populations of proliferating cells that vary in their capacity for self-renewal, and correlated this with marker profiles. This data supports previous reports suggesting that tanycytes act as neural stem cells in the hypothalamus. Finally, I have started work investigating the control of hypothalamic stem / progenitor proliferation by fibroblast growth factors. I demonstrate that FGF signalling is necessary for in vitro proliferation. Finally, my studies suggest that endogenous FGFs may regulate hypothalamic stem cell proliferation.

Chapter 1

Chapter 1 - Introduction

1.1 Normal Development of the Hypothalamus in the Anterior Neural Tube

The development of the nervous system appears to be relatively conserved between different vertebrate species. During gastrulation, underlying mesodermal tissue induces the overlying medial area of ectoderm to become a single columnar epithelial layer know as the neural plate [1]. Neural induction is thought to involve the sequential action of fibroblast growth factors (FGFs) and antagonists of bone morphogenetic proteins (BMPs), such as noggin and chordin, which prevent BMP signalling from inducing an alternative epidermal fate [2]. The neural plate then invaginates during neurulation, eventually forming the neural tube, with the roof plate dorsally and the floor plate at the ventral midline (Figure 1.1 A-C). The most anterior (future rostral) area of the neural tube becomes the prosencephalon, which will later form the forebrain, this is followed by the prospective-midbrain mesencephalon, then the rhombencephalon, which later develops into the hindbrain [3]. Most posteriorly (caudally) the spinal cord is formed (Figure 1.1 D). The prosencephalon can be divided into the telencephalon in the most anterior and dorsal region, while more caudally is the diencephalon. The hypothalamus forms from anterior ventral areas of the diencephalon whilst more dorsal and posterior regions form the thalamus; laterally the optic cups will go on to form the retina of the eyes. The anterior default theory of anterior-posterior (A-P) patterning suggests that neural tissue will develop anterior characteristics by default, unless it exposed to caudalising signals such as retinoic acid (RA), Wnts, BMPs or FGFs [2]. An early marker of anterior neural tissue is the transcription factor Six3, which is also expressed throughout the diencephalon.

The hypothalamus can be divided into two main developmental areas: ventral (hypothalamic floor plate derived tissue) and dorsal (basal plate derived tissue) (Figure 1.1 E). The dorsal region is $Shh^{+}/Nkx2.1^{+}$ and may contribute to the

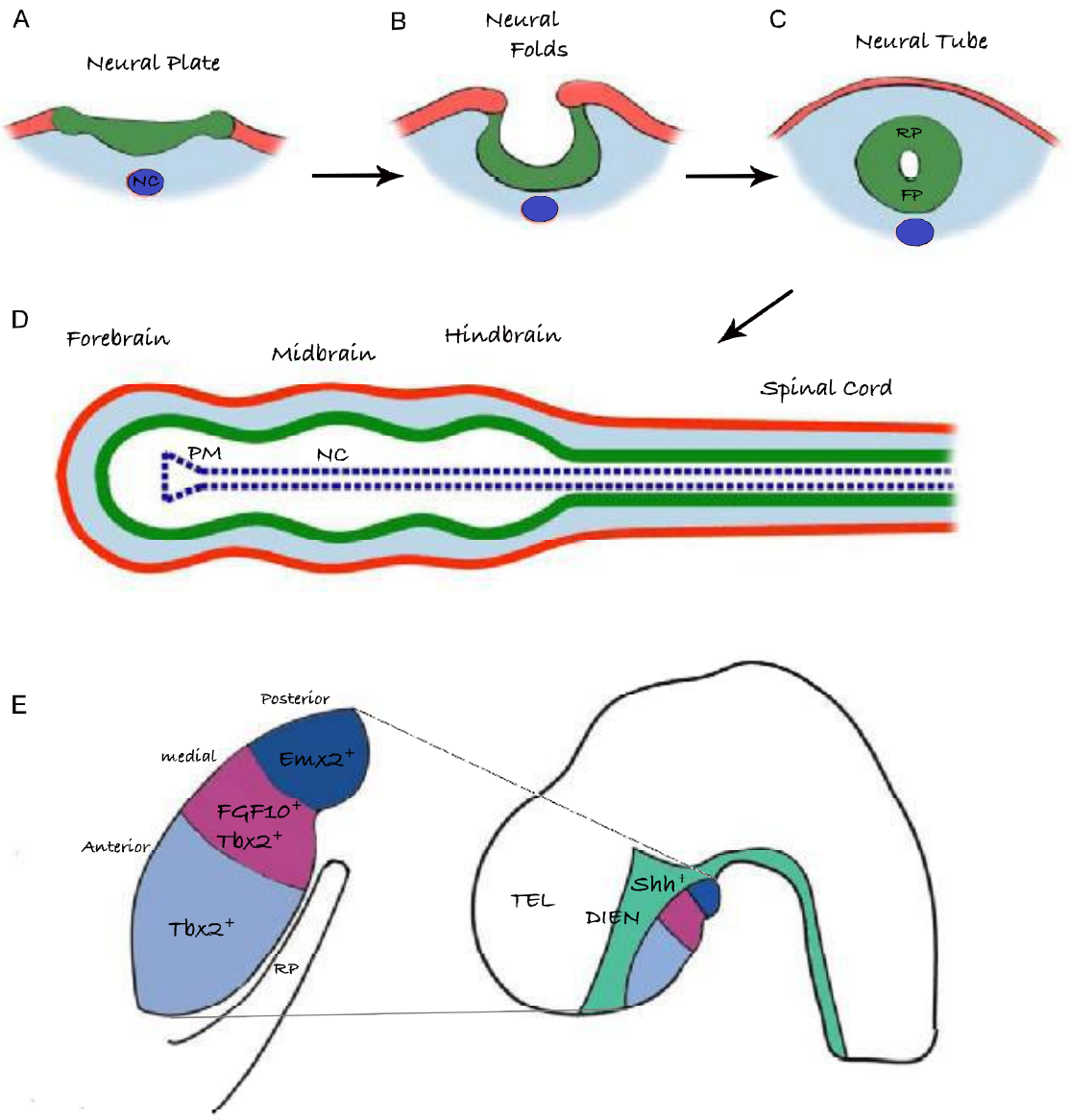

Figure 1.1: Development of the hypothalamus

A-D: Formation of the neural tube. A: Mesodermal (blue) signals induce the neural plate (green) neural tube (C). The floor plate (FP) is dorsal, and the floor plate (FP) is induced ventrally by signals from the notocord (NC, dark blue). D: The anterior end of the neural tube then widens into 3 vesicles, which will go on to become forebrain, midbrain and hindbrain. The dotted blue line indicates the position of underlying notocord and prechordal mesoderm (PM).

E: Specification of the hypothalamus. The hypothalamus forms from the ventral forebrain. The dorsal region is basal plate derived, and is induced to express Shh (green), while the ventral portion is derived from floor plate-like cells that downregulate Shh and upregulate Tbx2, FGF10 or Emx2 depending on anterior-posterior positioning (blue/pink). Ventrally, Ratheke's Pouch (RP) will go on to form the anterior pituitary gland. TEL = telencephalon, DIEN = diencephalon.

Adapted from C.Pearson (Unpublished)

retro- and supra-chiasmic nuclei [4]. Ventral hypothalamic cells originate from the same area as floor plate cells, but migrate rostrally [2]. These cells initially express Shh, but later downregulate it. This region probably gives rise to posterior and ventral hypothalamic nuclei in the adult. Evidence in the chick suggests that three anterior-posterior (A/P) domains exist – $Tbx2^+$ anterior and medial domains, and an $Emx2^+$ posterior domain (C. Pearson, unpublished). The medial area secretes FGF-10, and later gives rise to the infundibulum.

A variety of signalling factors are thought to be significant for development of the two regions of the hypothalamus. The floor plate-like cells are continuous with floorplate cells more caudally, and initially appear to be very similar in character. However, underlying the hypothalamic floor plate cells are a specialised mesodermal cell type known as prechordal mesoderm, which secretes factors including Sonic Hedgehog (Shh), Nodal and BMPs; these act as crucial inductive and patterning signals for the hypothalamic floor plate region. In particular, they mediate the transition from the Shh^+ floor plate-like identity to $Tbx2^+$ / $FGF10^+$ / $Emx2^+$ identity. Prior to their downregulation of Shh, the hypothalamic floor plate-like cells appear to secondarily induce expression of Shh in adjacent basal plate cells. It remains unclear whether Shh acts simply as a morphogen, as is the case in the posterior neural tube [5], or whether it acts in other ways, for example affecting proliferation. There may also be a significant role for the Wnt pathway, with evidence to suggest that Wnt inhibition induces hypothalamic identity, as well as being involved in later A-P regionalisation [6].

1.2 The Adult Hypothalamus

1.2.1 Structure and function of the adult hypothalamus

In the adult the hypothalamus is located in the ventral diencephalon, below the thalamus and on either side of the third ventricle (Figure1.2). Its nuclei occupy discrete anterior-posterior (A/P), dorsal-ventral (D/V) and medial-lateral (M/L)

territories: the M/L groupings are referred to as the periventricular, medial and lateral zones [3]. The median eminence (ME) protrudes from the ventral surface of the brain, merging into the infundibulum which connects the hypothalamus to the pituitary gland. The hypothalamus can be broadly considered as controlling body homeostasis via the integration of autonomic responses and endocrine function. This includes the regulation of blood pressure and electrolyte balance, body temperature, energy metabolism, reproduction and emergency stress responses [7].

In order to carry out these functions the hypothalamus receives a diverse range of inputs. It receives both direct and indirect sensory information including visceral, olfactory, visual and somatosensory inputs. It also receives afferents from other CNS regions including parts of the cortex and limbic system, in particular the hippocampus and the amygdala. The circumventricular organs located in the ventricles have no blood-brain barrier, allowing them to monitor the blood for factors such as toxins or osmolarity. In addition, lypophillic hormones such as thyroid hormones and steroids, and metabolic molecules like glucose, can directly access and influence hypothalamic cells.

Output from the hypothalamus includes both neuronal projections to other areas of the CNS and control of the endocrine system via the pituitary gland. Efferent connections can be both ascending to areas of the cortex or limbic system to influence behaviour, or descending to the medulla and spinal cord to act on the autonomic nervous system.

1.2.2 Hypothalamic Circuits:

One important role of the neuroendocrine hypothalamus is in the regulation of energy balance and feeding behaviour. Various nuclei at the level of the median eminence, including the arcuate (ARC), ventromedial (VMN), dorsomedial (DMN) and periventricular nuclei (PVN), and the lateral hypothalamic area (LHA), are involved in this circuit (Figure 1.3). Peripheral signals such as leptin,


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ghrelin and insulin indicate both the long and short-term energy status of the body [10]. The arcuate nucleus contains many leptin- and insulin-sensitive neurons. They can be split into two types – neuropeptide Y (NPY) releasing neurons, which also produce agouti related peptide (Agrp), and pro-opiomelanocortin (POMC)-containing neurons, which release a derivative of POMC known as melanocyte stimulating hormone (MSH). Both neural types project to all the other hypothalamic energy balance nuclei. The VMN is associated with satiety, as lesions can cause increased eating and obesity while stimulation has an opposite effect in decreasing appetite. The LHA is associated with hunger, as lesions can cause anorexia (loss of appetite) and adipsia (loss of thirst). It contains neurons that release two novel peptides: orexin (also known as hypocretin) and melanin concentrating hormone (MCH), both causing an increase in food intake. Second-order neurons from nuclei such as the VMN and LHA have a wide range of projections, both within the hypothalamus and to other regions such as the brainstem and cortex, which are important for implementing the homeostatic and behavioural responses required. Projections from the PVH influence both thyroid hormone and corticotrophin releasing hormone, with stimulation resulting in an increased metabolism and so higher energy expenditure. Thus a simple model of energy balance involves peripheral hunger and satiety signals acting on neurons of the ARC. Satiety signals reduce the release of NPY/Agrp whilst simultaneously increasing MSH released by projections to the LH, thereby reducing hunger. Conversely, hunger signals increase NPY release, and result in higher appetite and reduced energy expenditure (reviewed in [11]).

Control of the pituitary gland is divided into two distinct systems (Figure 1.4). Magnocellular neurons mainly located in the supraoptic and paraventricular nuclei send projections to the posterior pituitary, where they can release oxytocin and vasopressin (also know as antidiuretic hormone, or ADH) directly into the peripheral circulation. Oxytocin is involved with labour and lactation, while vasopressin is an antidiuretic responsible for water retention in the kidneys. The alternative system involves parvocellular neurosecretory neurons,

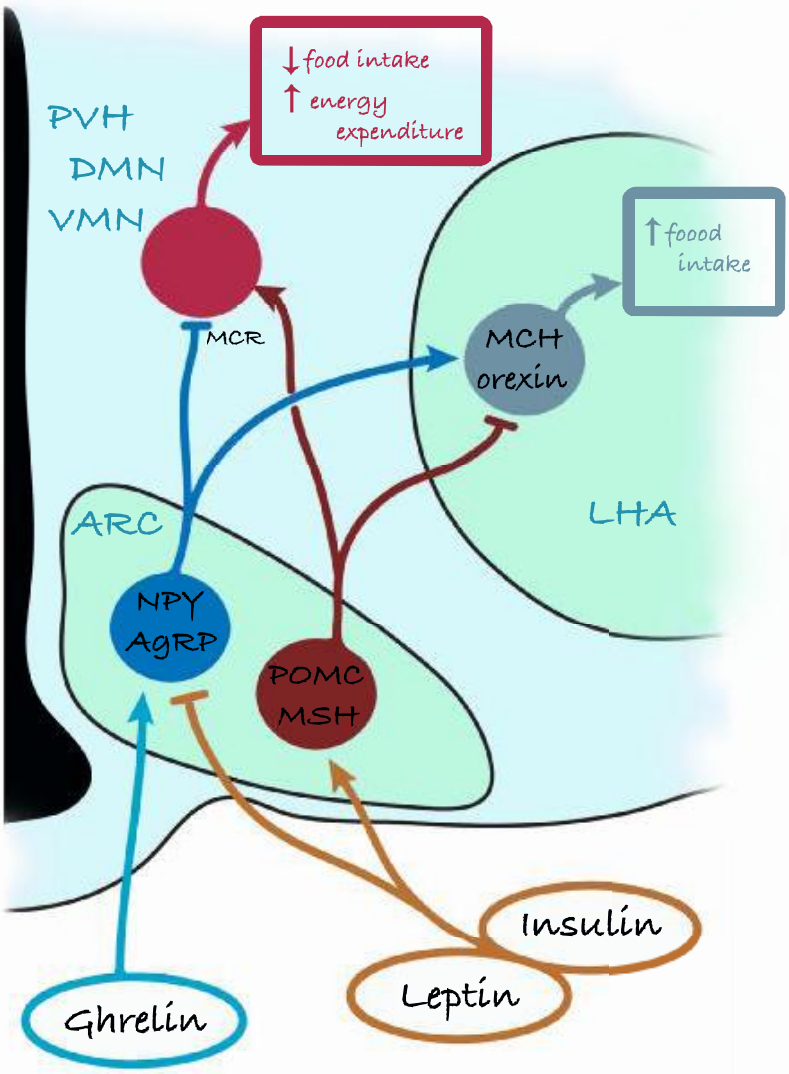

Figure 1.3: Hypothalamic energy homeostasis circuits.

neurons in other regions of the hypothalamus, leading to increased energy expenditure and a feeling of satiety. In the absence of food ghrelin from the stomach activates NPY/AgRP^{+} neurons, which project to MCH^{+} neurons in the lateral hypothalamus (LHA). These lead to feelings of hunger and increased food intake (blue circuit). Note that each pathway can inhibit the other at several stages.

PVH = paraventricular hypothalamus, DMN = dorsomedial nucleus, VMN = ventromedial nucleus.

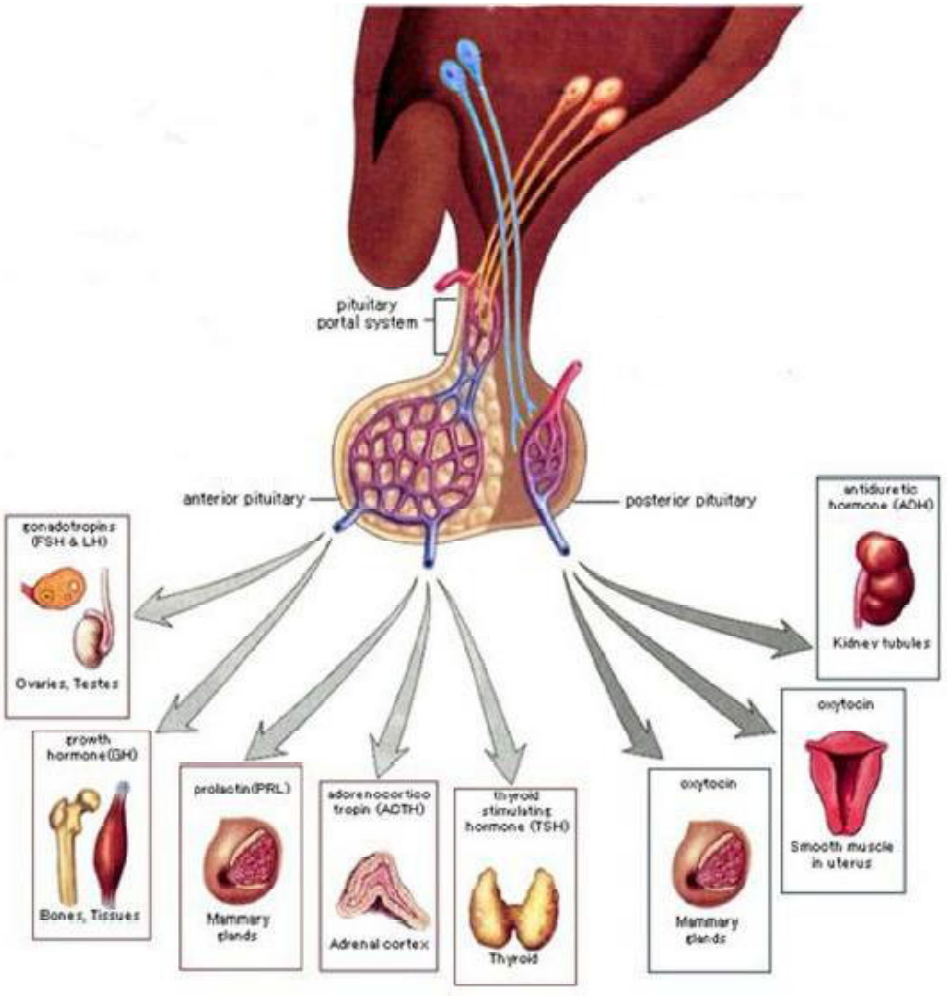

Figure 1.4: Hypothalamic control of pituitary hormones

Magnocellular (blue) neurons release oxytocin or ADH directly into the bloodstream of the posterior pituitary gland. Parvocellular (orange) neurons secrete releasing hormones into the portal veins of the pituitary, which carries them to the anterior lobe. Anterior pituitary cells respond by secreting their hormones directly into the systemic circulation.

Adapted from [12]

mainly located near the ventricle such as the pre-optic, arcuate, periventricular and paraventricular nuclei. These cells produce releasing (or prolactin inhibiting) hormones that are deposited into the portal veins of the infundibulum, which transports them to the anterior lobe of the pituitary. Here they control the secretion of a range of pituitary hormones such as thyrotropin, corticotropin, FSH, LH, growth hormone and prolactin.

1.3 Tanycytes

Tanycytes are a unique hypothalamic cell type, found in spatially restricted areas of the ventricular lining (reviewed in [13]). They are morphologically and functionally distinct from the ependymocytes that line the rest of the CNS ventricular system. Whilst they share some properties of both astrocytes and radial glial cells they display a unique set of morphological and functional characteristics. They are conventionally divided into 4 distinct subtypes, each with different properties and markers.

1.3.1 Location of tanycytes

Tanycytes are found in the ventral portion of the hypothalamus, at the approximate anterior/posterior (A/P) level of the median eminence. Their cell bodies are located in the ependymal zone surrounding the third ventricle, and they extend a long process into the parenchyma that comprises the non-ventricular regions of the hypothalamus. The four different subtypes are found sequentially along the dorsoventral axis (Figure 1.5). Each is defined by the target of its projection:

- **α1** are the most dorsally-located group, and project to the ventromedial nuclei of the hypothalamus.
- **α2** tanycytes lie adjacent to the arcuate nucleus, which is also the target of their projections. They can be further subdivided into dorsal and

ventral groups on the basis of marker expression (D McNay, personal communication).

- **β1** tanycytes have their cell bodies in the lateral evaginations of the infundibular recess. Their basal processes are found not on a neuronal target, but in the perivascular space surrounding the portal capillaries found in the lateral median eminence.
- **β2** tanyctyes are the ventral-most group, with their cell bodies in the floor of the infundibular recess. They project to the portal capillaries of the medial zone of the median eminence.

Dorsal to all the tanycyte subtypes, the ependymal zone of the third ventricle contains only specialized ependymal cells known as ependymocytes. These are a columnar ciliated epithelium without the long basal process found on tanycytes [14]. For clarity, throughout the rest of this thesis the terms 'ependymal cells' or 'ependymal lining' will be used as a non-specific term that encompasses both tanycytes and ependymocytes. Ependymocytes will always be referred to specifically by that name.

1.3.2 Development, radial glia and tanycytes

During embryonic development, two separate types of stem cell population can be distinguished: ventricular zone cells, regulated by FGF-2, and subventricular zone cells that respond to EGF (reviewed in [15]). The ventricular zone appears first, but regresses throughout development until it finally consists of only a single layer of cells around the ventricles, known as the ependymal lining. Early ventricular zone cells can give rise to radial glia, now known to act as both neurogenic and gliogenic stem cells throughout embryogenesis. The majority of radial glia eventually differentiate into astrocytes, and only persist in a more similar form in a few specialised locations of the adult brain. These 'modified radial glia populations' include the Bergmann glia of the cerebellum and the tanycytes of the hypothalamus (reviewed in [13]). The identification of tanycytes as a form of radial glia is based on a number of shared features, and many

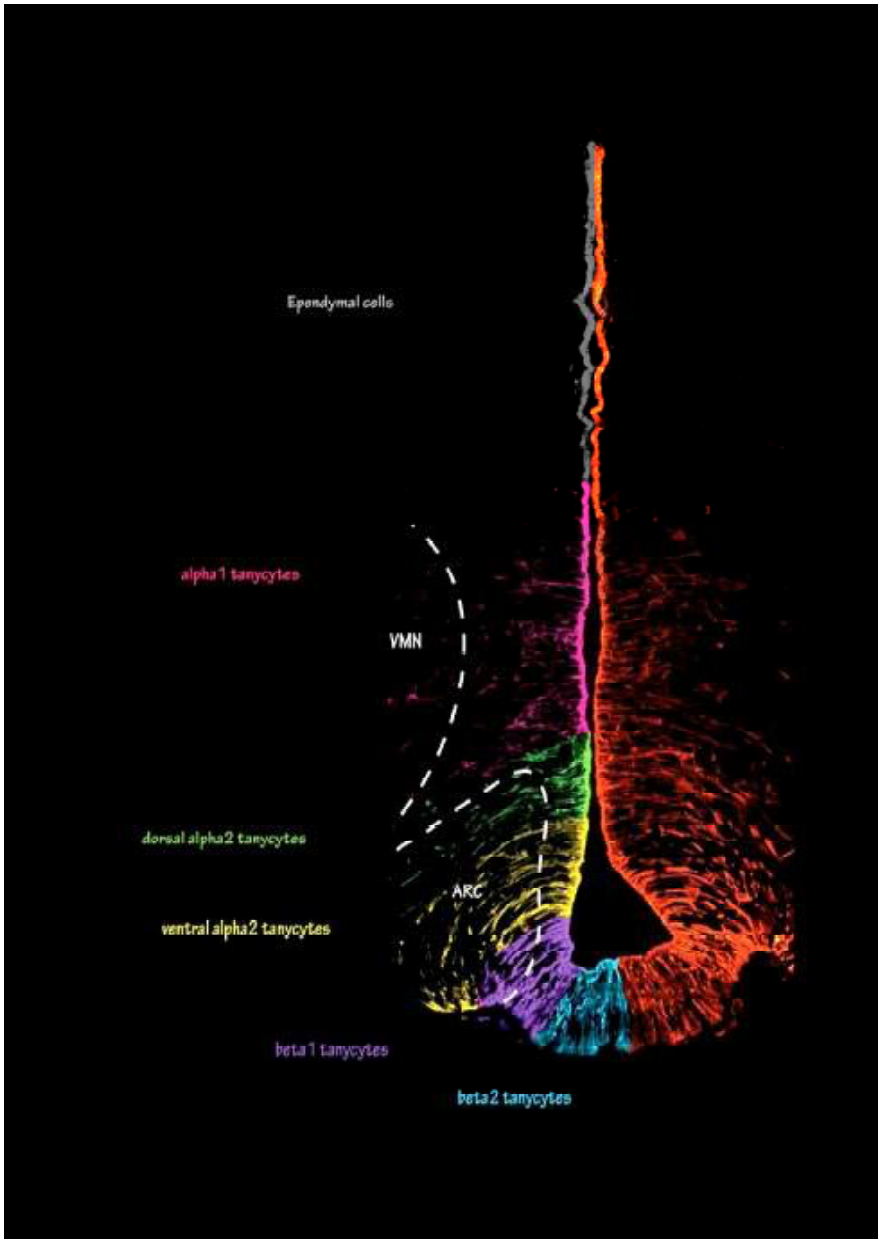

Figure 1.5: Tanycyte subtypes

Cells of the third ventricle, labelled with α-vimentin and coloured to show tanycyte subtypes. Pink = α1, green = dorsal α2, yellow = ventral α2, purple = β1, and blue = β2 tanycytes. Grey marks ependymocytes, which are not tanycytes. Approximate positions of the ventromedial nucleus (VMN) and arcuate nucleus (ARC) are shown.

S. Robins and D. McNay (see section 3.2.4).

authors additionally believe tanycytes are actually descended from embryonic radial glia [16]. Morphologically, tanycytes are very similar to radial glia, with the cell body located at the ventricular surface and a long process extending to the pial surface. Tanycytes express many of the markers shared by both radial glia and other immature astrocytes, such as vimentin and nestin . They also share a sensitivity to hydrogen peroxide, suggesting they may lack the robust antioxidant enzymes usually expressed in mature astrocytes [17]. In addition, tanycytes express a number of specific radial glia markers not usually found in astrocytes. These include the glutamate transporter GLAST and the radial glia marker RC1 [13].

1.3.3 Age related changes in tanycytes

As with many cell populations, tanycytes have been observed to undergo several changes throughout the lifespan of the organism. Scott and Sladeck [18] observed a physical thinning of the tanycyte layer in aged rats. Using scanning electron micrographs they showed that in young rats the tanycytes formed a tight continuous layer, thought to be important in the maintenance of the blood-CSF barrier in this region. In contrast, older rats had gaps between the tanycytes through which the underlying neuropil was visible, as well as a decrease in the number of apical microvilli.

1.3.4 Markers expressed by tanycytes

Tanycytes can be identified by the expression of a number of key molecules and markers (reviewed in [13]). All 4 subtypes express vimentin cytoskeletal filament. GFAP expression, seen in both astrocytes and radial glia, is controversial but likely to be expressed in the majority of subtypes. DARPP-32 is seen only in the β subtypes [19]. FGF10, which has been hypothesized to play a role in adult neurogenesis, is strongly expressed in ventral tanycytes [20]. The glucose transporters GLUT-1 and GLUT-2 are expressed in most subtypes, while the glutamate transporters GLAST (β only) and GLT-1 (α only) are more restricted. There is also some evidence that tanycytes express GHRH.

1.3.5 Proposed functions of tanycytes

1. **Barrier properties**
2. **Transport of CSF molecules**
3. **Synthesis and secretion of active molecules**
4. **Role in GnRH secretion**
5. **Role in thyroid regulation**
6. **Sensing of glucose levels**
7. **Role in axon regeneration**
8. **Neural stem cells**

Barrier Properties

The median eminence of the hypothalamus is an unusual area of the CNS, as it is largely lacking in a blood brain barrier (BBB). This can be demonstrated by simply injecting an intravenous tracer into the circulation, which can subsequently be found in the intercellular space of the median eminence. However, it is not found in other adjacent areas of the hypothalamus or in the third ventricle, indicating the presence of median eminence-arcuate nucleus and median eminence-CSF barriers[13] . This system allows neurons to secrete factors directly into the blood stream, as well as to allow sensory neurons to monitor levels of hormones and other chemicals in the circulation. The β2 tancyctes lining the floor of the third ventricle are linked by continuous tight junctions. These act to prevent the diffusion of substances between the ventricular CSF and the intercellular space of the median eminence. The lateral barrier separating the median eminence from the arcuate nucleus is likely to be formed by β1 tancyctes. They have been found to express the glucose transporter GLUT-1, a marker of the BBB.

Transport of CSF molecules

It is known that tanycytes have the capacity to absorb a variety of substances from their apical pole, which contacts the CSF. It is likely that they can also transport these substances basally, to be released at the process terminal. They

may also be able to transport cargo molecules in the opposite direction, from the terminals to the CSF. There is evidence of clathrin-mediated endocytosis in all tanycyte subtypes, and of caveolin-mediated endocytosis in the β tanycytes only.

Synthesis and secretion of active molecules

It is still unclear to what extent tanycytes are involved in the synthesis and secretion of molecules into the CSF or other compartments. However, some tanycytes do have structural features that would be consistent with this role. It has been shown by Prevot et al [21] that tanycytes synthesise and secrete prostoglandinE2 and then subsequently transforming growth factor β1 (TGFβ1) in response to TGFα, as part of their regulation of GnRH secretion.

Role in GnRH secretion

Gonadotropin-releasing hormone (also known as luteinising hormone releasing hormone, or LHRH) is a key regulator of sexual development and reproductive function. It is released from neurons found in the preoptic area, which extend their axons into the median eminence. GnRH is released into portal blood vessels prior to exerting its effects in the anterior pituitary. Although this process is largely under neural control, both glia and tanycytes are thought to play some role in its regulation. Under most hormonal conditions the GnRH neuron terminals do not extend all the way to the perivascular space surrounding the portal capillaries, but are surrounded by the end terminals of β tanycytes [22] . At the preovulatory surge, when large amounts of GnRH are released, the tanycytic endfeet undergo plastic changes. They retract away from the capillaries, allowing direct access of the GnRH terminals to the pericapillary space, thus facilitating GnRH release. A number of signalling factors are thought to be involved in this process, including TGFα, TGFβ1 and nitric oxide [23] . PSA-NCAM is also thought to be crucial to the plasticity of both the axonal and tanycytic processes, by reducing cell-cell adhesions and thus facilitating remodelling [24].

Role in thyroid regulation:

Thyroid hormone can exist in several different forms. Thyroxine (T4) has to be converted to its biologically active form, tri-iodothyronine (T3) in order to exert its effects. In the brain this conversion is principally mediated by type 2 iodothyronine deiodinase (D2), an enzyme which is abundantly expressed in all subtypes of tanycytes. It has been suggested that tanycytes extract T4 from either the bloodstream or the CSF, convert it to T3 and release it back into these compartments. This mechanism could present T3 to thyrotropin-releasing hormone (TRH) neurons, allowing a negative feedback regulation of thyroid hormone levels. This hypothesis is supported by the observation that under the atypical conditions of fasting or infection a fall in circulation thyroid hormone levels is actually followed by a reduction in TRH production. A model for infection, the administration of bacterial lipopolysaccaride (LPS) produces a 400% increase in tanycytic D2 activity. A more modest increase is also induced by fasting. This could lead to a perceived increase of T3 levels by TRH neurons and thus reduced thyroid hormone secretion, despite lowered systemic T4 levels. The reduced levels of secreted thyroid hormones at times of stress would result in reduced energy expenditure, a potentially important survival mechanism (reviewed by [25]).

Sensing of glucose levels:

The glucose transporters GLUT1 and GLUT2 are expressed in both α and β tanycytes, as is the K-ATP channel subunit Kir6.1 [26] . In the pancreas these molecules are linked to glucose sensing mechanisms, and a similar role has been proposed in the hypothalamus. They are present mainly at the ventricular surface and so would be able to detect glucose levels in the CSF.

Role in axon regeneration:

Whilst regrowth and repair of damaged tissue in the adult CNS is notably scarce due to reactive astrocytes forming a glial scar impermissive to axon regrowth, the hypothalamus is one of the few areas to have demonstrated some regenerative capacity. Although new cells are not necessarily generated

(although see section 1.4.3 later), it has been shown that neurohypophysial axons in the region of the median eminence can regenerate after undergoing axotomy. Chauvert, Prieto and Alonso [27] theorized that tanycytes may be responsible for this phenomena. To test this they lesioned the hypothalamus at two dorsoventral levels: dorsomedial lesions were located in the area containing only ependymocytes, while ventromedial lesions would have been in a region roughly corresponding to the alpha tanycytes. They found that TH^+ and $5\text{-}HT^+$ axons regrew across the ventral lesion, which contained many $vimentin^+$ tanycytic processes, but were unable to penetrate the glial scar surrounding the dorsal lesions. They noted an inverse correlation between the presence of reactive astrocytes and the ability of axons to pass through an area. In a separate study the same authors showed that tanycytic cultures in vitro supported both the survival and axonal growth of co-cultured neurons [28]. They suggested that neural regeneration in the CNS is normally inhibited by reactive astrocytes or oligodendrocytes, but in the region of the mediobasal hypothalamus tanycytes may actively support this process, either by directly supporting axons or by influencing the organization of the astrocytes.

In an extension of these studies, they investigated whether transplanted tanycytes could aid axon regeneration in spinal cord injury [29]. Whilst transplanted cortical astrocytes resulted in the formation of a glial scar that was totally impenetrable to regenerating axons, transplanted tanycytes resulted in a smaller scar that was transected by numerous axon types. As the glial scar is actually formed by endogenous astrocytes, it was concluded that the tanycytes were releasing factors that influenced the reactive astrocytes to alter the composition of the scar. In addition they are likely to express a range of neurotrophic factors, cell adhesion molecules and extracellular matrix molecules conducive to axon regeneration.

1.3.6 Tanycytes as neural stem cells:

Evidence is mounting from various sources that in addition to the roles documented above, tanycytes may act as neural stem cells in the adult brain. I

will go on to discuss the evidence for this in detail in section 1.6, but first will provide a general overview of the adult neural stem cell field.

1.4. What is a Stem Cell?

Although stem cell research is a widely-studied field, there is very little consensus on what the term 'stem cell' should actually mean. This is largely due to conflicting ideas between the disciplines of developmental versus adult stem cell research. As stem cells probably fulfil fairly disparate roles at different times in the development and life of an organism, it is not surprising that there is so much disagreement on what the fundamental defining features of a stem cell should be [30]. Stem cells could be considered as having two main functions: firstly providing a way of generating a large repertoire of cell types and secondly acting as a reservoir of developmental potential to be called upon if renewal or regeneration are required [1]. This would lead to a very broad definition that could include any relatively unspecialized cell that could produce progeny that differentiates into a more specialized cell type. However many people feel that the definition of a stem cell should be much more rigorous. Much of the opinion from people working with adult stem cells uses the characteristics of haematopoietic stem cells (HSCs) as a model, as they were the earliest to be discovered and most thoroughly characterized stem cell system. Researchers in this field tend to define self-renewal and multilineage differentiation as absolutely essential properties of a stem cell. Some consider that asymmetric division should be a defining feature; the alternative argument is that if stem cells are considered as a population rather than as individual cells then such asymmetric division is unnecessary for their renewal. Developmental biologists have also argued that there are many examples in early development of symmetrical division to amplify a population of stem cells, and so asymmetric division cannot be considered a defining feature. Many go further, and refute the requirement for self-renewal in any capacity as it appears to often be unnecessary in development as well as nonvital adult systems [31]. One fairly

sensible view on neural stem cells is that multipotent (i.e. capable of generating neurons, glia and oligodendrocytes, the three neural cell lineages) self-renewing cells should be considered as stem cells, while cells only capable of differentiation without indefinitely replacing themselves, whether multipotent or not, should be termed progenitor cells instead [32].

Another issue is whether stem cells should be assessed and defined as individual cells, as a population, or as a whole interactive system of different cell populations. The concept of stem cell niches that support and control stem cells has been studied in some depth. A stem cell niche could be considered as 'a specific location in a tissue where stem cells reside for an indefinite period of time and produce progeny cells while self-renewing' [33], although this definition probably reflects the fact that the niche is a concept more associated with adult rather than developmental stem cells. This niche may take a number of different forms in different tissues and systems. The most basic form is known as a simple niche, examples of which can be found in gonadal, epithelial and digestive tissues. In this system each stem cell is tightly anchored by specific junctions to a stromal partner cell. As the cell divides one daughter may remain connected to the stromal cell and receive signals inhibiting its differentiation, while the outer daughter is free to begin differentiating and migrate out of the niche. More complex niches may involve multiple stem cell types within one niche, such as occurs in the hair follicle bulge, or more controlling cells to influence the stem cell. This may well be the case for neural stem cells (NSCs) in the subventricular zone (SVZ), which can interact with other astrocytes, neuroblasts, ependymal cells and endothelial cells as well as the basal lamina.

1.5. Adult Neural Stem Cells and Neurogenesis

For many years the lack of substantial tissue repair or recovery of function after brain injury led to the assumption that new neuronal tissues were never produced in adult mammals. Repair mechanisms were thought to be limited to

postmitotic processes such as outgrowth of new axon terminals or synaptic reorganisation. Since the development of methods using [H^3]-thymidine to label dividing cells in the 1950s, evidence has been mounting to the contrary. More recently it has been shown that while neurogenesis is probably not possible in every area of brain tissue there are a few specific sites containing stem cell like cells, capable of proliferating to generate a variety of mature CNS cell types [34].

A variety of techniques can be used to investigate the capacity for proliferation and neurogenesis in adult organisms. An important in vivo method is the labelling of dividing and newly-generated cells with exogenous nucleotides such as [H^3]-thymidine or the more-commonly used BrdU [34]. As BrdU is detected by immunohistochemistry it can be used in conjunction with antibody staining to confirm colocalisation with specific cell type markers. Marker expression can in itself be very useful, as there are a wide range of markers known to be fairly specific to different stages of neuronal differentiation [36], such as those illustrated in Figure 1.6 [35]. For example, Sox transcription factors, GFAP and nestin may be expressed in neural stem or progenitor cells, TuJ1 and polysialylated neural cell adhesion molecule (PSA-NCAM) are found in immature neurons and MAP-2 and NeuN identify fully mature neurons. The main drawback to this approach is in the specificity of some of the markers, for example GFAP is expressed in mature non-mitotic astrocytes as well as stem cells, and PSA-NCAM can be re-expressed in some mature neurons under certain conditions. For this reason it may be necessary to combine several markers together to confirm the identity of a cell.

One commonly-used in vitro method of testing the proliferative and neurogenic capacity of an area of CNS is the neurosphere assay. Primary cells are cultured at low density under conditions such that individual cells form spherical clonal colonies [32]. Both non-renewing proliferative progenitors and self-renewing NSCs can be cultured as neurospheres, however only stem cell-containing cultures can be repeatedly passaged. The frequency of colony-forming cells can

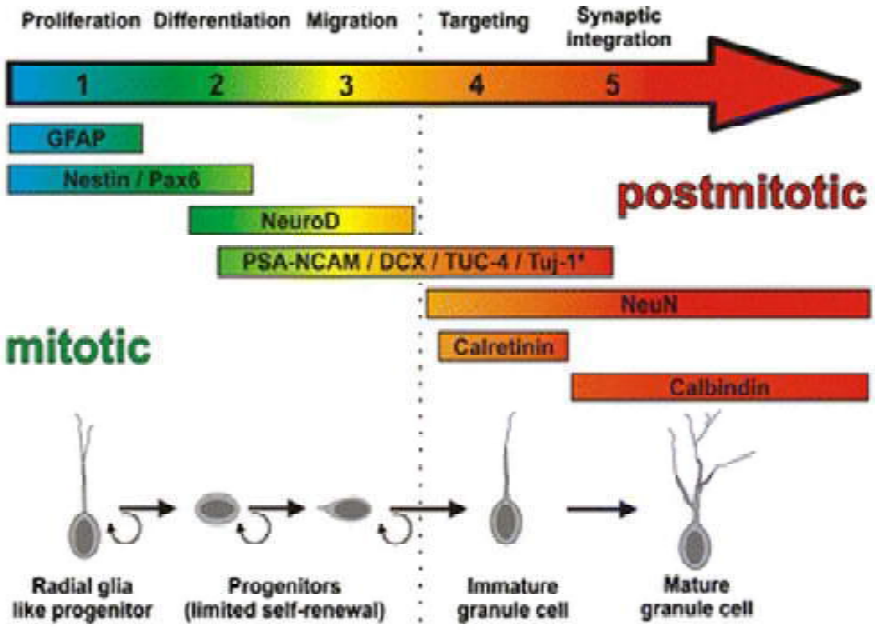

Figure 1.6: Markers for determining stages of neurogenesis

Hippocampal neurogenesis is well-characterised, and the markers expressed at every stage of neurogenesis have been assessed. Each stage – proliferation, differentiation, migration, targeting and synaptic integration – results in cells expressing a unique set of proteins, making antibodies for these markers extremely useful tools for studying neurogenesis.

Taken from von Bohlen et al, [35].

be determined by a limiting dilution neurosphere assay, in which cells are plated at reducing densities and the number of neurosphere negative wells used to calculate the proportion of proliferative cells. Cells cultured either as neurospheres or as an adherent monolayer usually require growth factors to maintain them, the most commonly used being EGF and FGF-2, although Shh may also be effective [34]. Removal of these factors can be used to induce differentiation for analysis of the potential cell types generated, including neurons [32]. Cultured cells can also be labelled and transplanted back into embryonic or adult animals to investigate both the effect they have on endogenous neurogenesis, and the manner in which they can be affected by different environments.

1.5.1 Adult neural stem cells in non-mammalian vertebrates

Many vertebrate species demonstrate considerable capacity for neurogenesis, as both a constitutively active process and for regeneration after injury. In fact there seems to be a phylogenetic reduction of both neurogenesis and regenerative capacity, with high levels of proliferation seen in fish, reptiles and amphibians, less in birds, and just a few very restricted areas in mammals (Figure 1.7) [36]. One reason for this is likely to be that fish, amphibians and reptiles have brains that continually increase in size throughout life. Some of this brain growth relates to their continual increases in body size, and so expanding sensory areas. Although new cells are not added universally to all areas of the CNS, in these species they are found in a wide range of structures. In the zebrafish precisely 16 constitutively active zones of proliferation have been identified in the CNS [37]. These include zones in the telencephalon, the thalamus, the hypothalamus and the cerebellum. Newborn cells did not necessarily remain in the proliferative zones, but migrated to diverse locations. Double labelling in the hypothalamus indicated that the majority of these newborn cells became neurons rather than glia. Various specific neuronal types appropriate to that region were also identified, including dopaminergic and serotinegic neurons. Teleost fish also show a remarkable level of regenerative capacity. They can regenerate after incisions to the brain, removal of entire


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regions or transection of the spinal cord. It has been shown that this process is dependent on the nearby proliferative zones, and that progressive removal of these zones reduces the capacity to replace lost tissue (reviewed in [36]).

Avian brains do not increase in size throughout the animal's life, and it is thought that in areas such as the hippocampus the gain of new neurons roughly balances the gradual loss of old neurons, resulting in no net changes [38]. Proliferation is seen around the lateral ventricle in the telencephalon, with neuroblasts migrating away along radial glia processes to a diffuse area. In songbirds neurogenesis has been correlated with song patterns, particularly with neurons integrating into the high vocal centre (HVC) [39]. Neural integration and survival in this area often varies seasonally, possibly indicating a hormonal influence.

1.5.2 The mammalian 'neurogenic niches:' SVZ and SGZ

Only two areas of the mammalian brain have been unequivocally shown to contain a stem cell niche: the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus [40]. Analogous regions of the brain have been shown to be proliferative in non mammalian vertebrates, although they also contain many other neurogenic zones [36]. These two regions have been extensively studied, and the cell types involved established (Figure 1.8). In the SVZ, the primary stem cell is known as a B cell. It is located next to the ependymal cells lining the ventricle, and often extends a process to contact the CSF. It is astrocytic in character, both in regard to GFAP expression and ultrastructural organisation (reviewed in [41]). The developmental origin of these astrocytic stem cells is thought to be radial glial cells. B cells are relatively quiescent cell types, which give rise to rapidly proliferating type C cells. These in turn produce immature neuroblasts (A cells), which migrate along the rostral migratory stream to differentiate into interneurons in the olfactory bulb. In the SGZ the primary stem cells are also astrocytic. In this case they possess an extended radial process, and so are

sometimes referred to as radial astrocytes. They give rise to type D progenitor cells, which then migrate the short distance into the granule cell layer and mature into functional granule cell neurons. Several types of experiments were used to elucidate the identity of the neural stem cells in these regions. Mouse mutants have been used to track GFAP-expressing cells and their progeny [42]. A day after labelling, only type B cells were labelled, but after a further 2.5 days labelled neuroblasts were also observed. After two weeks, labelled olfactory interneurons started to appear. An alternative approach from the same lab was to look at whether B cells could repopulate the niche after removal of other cell types. The anti-mitotic chemical cytosine-β-arabinofuranocide (Ara-C) was used to depopulate the niche by eliminating all the rapidly dividing cell types. Only type B cells remained, and were observed to proliferate over the subsequent few days to replace the lost C and A cells.

An important question is how adult neurogenesis is regulated, and already a huge variety of factors have been shown to influence the frequency of neurogenesis in the SGZ and SVZ. The stem cell properties of NSCs are likely to be regulated by a combination of internal and external factors. It has been observed that the tendency for embryonic cortical neural stem cells to give rise to different progeny at different developmental timepoints is maintained in the absence of the stem cell niche, suggesting regulation by factors intrinsic to the stem cell [43]. Subsequent reintroduction of niche cells from an earlier timepoint failed to regress the later stage cortical stem cells to their previous potency. Several mechanisms may coincide to coordinate cell-intrinsic regulation. There is likely to be a level of epigenetic control, with an important role for proteins involved in histone modification or DNA methylation [44]. In addition, stem cells often express unique patterns of transcription factors, controlling the balance between self-renewal and differentiation. One of the most thoroughly-characterised of these is Sox2, which is thought to play a role in maintaining undifferentiated stem cells in both developmental and adult systems [45],[46]. Adult mice deficient for Sox2 have a variety of CNS deficits, including a reduced capacity for neurogenesis [47].

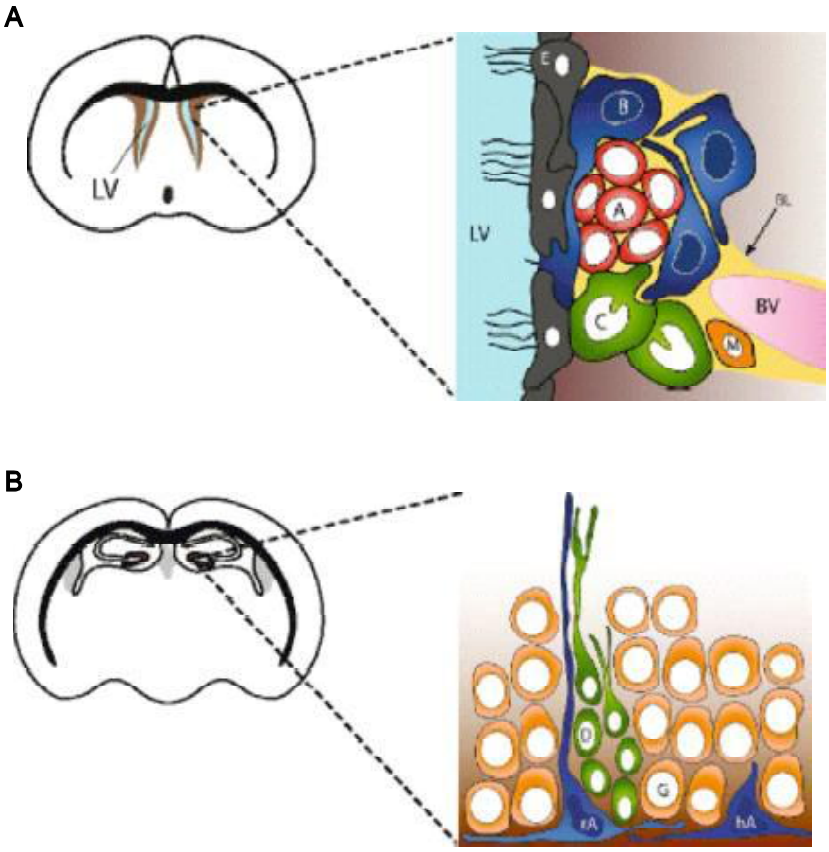


Figure 1.8: The mammalian neurogenic niches

A. Architecture of the subventricular zone (SVZ) in mouse brain. The SVZ contains type B cells (B), which correspond to the in vivo neural stem cells and have astrocytic characteristics. Also present are rapidly dividing type C cells (C), the transit-amplifying progeny of B cells. C cells in turn give rise to neuroblasts (or type A cells, A), which migrate to the olfactory bulb and differentiate into neurons. The cells of the SVZ have extensive contact with the basal lamina (BL, arrow) and microglia (M) and also lie near blood vessels (BV). SVZ cells additionally make contact with the multiciliated ependymal cells (E) that line the lateral ventricle (LV)

B. Architecture of the subgranular zone (SGZ) in mouse brain. The SGZ contains radial (rA) and (hA) horizontal astrocytes. Radial astrocytes have long radial processes that penetrate the granular layer and tangential processes that are oriented parallel to this layer. These astrocytes give rise to type D immature precursors (D), which divide and mature into new granule neurons (G). D cells develop apical processes that become the dendrites of the new granule neurons

Taken from Ihrie and Alvarez-Buylla [41]

However, it is also clear that many factors present in the niche are capable of directing the behaviour of stem cells. It is thought that under normal conditions, stem cell populations are relatively quiescent, requiring particular signals to undergo renewal or differentiation. These signals may take the form of soluble factors, membrane-bound molecules and cell-contact signals, or extracellular matrix components. The presence of growth factors or signalling molecules may also influence other aspects of stem cell function, such as the fate of their progeny. These growth factors are generally thought to be secreted from the astrocytes forming the stem cell niche. Fibroblast growth factors (FGFs), in particular FGF-2, are known to play an important role in stimulating proliferation, and their infusion into the ventricles can dramatically increase levels of newborn cells (reviewed in [40]). Although epidermal growth factor (EGF) can also induce proliferation, the ligand is not present in the SVZ. Instead it has been suggested that TGFα acts as the endogenous ligand, as it is also capable of activating the EGFR [48]. Shh is another soluble factor proposed to regulate both the SZV and SGZ niches. In both of these regions addition of exogenous Shh increases levels of proliferation, whilst cyclopamine-induced inhibition of Shh signalling reduced them [49], [50]. These results, plus the presence of various components of the Shh signalling pathway such as the receptor Patched and downstream effector Gli1, suggest that Shh is an endogenous stem cell regulator involved in constitutive neurogenesis. In addition, exogenously applied Shh is able to induce neurogenesis in the cortex, a region generally considered to be non-neurogenic [51]. The list of signalling factors shown to be capable of influencing adult NSCs is extensive, and includes Wnt3, BDNF, CNTF and Notch [52],[53],[54],[55]. However not all of these have yet been proved to be present and active in vivo under normal conditions. One interesting question raised by the involvement of signalling molecules such as Shh, Wnts and FGFs is the extent to which adult stem cell systems recapitulate developmental routes for proliferation and differentiation. Many of the molecules implicated in the control of adult NSCs were first discovered in the embryo, where they are involved in neural growth and patterning. As yet there is no good answer to this, although there is increasing

evidence that many of the same mechanisms are active in adult and embryonic systems.

Many more systemic factors have also been demonstrated to affect neurogenesis, for example it tends to decrease with age, stress and some drugs of abuse, and increase in response to hormones such as oestrogen, increased environmental stimulation and some degenerative neurological diseases like Alzheimer's disease (reviewed in [34]).

Despite the extensive research on adult neurogenesis its function still remains unclear. Studies in songbirds have suggested a role in learning and memory. One possibility is that the unique properties of new neurons allow them to fulfil separate functions. This would be supported by the observation that new neurons have a lower LTP/LDP induction threshold relative to older neurons. Their ability to extend new axons and dendrites in the mature CNS may also make them important to structural plasticity in the brain [34].

1.6 Stem Cells and Neurogenesis in the Adult Hypothalamus

In order for a region to be considered neurogenic, two significant conditions need to be fulfilled: firstly, the region must contain a self-renewing population of proliferative cells, and secondly these cells must be capable of giving rise to fully functional neurons, which integrate into existing neural circuits. As previously mentioned, adult neurogenesis appears to be more widespread in fish, reptiles and birds than in mammals. There is good evidence that in zebrafish the hypothalamus constitutes one of the major proliferative zones [37]. This area appears to exhibit both constitutive neurogenesis and regenerative capacity. The source of proliferative cells appears to be the ventricular lining, also known as the ependymal lining of the third ventricle. In mammals the picture is less clear, but an increasing amount of evidence from a

variety of different approaches suggests that the mammalian hypothalamus may also contain a source of neural stem cells. This evidence is summarised below.

1.6.1 In vitro evidence

Evidence from in vitro studies can provide large amounts of information about the potential of cells in a given region. Several sources suggest that the hypothalamus retains the capacity for proliferation, although not all authors conclude that it is a constitutively active proliferative region. It is a popular view that non-neurogenic regions can sometimes be induced to produce new cells in response to artificial conditions or injury. Whilst in vitro data cannot be taken conclusively to suggest that in vivo proliferation and differentiation occurs, it provides a tantalizing clue that it is possible given permissive conditions. One of the earliest reports of the culture of cells from the adult hypothalamus comes from Evens et al [56] using an adherent monolayer approach in the rat. They were aiming to culture pre-existing matured neurons, but found that the majority of cells were proliferative and exhibited the properties of more immature neural cell types. These cells were characterised by immunostaining for α-internexin, a marker of immature neurons, occasional vimentin or nestin co-labelling, and the absence or glial or oligodendrocyte markers. They also had electrophysiological characteristics of immature neurons, with the suggestion that they expressed both Ca^{2+} and Na^{+} channels giving rise to voltage-gated currents. Despite this neuronal character, the cells were found to be proliferative, and could incorporate BrdU and be expanded in culture. The authors concluded that these cells could either be the result of dedifferentiation of mature neurons, or of a resident stem cell population that had partially differentiated. Adherent monolayer techniques have also been used to make specific cultures of hypothalamic tanycytes [17]. Tanycytes were identified as being vimentin^{+}. They were reported to be functional for both the in vitro and in vivo support of regenerating axons, and could be transplanted into a rat model of spinal cord injury [29].

Finally, several groups report that the hypothalamus can be cultured as neurospheres [57-59]. The most thorough of these is Xu et al [59], who derived neurospheres from adult rat brain. They showed that primary spheres could be dissociated and regrown as secondary spheres (although they make no mention of whether more passages are possible), and that neurospheres could be generated under clonal conditions. This involved growing them at a density of precisely one cell per well, to ensure that each sphere was the progeny of a single original cell. Neurospheres could also be differentiated, and produced cells belonging to all three neural lineages: TuJ1$^+$ neurons, GFAP$^+$ glia and RIP$^+$ oligodendrocytes. In summary, the in vitro studies mentioned above have demonstrated that there are cells with some capacity for self-renewing proliferative divisions within the hypothalamus. Furthermore, these cells have the potential to form cells from all three neural lineages, including neurons. What is not clear is how far the self-renewal capacity extends, and whether these are true stem cells or progenitors. Furthermore, the precise location of such cells within the hypothalamus remains unclear.

1.6.2 Evidence from marker expression

Another clue to the proliferative status and neurogenic of a region is the presence of putative stem cell markers. Various proteins have been detected in the adult hypothalamus that might indicate neural stem cells. Sox2 has been suggested to be a robust general marker of NSCs [46], and has been observed in restricted zones of the rodent hypothalamus [46]. Musashi, another stem cell marker, has also been observed to be present (K. Ohyama and M. Placzek, unpublished observations). Tanycytes of the third ventricle are known to strongly express nestin [60], a cytoskeletal protein associated with NSCs in other systems [56, 61]. The polysialylated form of neural cell adhesion molecule (PSA-NCAM) has also been observed in the area of the arcuate nucleus/ median eminence [24]. This form is more commonly seen in the embryo, and is linked neural plasticity. However, as this area is known to undergo many plastic changes related to hormone regulation its presence may not be related to the formation of new neurons. There is limited data on the distribution of cell cycle

markers, although there is one report showing occasional expression of Ki67 in the mouse hypothalamus [62]. In addition, a study in the pig reported the presence of proliferating cell nuclear antigen (PCNA) in the vasopressin and oxytocin containing nucleus (VOC) [63]. This region is known to undergo dramatic growth at puberty in pigs, and to continue to increase in size throughout reproductive age.

1.6.3 In vivo BrdU evidence

Incorporation of synthetic nucleotides is a very reliable method for studying neurogenesis. It relies on the principle that only dividing cells are involved in active DNA synthesis, and so all labelled cells must have been in the synthesis stage of cell cycle during the period of administration. The presence of BrdU under normal conditions is good evidence for constitutive proliferation, and it is also a useful tool to investigate the effect of mitogenic factors. Although early BrdU studies failed to pick up substantial newborn cells in this region, this may be attributable to the method of delivery. A study by Chouaf-Lakhdar et al [60] demonstrated that when BrdU was injected into the brain ventricles, as opposed to the traditional technique of interperitoneal administration, proliferating cells could be seen throughout the third and fourth ventricles. All studies using this method reported the presence of a number of proliferating cells in the hypothalamus [60],[62],[53]. One study estimated the number of newborn cells produced over the course of seven days to exceed 20 000, representing a significant level of constitutive proliferation [62].

In addition to simply demonstrating the presence of proliferation, BrdU labelling can be combined with marker expression to examine the fate of newborn cells. This approach was used by Kokoeva et al, who reported that many $BrdU^+$ cells co-expressed Hu or doublecortin, indicating mature or immature neurons respectively [62],[64]. Some newborn cells in the arcuate nucleus were observed to express NPY or POMC, indicating that they matured into types of neuron appropriate to their location. They also observed new APC^+ oligodendrocytes, but very few $GFAP^+$ glia. They examined $BrdU^+$ cells up to 7

weeks after BrdU administration and saw very little reduction in numbers over time, suggesting that the majority of cells survive for a reasonable period of time.

It is clear from the studies above that the hypothalamus contains cells that constitutively proliferate under normal conditions. Furthermore, at least two lineages of differentiated cells are produced, including subtypes of neurons specific to the hypothalamus. Although these neurons have not yet been proved to form appropriate synapses and contribute to hypothalamic function, they appear to survive for a significant period of time, which is sufficient in theory for them to fully mature and integrate into existing neural circuits.

1.6.4 Site(s) of proliferation

By combining the data from the three approaches detailed above, it is clear that there are proliferating cells in the hypothalamus, which generate new neurons. The presence of this novel neurogenic niche raises many questions, such as what cell types are involved, how they are regulated, and where the stem and progenitor cells are located.

There are two main theories concerning the site of neural stem / progenitor cells (NS/PCs) in the hypothalamus: that they reside scattered throughout the hypothalamic parenchyma, or that they are contained in the ependymal lining of the third ventricle. It has also been suggested that there may be a subependymal zone in the hypothalamus, similar to that in the SVZ, that could be a source of proliferating cells [60]. However, there is little anatomical evidence that such a distinct zone exists. Proliferation has been observed in both the tanycytic region of the ventricular wall, and the hypothalamic parenchyma. Both Pencea et al [53] and Kokoeva et al [62] report seeing the majority of $BrdU^+$ cells in the parenchyma of the hypothalamus. While this in itself is no proof of parenchymal proliferation – the cells could have easily migrated from the nearby ventricle – they also both report often seeing the cells in pairs. As the $BrdU^+$ cells are relatively infrequent, it seems likely that closely-

opposed pairs will have recently originated from division of the same cell. In addition, Kokoeva et al note this phenomenon at even the earliest timepoints after BrdU administration, when there may not have been sufficient time for extensive migration.

The alternative argument, that the stem cells are found in the ventricular lining, is focused on the idea that hypothalamic tanycytes may be acting as stem cells. This idea stems from the observation that in other adult systems glia seem to act as the original stem cell, coupled with the well-proven fact that in development radial glia commonly divide to produce new neurons [41]. As tanycytes are so closely linked to radial glia, it seems logical that they could also possess proliferative potential. Neither of the two studies quoted above as observing parenchymal proliferation ruled out this option, as $BrdU^+$ cells were also observed in the vicinity of the third ventricle [53, 62]. Another study has gone further, and specifically identified BrdU labelled cells as $nestin^+$ tanycytes [60], although this study also noted that ependymocytes in other regions of the third ventricle ependyma were could proliferate. In order to test the theory of tanycytes as stem cells, Xu et al [59] carried out experiments to label the ependymal layer of the third ventricle with a GFP-recombinant adenoviral vector (vGFP). Injection of vGFP into the fourth ventricle resulted in the labelling of both tanycytes and ependymocytes 48 hours later, but not of parenchymal cells. Over the subsequent weeks increasing numbers of $vGFP^+$ cells were seen within the parenchyma, located along tanycytic processes. The authors interpreted this as an indication that tanycytes were proliferating at the ependymal lining and then migrating inwards along the processes of other tanycytes. This closely mimics the role of radial glia in development. By 2-4 weeks after the injection GFP^+ cells with a neuronal morphology were seen throughout the hypothalamus. The authors also showed that ependymal cells could be the source of neurospheres grown in vitro. They injected DiI into the ventricles where it was incorporated solely into the ependymal lining. The DiI^+ cells were then isolated by FACS sorting and grown into neurospheres.

However, the authors made no mention of whether neurospheres could ever be derived from the DiI^- fraction.

It is worth noting that these two theories on the location of proliferative cells are in no way mutually exclusive, and may in fact complement one another. If hypothalamic neurogenesis resembles that seen in other brain areas, then it would not be surprising to see different stages of cells in different locations. It may be that the stem cells reside in one specific location such as the ependymal lining, and give rise to neural precursors that both continue to divide and migrate away from the ventricle, resulting in parenchymal proliferation. Precedents from other neurogenic regions would suggest that the stem cell is likely to divide less frequently than the precursor, accounting for all the results reviewed above.

1.6.5 Mitogenic factors

Many different factors have been suggested as mitogens in other stem cell systems, and only a few of them have been tested in the hypothalamus. Nevertheless, it is clear that several neurotrophic factors are capable of inducing hypothalamic proliferation. How many of these pathways are active in vivo is a question that has yet to be addressed. Pencea et al [53] infused BDNF in the lateral ventricles of adult rats and observed newborn $BrdU^+$ cells in the region of the hypothalamus, as well as around the infused ventricle and in the thalamus. The authors noted that the newborn cells were not evenly spread throughout the hypothalamic parenchyma, but were densest in specific nuclei. The parvocellular region of the paraventricular nucleus was observed to have many $BrdU^+$ cells, whilst the periventricular nucleus immediately adjacent to the third ventricle had fewer new cells. Many of the cells could be double-labelled with MAP2 or TuJ1, indicating that a significant proportion of these cells differentiated into neurons. Interestingly, the hypothalamus was the only structure examined in which the BDNF infusion actually altered the proportion of neurons, increasing it from 21% in control PBS-infused brains to 41% after BDNF treatment. The effect of CNTF in vivo was examined by Kokoeva et al

[64]. Mice infused with CNTF for 7days showed a 4-5 fold increase in the number of $BrdU^+$ cells seen in the neuroendocrine hypothalamus, the area around the median eminence. They also observed a shift in cell fate, with 42% of new cells becoming neurons in the CNTF treated animals compared to just 21% in control animals. Finally, a range of growth factors were tested by Xu et al, although their study was a little different as it utilised intraperitoneal rather than intracerebral BrdU. They found a slight but non-significant effect from injecting N2, BDNF or EGF, and a dramatic increase in new cells when FGF-2 was injected. The greatest effect was found using a combination of FGF-2 + EGF. The discrepancy in the effect of BDNF between this study and the one carried out by Pencea et al may be accounted for by the fact that Xu et al administered only a single dose of growth factor, while the previous study had infused it continuously over the course of 12 days.

1.6.7 Possible functions of neurogenesis

If there is, as seems likely from the evidence above, neurogenesis in the adult hypothalamus, then it is reasonable to assume that it will serve a specific purpose. There are several reasons that might logically account for producing new neurons. One is the idea of replacing old neurons as they are lost. If this were true, then it might be expected that the brain would show considerable regenerative capacity after damage, which does not seem to be the case. It is possible that it could be a system for replacing very limited numbers of neurons over a long time period, and that it does not contain the capacity to deal with larger scale neuronal loss. However, although like the rest of the CNS the hypothalamus can never truly regenerate, there is evidence that it has some capacity for recovering from injuries such as hypophysectomy [65],[29]. To what extend this relies on neurogenesis rather than axon regrowth is not clear, but it is reported that neuron-like cells emerge onto the ventricular surface after axotomy [66]. Another possibility is that the new neurons have different functional properties from more mature cells, and so add a different kind of processing into the circuit. This has been demonstrated in the hippocampus [67], but as of yet there have been no studies into the electrophysiological

properties of young hypothalamic neurons. A third option is that new neurons are added into hypothalamic circuits in order to alter their overall properties. This may be particularly relevant to the hypothalamus, as many aspects of body homeostasis change gradually and permanently over time. A couple of studies lend support to this idea. The VON of the pig hypothalamus changes in size over the pigs lifespan, apparently in parallel to its reproductive status. It undergoes dramatic growth at the onset of puberty, and then continues to increase more sedately throughout reproductive age. It has been suggested that the growth is due to the addition of new neurons relating to reproductive function [63]. In mouse it may be that new neurons have a role to play in the regulation of energy balance. Kokoeva et al noted in their study of CNTF that it also has the interesting side effect of causing weight loss in both mice and humans [64]. Crucially, this weight loss persists after the CNTF treatment is stopped, something that is not seen with any other factor yet discovered. The weight loss was caused by the mice voluntarily decreasing their food intake. To test their theory that CNTF-induced neurogenesis was adding new neurons into the energy balance circuits of mice, they looked at the type and location of new cells. New cells were seen in the arcuate, ventromedial and dorsomedial nuclei, all of which are integral to the control of feeding and metabolism. Roughly a quarter of all newborn cells in this region responded to leptin administration by phosphorylating STAT3. As leptin is an important long-term signal of fat levels in the body, an increased sensitivity would explain why these mice lost weight. To confirm that this effect was a direct consequence of the neurogenesis, the experiment was repeated in the presence of the mitotic inhibitor Ara-C. This resulted in an almost total loss of proliferating cells, and in the loss of the long term effects on weight loss. It is worth noting that a short-term effect on weight loss was still seen both in the Ara-C mice and in leptin-null Ob/Ob mice, suggesting that this occurs by some other mechanism.

1.7 Thesis Hypotheses

The data reviewed above strongly suggests the presence of a stem cell niche in the adult mammalian hypothalamus. There is evidence for both constitutive proliferation of endogenous cells, and differentiation of their progeny into new neurons. However, the different strands of information are not well co-ordinated, and there are many more questions to be answered. Although it is well-established that the hypothalamus can form neurospheres, the precise location of proliferative cells has not been determined, beyond the theory that tanycytes may play a role. Other regions of the hypothalamus, and the possibility of A/P or D/V differences in hypothalamic stem cells, have not been specifically examined. There is also very little known about how the hypothalamic stem cell niche compares to other neurogenic adult niches, the factors that might govern hypothalamic cell proliferation, or the stages which cells progress through from stem cell to differentiated neuron. Bearing these questions in mind, in this thesis I aim to:

- Perform a thorough and systematic investigation of the stem cell associated markers present in both the adult mouse hypothalamus and in neurospheres derived from this same region, and compare and coordinate the two.
- Compare hypothalamic neurospheres with those from other proliferative regions, to look for characteristics unique to the hypothalamic niche.
- Test the different regions of the hypothalamus for neurosphere-forming capacity, in order to map the areas with proliferative potential.
- Initiate a collaboration to examine hypothalamic NS/PC proliferation in vivo.
- Investigate whether the growth factors FGF and EGF are required by neurospheres, and whether FGFs act as endogenous regulators of hypothalamic NS/PCs.
- Devise and test a new ex vivo culture system for hypothalamic tissue that can be used for the study of hypothalamic NS/PCs.

Chapter 2

Chapter 2 – Materials and Methods

2.1 Mice

All mice used (excluding transgenic mice, which were kept and processed at other institutions, under collaborator's licenses) were female C57/Black6 mice of between 6 and 12 weeks of age (post pubescent young adults). Mice were sacrificed using a lethal overdose of inhaled isofluorane anaesthetic, followed by cervical dislocation.

2.2 Histological techniques

2.2.1 Fixation of tissue:

Two alternative fixative solutions were used. A solution of 4% paraformaldehyde in 0.1M phosphate buffer (4% PFA) was used for tissue intended for in situ hybridization (ISH), or for immunofluorescence (IF) for cytoskeletal or cell surface antigens. Tissue was incubated with 4% PFA at 4°C. MEMFA fixative was used where IF was to be performed on nuclear antigens. Tissue was incubated at room temperature (RT) in the dark.

Adult brains were fixed using a standard perfusion method, kindly performed by A. Furley. Following perfusion, the brain was swiftly removed from the skull and post-fixed. The post-fix lasted approximately six hours in 4% PFA, or four hours in MEMFA. Undifferentiated neurospheres were fixed for 40-60 minutes, and differentiated neurospheres were fixed for 15-25 minutes.

2.2.2 Sectioning of tissue:

Tissue from the adult CNS and undifferentiated neurospheres were analysed as cryosections. After fixation, tissue was incubated in a 30% (w/v) sucrose

solution overnight. It was then embedded and frozen in OCT compound (VWR). At this point, the tissue could be wrapped in an airtight covering and stored at -20°C without any subsequent loss of signal. A Bright cryostat was used to cut the tissue, and sections were collected on Superfrost slides (Thermo Scientific). CNS tissue was usually cut to 15-25μm thick for IF, and 30μm for ISH. Neurospheres were cut to 5-15μm. Sections were air dried for 1 – 4 hours, depending on thickness.

2.2.3 Immunofluorescence:

Standard methods were used for the IF detection of proteins on sectioned tissue and differentiated neurospheres [68]. Adult tissue was sometimes pre-treated in a blocking solution of phosphate buffered saline (PBS) + 1% heat inactivated normal goat serum (HINGS) + 0.1% Triton X-100 for one hour, as this was found to improve signal, but this step was not found to be necessary for other tissues. All tissues were incubated in primary antibody diluted in blocking solution overnight at 4°C. A solution of secondary antibody was then applied for 30-60 minutes at RT, with 4',6-diamidino-2-phenylindole (DAPI) added to visualise the cell nuclei (shown in blue throughout this thesis). Slides were mounted using Vectashield mounting medium (Vector Laboratories) and glass coverslips. The antibodies used are listed in Table 2.1. Where antibodies had not been used before in the lab, I determined the optimal conditions myself. To rule out non-specific antibody binding, secondary antibodies were incubated with tissue in the absence of primary antibody exposure (Figure 2.1). Some non-specific labelling was seen using mouse secondary antibodies in the ME and ARC regions, thought to be related to blood vessels (Figure 2.1 A). All other antibodies did not produce non-specific binding, either on CNS sections or neurospheres (Figure 2.1 B-F).

2.2.4 In situ hybridization:

In situ hybridization was performed on cryosectioned tissue, using a modified version of a previously used protocol [69]. Briefly, slides were post-fixed in 4% PFA for 10 minutes. Adult sections were then incubated with a 5μg/μL solution

Primary Antibody	Dilution	Source
AVP	1:500	gift from H. Kawana
BLBP	1:500	Chemicon
BrdU	1:200	Novocastra
DARRP-32	1:200	Chemicon
ChAT	1:100	Chemicon
GFAP	1:50	BD Pharmingen
GHRH	1:600	Chemicon
GLAST	1:4000	Chemicon
Ki67	1:500	Novocastra
MAP2	1:1000	Sigma
nestin	1:800	BD Pharmingen
Nkx2.1	1:1000	Upstate
Nkx2.2	1:50	DS Hybridoma Bank
Nkx6.1	1:50	DS Hybridoma Bank
nodal	1:1000	M. Placzek
NPY	1:100	Immunostar Inc
Pax6	1:50	DS Hybridoma Bank
PSA-NCAM	1:200	Chemicon
pErk1/2	1:50	Cell Signalling Technologies
RIP	1:10	DS Hybridoma Bank
RC2	1:5	DS Hybridoma Bank
Six3	1:1000	M. Placzek
Sox1	1:400	gift from L. Pevny
Sox2	1:1000	Chemicon
Sox3	1:500	gift from T. Edlund
Sox1+3	1:500	gift fromH. Kondoh
TuJ1	1:1000	Calbiochem
TH	1:100	Chemicon
vimentin	1:1000	Sigma

Secondary Conjugate	Dilution	Source
Cy3	1:200	Jackson Immunoresearch
FITC	1:200	Jackson Immunoresearch
Alexa594	1:500	Molecular Probes
Alexa488	1:500	Molecular Probes

Table 2.1

Primary and secondary antibodies

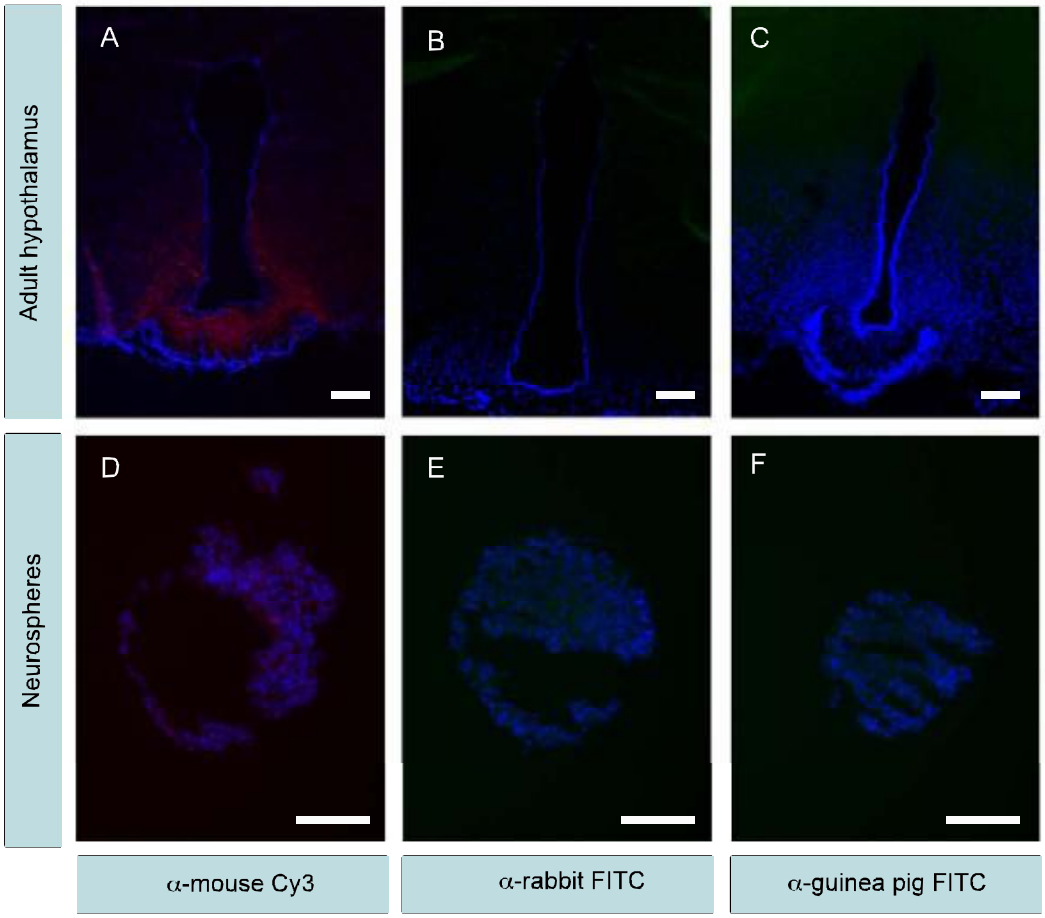


Figure 2.1: Negative controls for antibodies.

Both adult sections and undifferentiated neurospheres were tested for non-specific antibody binding, by testing secondary antibodies alone (with DAPI counterstain in blue).

A-C: Some non-specific binding of mouse secondary antibody was detected in the ME region (A), while rabbit (B) and guinea-pig (C) secondary antibodies did not bind.

D-E: There was no non-specific binding of mouse (D), rabbit (E) or guinea-pig (F) secondary antibodies to neurospheres.

of proteinase K for 15 minutes at RT, to help permeabilise the tissue. Sections were then post-fixed again. Slides were incubated for 10 minutes with an acetylation mix of triethanolamine (Fluka) and acetic anhydride (Sigma), before a minimum of 1 hour incubating in prehybridisation solution at 68°C. RNA probe was diluted in prehybridisation solution, and incubated overnight on the slides at 68°C. The next day the slides were washed twice in formamide/SSC based solutions, before blocking with 10% HINGS in TBST. They were incubated at RT with an AP conjugated α-DIG antibody, for a minimum of 80 minutes and up to overnight. Slides were developed using a solution of NBT and BCIP (both from Roche) diluted in NTMT. Where colour development took several days or weeks, fresh development solution was added at least once a day.

Template DNA was used to generate digoxygen labelled antisense RNA probes using conventional methods. The templates and enzymes for linearization and transcribing are listed below.

Probe	Linearised with	Transcribed with
FGF2	BamH1	T3
FGF10	BamH1	T3
FGF18	EcoR1	SP6
FGFR1	BamH1	T7
FGFR2	EcoR1	T7
FGFR3	Xho1	T7
FGFR4	HindIII	T3
Six3	HindIII	T3
Shh	HindIII	T3

Table 2.2 Generation of RNA probes

2.2.5 Nissl staining:

Nissl staining was performed on cryosectioned tissue, according to standard techniques [70].

2.2.6 Image acquisition:

Standard fluorescent images were taken using one of two systems: an Olympus BX60 running Spot software (Diagnostic Instruments Inc), or an Olympus BX51 using Openlab software (Improvision). The BX60 was also used for colour images of ISHs or nissl staining. Optically sectioned fluorescent images were taken using a Zeiss Apotome microscope with Axiovision software (Zeiss).

2.3 Neurospheres

The neurosphere protocol was adapted from methods used by Molofsky et al [71]. The dissections were altered for hypothalamic tissue, and methods were adapted to take account of the equipment available. The method of mechanical dissociation was also altered, along with passaging and differentiation protocols.

2.3.1 Dissection of tissue:

The head was removed and doused in 70% IMS to improve sterility and prevent hairs from sticking to the instruments. The skin and fur were removed from the head, and the skull opened by making a coronal cut to the posterior skull above the cerebellum followed by a saggital cut along the midline. The dorsal aspect of the skull could then be removed with strong forceps, and the brain lifted out. The optic nerve was usually cut just anterior to the optic chiasm. The brain was immediately transferred to ice cold L-15 medium (GIBCO), and the meninges were removed.

For the standard whole hypothalamus dissection, the brain was laid on its dorsal surface and the tissue to the anterior, posterior and lateral sides of the hypothalamus removed. The tissue was then turned, and cut dorsal to the third ventricle, leaving a cube roughly approximating to the hypothalamus.

Dissection needles were then used to remove the more lateral tissue, leaving the area immediately surrounding the third ventricle (Figure 2.2 A).

For hypothalamic subdissection experiments the brain was cut into thin coronal slices corresponding to the anterior, median eminence and posterior regions (Figure 2.2 D). Dissection needles were then used to remove the appropriate pieces of tissue, using the third ventricle as a guide (Figure 2.2 E). For α and β tanycyte subdissections the β region was first removed from the ventral aspect of the complete brain, before cutting a coronal section and removing the α and ependymocyte regions in the same way as for the original subdissections (Figure 2.2 F).

To generate SVZ neurospheres a slice of tissue anterior to the hypothalamus was used. The lateral ventricles were easily identifiable on these slices, and the lateral area of grey matter containing the SVZ was extracted using needles (Figure 2.2 B).

Spinal cord (SC) dissections were generally taken from the thoracic region between the forelimbs and the middle of the back. The skin was removed from the back, and the muscle to either side of the spinal column was cut. A transverse cut through the spinal column was made as posteriorly as possible, and watchmakers scissors were used to cut up each side of the vertebrae. The dorsal part of the vertebrae was lifted away exposing the spinal cord. This was removed into ice cold L-15 (GIBCO), where the spinal nerve roots and meninges were removed. The lateral edges were trimmed, and any other obvious areas of white matter removed as far as possible (Figure 2.2 C).

2.3.2 Derivation of primary neurospheres:

Dissected tissue was incubated for 20 minutes in trypsin solution (Calbiochem), before trypsin inhibitor (Sigma) and DNase (Sigma) were added and the tissue centrifuged out. It was resuspended in BSA-containing medium, and mechanically dissociated using a sequence of 25 and 30 gauge needles. For

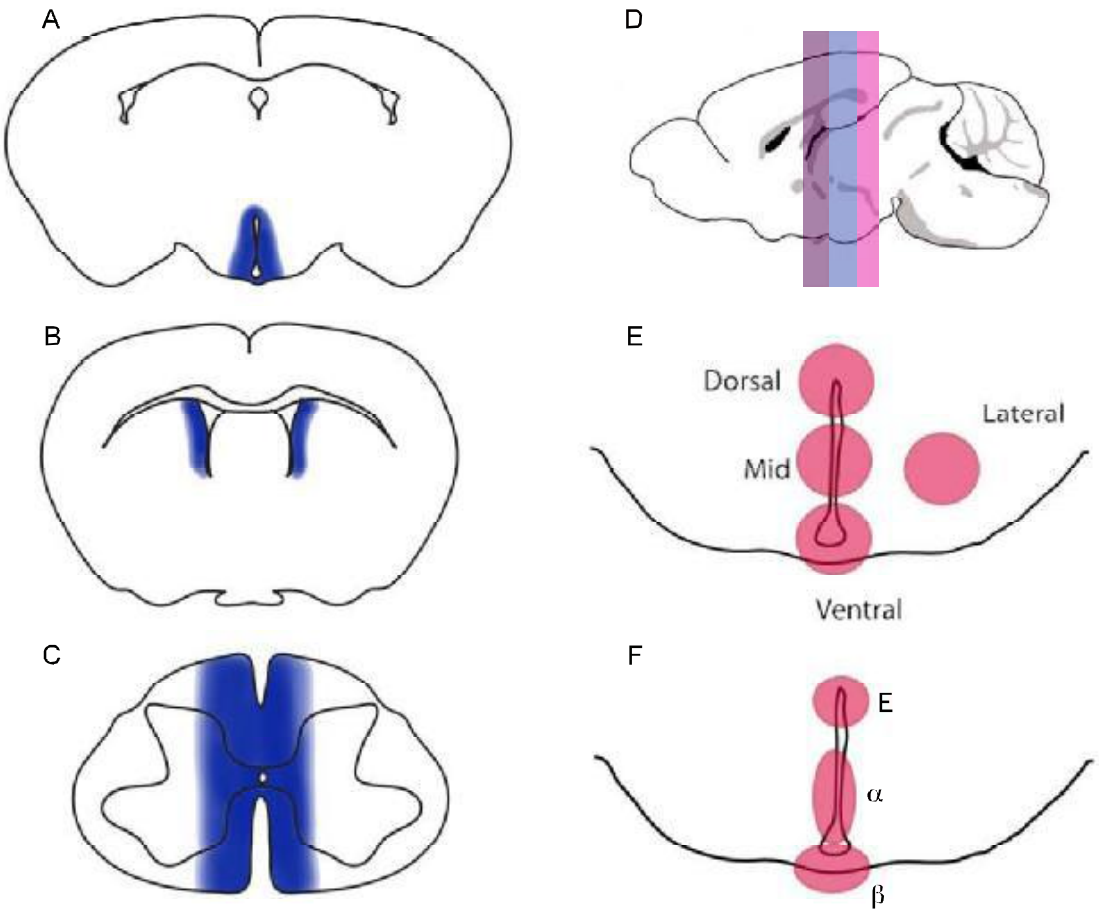


Figure 2.2

Dissections for neurospheres

A-C: Cartoons to illustrate how different brain regions are dissected out for deriving neurospheres. A. Hypothalamus. B. Subventricular Zone. C. Spinal Cord.

D-E: Cartoon illustrating neurosphere subdissections. D shows the position of the three coronal slices (purple =anterior, blue = ME and pink = posterior), and E shows how each one is subdivided into four pieces using the third ventricle as a guide.

F: Adaptation of the standard subdissections to distinguish a and b tanycytes, and ependymocytes.

whole hypothalamus / SVZ / SC preparations the resulting single cell suspension was filtered through a 40μm nylon mesh (VWR). This step was omitted from subdissections due to the small amounts of tissue involved. Cells were counted on a haemocytometer using standard trypan blue exclusion to identify viable cells. Cells were plated onto ultra-low binding plates (Corning) designed to prevent attachment. 6 well plates received 25000 cells per well (c/w), while 24 well plates were given either 6700 c/w (the density equivalent of 25000c/w in 6 well plates) or 4000 c/w for subdissection experiments.

2.3.3 Media and neurosphere culture:

The standard media used was DMEM:F12 (GIBCO) supplemented with penicillin/streptomycin, L-glutamine, B27 and N2 supplements (GIBCO), plus 20ng/ml each of FGF-2 and EGF (Fisher). A later version of this media (termed 'low insulin media') used an alternative homemade N2 that omitted the insulin and replaced it with IGF (Sigma). This version also included 5μg/ml of heparin. Plates were incubated at 37C with 5% CO2. In standard medium they were grown for 14 days before counting, fixing, passaging and/or differentiating. In this protocol, they were usually fed with 1/5 volume of medium containing fresh FGF and EGF approximately every 5 days. Growth was considerably quicker in low insulin medium, and neurospheres were processed 7-10 days after plating. In this case, extra medium was added after 7 days to prevent the wells drying out, but extra FGF and EGF were not added.

2.3.4 Passage of neurospheres:

To passage neurospheres two alternative methods were used. Neurospheres grown in standard medium were passaged by simple mechanical dissociation, using 25 and 30 gauge needles. Those grown in low insulin medium were passaged by a more efficient method, in which they were incubated in TrypLE enzyme solution (GIBCO) before mechanical dissociation. Spheres were then repeatedly passed through the needles until a suspension of single cells was formed. Dissociated cells were either divided between all the wells (where a set

number of neurospheres had been dissociated) or counted and replated at the original density.

2.3.5 Analysis of neurosphere size and number:

Neurosphere counts were done on a standard dissection microscope (Zeiss / Leica), with the magnification set to 1.6x. Every sphere visible at this magnification was counted: this was likely to include some spheres under 50μm diameter, but not the very smallest spheres which could only be observed using a higher magnification. Every well was counted and recorded separately to provide technical replicates. Neurosphere sizes were assessed using the 10x lens of a higher magnification microscope (Leica), equipped with an eyepiece graticule to accurately measure neurosphere diameter. Five size categories were established (<50μm, 50-100μm, 100-200μm, 200-400μm and 400μm+), and the number of neurospheres noted for each. Due to many experiments involving a low number of neurospheres, data was pooled from several wells. A minimum of 50 spheres were assessed for each condition, if this was not possible then that condition was not included in data analysis. If more than 300 spheres were available then not every well was counted, however once a well was started it was always completed.

2.3.6 Differentiation of neurospheres:

Differentiation of neurospheres was performed in 8 well chamber slides (SLS). They were coated with 150μg/mL poly-D-lysine (Sigma), followed by 100μg/mL fibronectin (Sigma) prior to use. The same neurosphere medium as spheres were grown in was used, but containing only 10ng/ml of FGF-2 and no EGF. A single neurosphere was transferred to each chamber, and was incubated for 6-7 days before fixation and processing.

2.4 Slice cultures

There were no records of previous hypothalamic slice cultures, so a protocol for organotypic cerebellar cultures was used as the basis for my methods [72]. As I was optimising this new technique, my methods varied slightly from experiment to experiment. The main procedures are detailed below.

2.4.1 Obtaining slices:

Brains were obtained from young mice as previously described. The anterior and posterior extremities were trimmed, leaving the hypothalamic region. This was then embedded in a solution of 1.4-2.0% low gelling point agarose. Two alternative methods of creating slices were then tested. The first method used a tissue chopper. This was easy to sterilize, but did not always cut the tissue cleanly. A high speed setting was required to reduce the risk of the tissue sticking to the blade, and this may have caused tissue damage. The alternative method was to cut the slices on a vibratome. This was harder to keep sterile, although no problems with infections were ever subsequently observed in the cultures. This method proved to be more reproducible over time, and is thought to cause less trauma to the tissue.

Slices were usually cut 250μm thick, although thick slices of 1000μm were also tested. The agarose was removed from each slice with forceps, and it was then transferred to a membrane insert (Millipore), which was placed in a 6 well plate containing 1100μL of culture medium per well. The inserts provided structural support for the slices, and allowed media to diffuse over the slice without submersion. Usually two or three slices from different A/P levels were placed on each insert.

2.4.2 Culturing slices:

Typically, slices were cultured for between two and five days. They were kept in humidified cell culture incubators at 37°C with 5% CO_2. Culture medium was generally the same as the standard medium formulation used for neurosphere

cultures: DMEM:F12 supplemented with B27 and N2. FGF-2 and EGF were not added. The medium was either replaced with fresh solutions or topped up every one to two days.

2.4.3 BrdU labelling of slices:

Cultures were usually set up at least 24 hours before BrdU was added. A day after plating, BrdU was added to the existing culture medium at a final concentration of 10μM. Slices were left in BrdU for a minimum period of overnight to allow time for it to be incorporated into synthesizing DNA, before being fixed and processed. For longer experiments, it was also included in subsequent media changes at the same concentration.

2.4.4 Electroporation of slices:

Slices were typically cultured for 24 hours before electroporation. Standard DNA constructs encoding either red fluorescent protein (RFP) or green fluorescent protein (GFP) were used (gifts from L. Manning and K. Ohyama), enabling successfully electroporated cells to be identified using a fluorescent microscope. Slices remained on the inserts during electroporation. The electrodes (Genetronics Inc) were placed outside each slice, parallel to the third ventricle. A few microlitres of 10μg/μL DNA was injected into the third ventricle, and the current applied as quickly as possible before it could disperse. Guidance on the levels of current required was taken from Murphy and Messer [73]. Nine 100mV pulses were administered, each 50ms long and approximately a second apart using a TSS10 electroporator (Intracel). Slices were then returned to the incubator for at least 24 hours, to allow the construct to be expressed.

2.4.5 Processing of slices:

Thick slices were sectioned, and processed with antibodies in the same way as other sectioned tissues. Thin slices were processed as wholemounts. They remained on the inserts where possible for structural support, and were fixed in either 4% PFA or MEMFA for 1 – 2 hours.

BrdU requires antigen retrieval before it can be detected with antibodies, and for this two alternative methods were tested. The first was adapted from Choi et al, and involved incubating the slices in 2M HCl for 30 minutes [72]. The other used DNase I instead of HCl. Slices were incubated with 45units/mL DNase I at 37°C, for a range of times between 30 minutes and 5 hours. Care needed to be taken with the incubation times – 3 hours gave good results, while 5 hours resulted in extensive tissue damage. Both methods were ultimately capable of detecting BrdU incorporation, but they varied in the level of background and compatibility with other antibodies. HCl gave slightly weaker labelling of other antigens, but with very low background. The BrdU labelling itself was very clear, and this is the method that was adopted for subsequent use. Slices being processed for vimentin detection also required antigen retrieval: for this they were incubated in citrate buffer at 95°C before antibody labelling.

All wholemount slices were incubated in blocking solution for several hours, before adding the primary antibodies overnight. The next day they were washed in PBS for several hours, and then secondary antibodies were applied overnight. On the final day the slices were given 1 hour in PBS + DAPI, then two more long PBS washes. Slices were mounted on glass slides, with a frame of electrical tape to prevent them being squashed.

2.5 In vivo BrdU experiments

All in vivo BrdU experiments were performed by D. McNay or M. Kokoeva, working in the Division of Endocrinology, Diabetes and Metabolism, Harvard Medical School, Boston. The results shown in Figure 6.7 were labelled with antibody and analysed by myself, those in Figure 6.8 were entirely the work of those mentioned above. The method was the same as that previously published by M. Kokoeva [62, 64]. Briefly, mice were surgically fitted with an osmotic minipump, with a cannula leading into one of the lateral ventricles. The pumps

released 12μg/day of BrdU into the ventricle, alone (for control mice) or in combination with 0.75μg/day of FGF-2 for a period of 7 days. 1-6 weeks after the start of the experiment, the mice were sacrificed, and antibodies were used to examine the distribution of newborn $BrdU^+$ cells.

Chapter 3

Chapter 3: Marker analysis of the adult mouse hypothalamus

3.1 Introduction

The adult mouse hypothalamus is relatively poorly characterized with respect to markers related to proliferation and neurogenesis. There are many markers whose link with these processes has been firmly established through studies in the SVZ and SGZ. In both of these areas, the various stages of stem and progenitor cells can be distinguished based on marker expression. Many markers are common to both neurogenic niches, and so it is logical that they may also be expressed in a hypothalamic stem cell niche. In this chapter I intend to test three hypotheses:

1. That marker analysis will suggest the presence of neural stem/progenitor cells (NS/PCs) in the adult hypothalamus.
2. That marker analysis will suggest which region(s) of the hypothalamus contain NS/PC populations.
3. That marker analysis may indicate NS/PCs with a unique hypothalamic regional identity.

3.2 Results

3.2.1 Structural features of the mouse hypothalamus

To begin, I wanted to establish the major morphological features of the mouse hypothalamus, to ensure that regions could be correctly identified and the markers precisely located. Sections of adult brain were taken approximately every 300μm along the anterior/posterior axis (A/P axis) and stained with Nissl stain to mark the cell bodies. The major hypothalamic nuclei were clearly visible on these sections, and could be identified using online brain maps. In

addition to the nuclei marked in Figure 3.1, it could be seen that the ependymal lining of the third ventricle is a single layer of tightly-opposed cell bodies for most of its length, although it may widen slightly near the median eminence. Various morphological features identified on these sections, such as the optic chiasm and the median eminence, could also be identified on a wholemount ventral view of the brain by light microscopy. This provided useful reference points for later dissections of the hypothalamus (see chapters 4 and 5).

3.2.2 Stem cell markers

Many different molecules have been suggested to act as markers of adult neural stem cells. They include extracellular and intracellular signalling molecules, transcription factors and components of the cytoskeleton. No single factor has yet been shown to be expressed exclusively in NSCs, however there are many that are generally associated with stem cells in the known neurogenic niches.

Sox transcription factors:

The Sry-related high mobility group (HMG) of transcription factors, known as Sox transcription factors, are widely expressed throughout development. There are 26 known vertebrate Sox proteins, divided into 7 subgroups [74]. The SoxB1 subgroup comprises Sox1, 2 and 3. All three of these have been implicated in the maintenance of neural stem cells by counteracting the differentiation effect of proneural proteins [45]. They are some of the earliest proteins expressed by the neuroectoderm, and continue to be expressed in neurogenic cells right through to adulthood. A study in Sox1-GFP mice suggested that all the neurosphere-forming cells in the embryonic mouse brain were $Sox1^+$ [75]. In the adult mouse brain, Sox2 is expressed in all known neurogenic regions, including the SVZ, the SGZ and the olfactory bulb [46]. It was also noted to be present in the central canal of the spinal cord, the cerebellum, scattered cells of the cortex and the ependymal lining of the third ventricle. In addition, all neurospheres generated from these mice expressed Sox2, which was

Figure 3.1

Structural features of the adult mouse hypothalamus

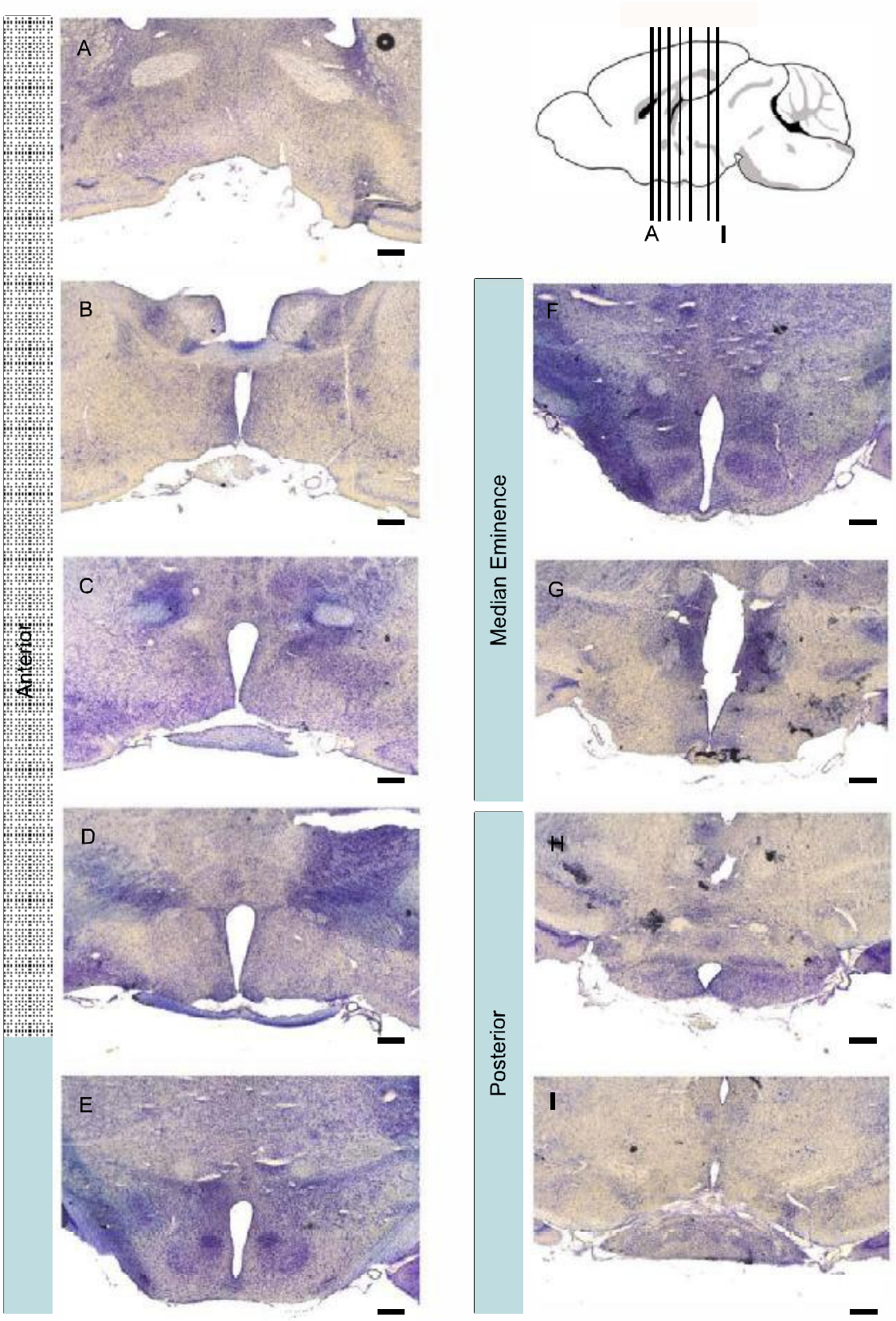
A-I: Nissl stained sections at the levels indicated in the cartoon. Scale bars = 200μm.

J: Enlargement of picture D, showing how the nissl staining can be used to identify the positions of major hypothalamic nuclei in the anterior hypothalamus.

K: Enlargement of picture F, showing how the nissl staining can be used to identify the positions of major hypothalamic nuclei in the mid/posterior hypothalamus.

Abbreviations:

PVH = Paraventricular Hypothalamic Nucleus
PVa = Periventricular Hypothalamic Nucleus (ant)
SCN = Suprachiasmatic Nucleus
AHN = Anterior Hypothalamic Nucleus
SO = Supraoptic Nucleus
LHA = Lateral Hypothalamic Area
ARC = Arcuate Nucleus
VMH = Ventromedial Hypothalamic Nucleus
DMH = Dorsomedial Hypothalamic Nucleus
TU = Tuberal Nucleus
ZI = Zona Incerta
3V = Third Ventricle
Fx = Fornix
ON = Optic Nerve
Mtt = Mammilothalamic Tract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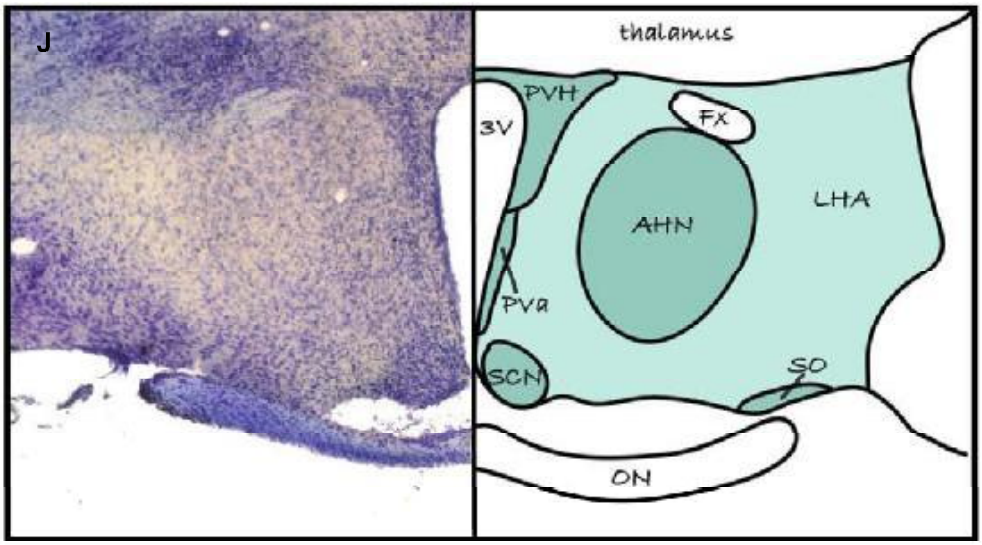
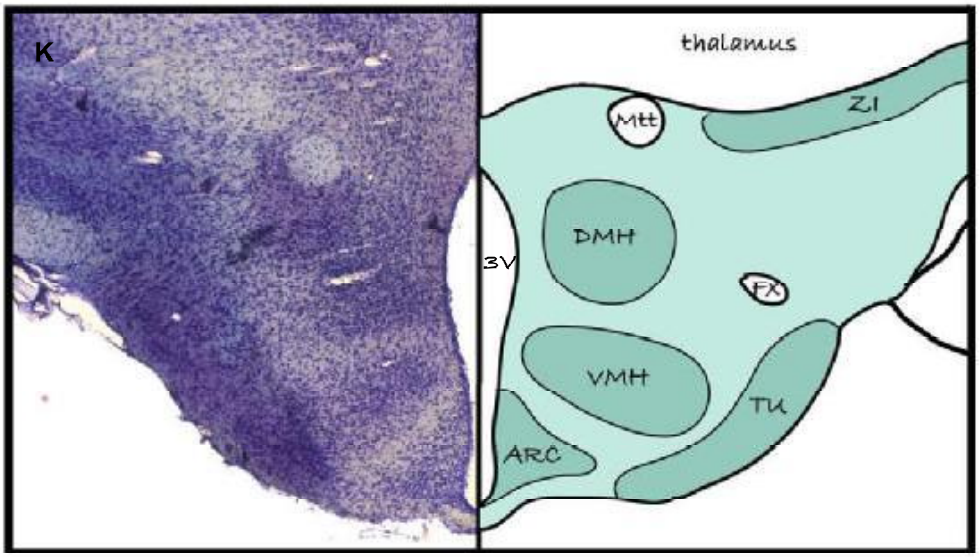
Anterior
Median Eminence
Posterior





Abbrieviations: PVH = Paraventricular Hypothalamic Nucleus, PVa = Periventricular Hypothalamic Nucleus (ant), SCN = Suprachiasmatic Nucleus, AHN = Anterior Hypothalamic Nucleus, SO = Supraoptic Nucleus, LHA = Lateral Hypothalamic Area, ARC = Arcuate Nucleus, VMH = Ventromedial Hypothalamic Nucleus, DMH = Dorsomedial Hypothalamic Nucleus, TU = Tuberal Nuxcleus, ZI = Zona Incerta, 3V = Third Ventricle, Fx = Fornix, ON = Optic Nerve, Mtt = Mammilothalamic Tract.

downregulated upon differentiation. These results led the authors to suggest that Sox2 could be a universal marker for neural stem cells.

My analysis of SoxB1 expression in the adult mouse hypothalamus indicates that all three SoxB1 proteins are expressed in approximately the same cells. They are found in the ependymal lining of the third ventricle throughout the A/P axis (Figure 3.2 and 3.3). Expression is most dense in the ventral two thirds of the ependymal cells, where every single cell appears to express the three proteins (Figure 3.2 H and 3.3 I, K). In the dorsal-most third, Sox^+ and Sox^- cells are intermingled (Figure 3.2 I and 3.3 J). In addition to the ependymal expression, varying levels of Sox protein are seen in cells scattered throughout the parenchyma. For the most part these Sox^+ cells appear to be scattered randomly, with no bias towards specific nuclei, or the A/P or D/V axes. The exception to this is the suprachiasmatic nucleus of the anterior hypothalamus, which demonstrates particularly dense Sox expression (Figure 3.2 B, C and Figure 3.3 B, H). In addition, I examined the expression of eGFP in a Sox2-eGFP mouse line. The expression of eGFP generally mirrored that detected by Sox2 antibodies. The one important exception to this is the ventral portion of the ependymal lining at the level of the median eminence, which surprisingly did not express eGFP (Figure 3.2 J). Double-labelling of Sox2-eGFP sections with Sox2 antibody indicated that Sox2 was present, even in the $eGFP^-$ region, suggesting that in these cells Sox2 may be controlled by a different promoter.

GFAP:

Glial fibrillary acidic protein, or GFAP, was first identified as a marker of mature astrocytes. It is an intermediate filament, forming part of the cytoskeleton. Since the theory that adult NSCs are actually astrocytic in character has gained credibility, there has been much interest in its potential as a stem cell marker. B cells, the NSCs of the SVZ, express GFAP as well as being morphologically reminiscent of astrocytes [41]. However, there is no suggestion that all $GFAP^+$ astrocytes are capable of acting as stem cells, even when placed in a permissive environment. This means that GFAP is a highly non-specific marker, found in

Figure 3.2

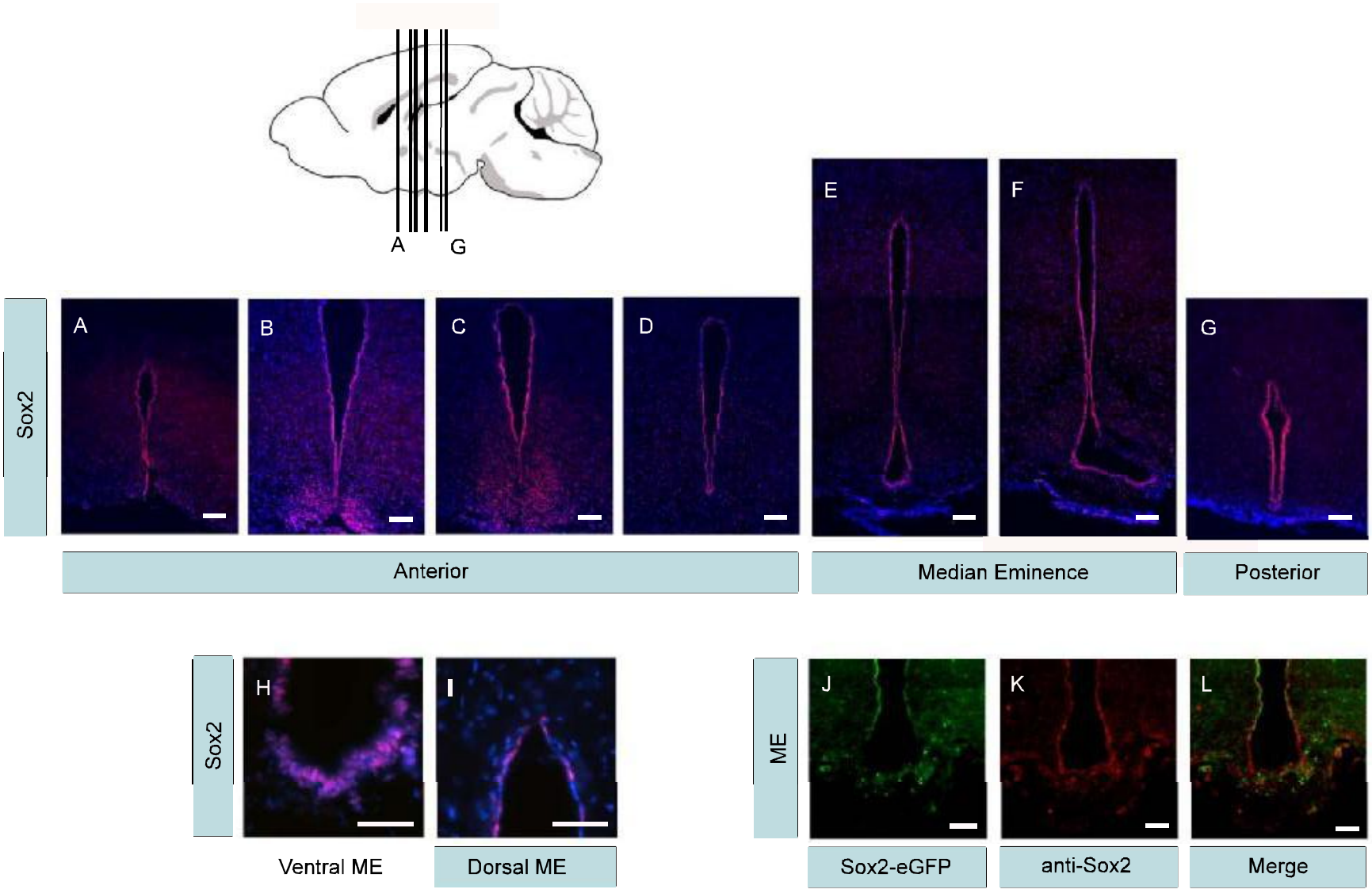
Expression of Sox2

A-G: Distribution of $Sox2^+$ cells along the A/P axis of the hypothalamus. Coronal sections were taken at the levels indicated on the cartoon (red = α-Sox2, blue = DAPI). Scale bars = 100μm.

H: High power magnification at the level of picture E, showing that at this point the ventral ependymal layer is up to three cells thick, with every cell expressing Sox2. Scale bars = 50μm.

I: High power magnification at the level of picture E, showing that the dorsal ependymal layer is only a single layer of cells, and contains both Sox2 positive and negative nuclei. Scale bars = 50μm.

J-L: High power magnification of a Sox2-eGFP mouse. These mice have a GFP negative region at the ventral ependymal layer at the level of the ME, however α-Sox2 labelling indicates that Sox2 is still present in this region (Green = GFP, red = α-Sox2). Scale bars = 50μm.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G
Sox2
A
B
C
D
E
F
G
Anterior
Median Eminence
Posterior
Sox2
H
I
Ventral ME
Dorsal ME
ME
J
K
L
Sox2-eGFP
anti-Sox2
Merge

Figure 3.3

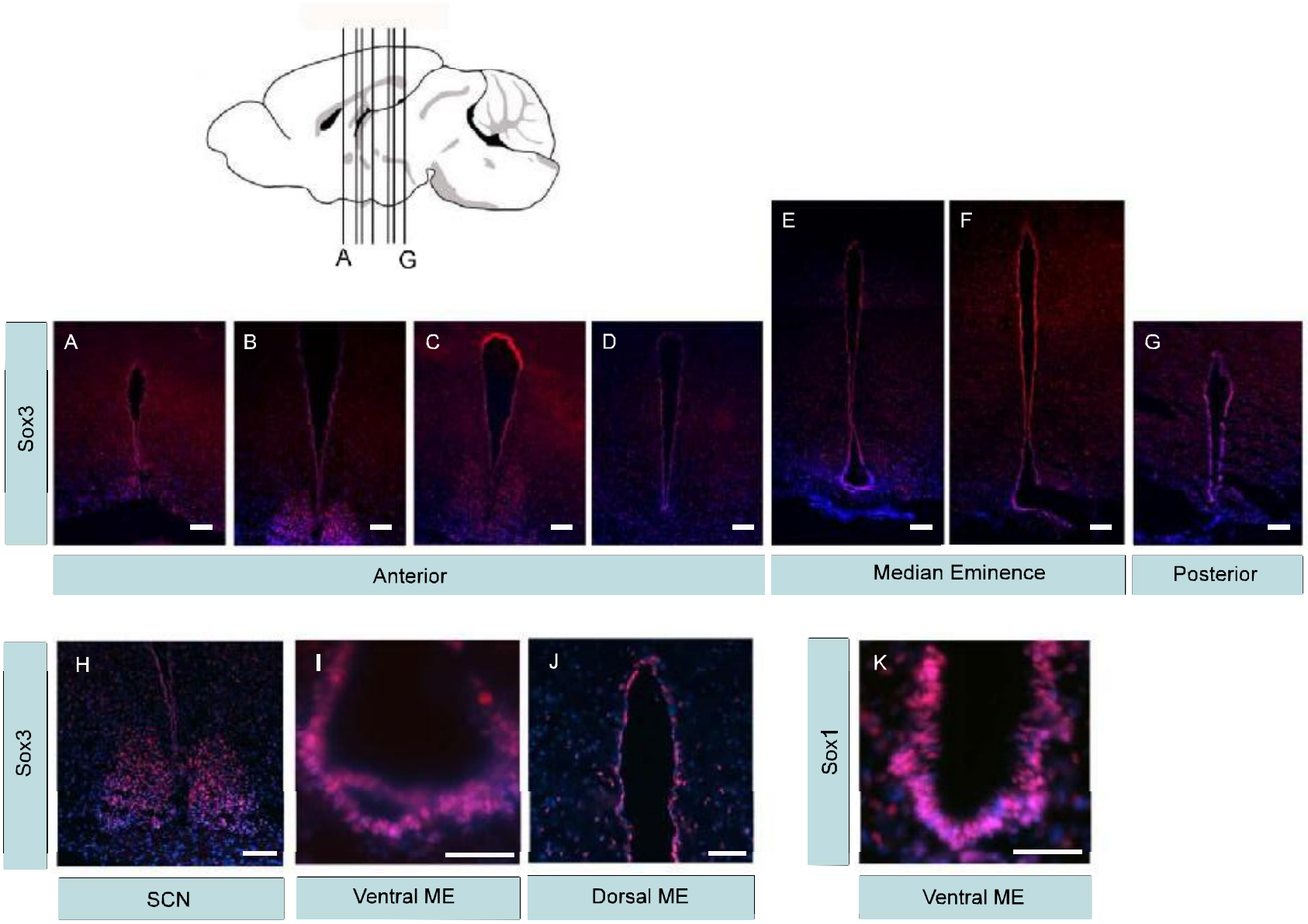
Expression patterns of Sox1 and Sox3

A-G: Sox3 expression along the A/P axis of the hypothalamus. Coronal sections were cut at the levels indicated in the cartoon. Scale bars = 100μm.

H: High power magnification showing Sox3 expression in the suprachiasmatic nucleus. Scale bar = 100μm.

I,J: High power magnification showing Sox3 expression in the ventral (I) and dorsal (J) ependymal layer at the level of the median eminence. Scale bars = 50μm.

K: High power magnification showing Sox1 expression in the ventral ependymal layer at the level of the median eminence. Sox1 expression was found to be indistinguishable from Sox3 expression throughout the hypothalamus. Scale bar = 50μm.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J
SCN
Ventral ME
Dorsal ME
Sox1
K
Ventral ME

many non-proliferative cells throughout the adult brain. The hypothalamus generally contains fewer astrocytes than other brain regions, making it an ideal region to use this marker. The issue of whether GFAP is expressed in tanycytes is controversial, with different groups variously reporting it present, absent, weakly expressed ([76], [77], [14]), or limited to specific subtypes (D. McNay - personal communication).

In the hypothalamus, GFAP expression is largely confined to the area around the third ventricle and the ventral surface of the brain (Figure 3.4). It is likely that it is in the ependymal cells themselves, although it should not be ruled out that it could be in the cells immediately adjacent laterally. Either way, it is clear that $GFAP^+$ processes contact the ventricular surface and the CSF. GFAP is seen around the entire A/P axis of the ventricle. For most of this length it is evenly distributed along the D/V axis. The only exception is towards the ventral part of the ventricle around the ARC and ME, where labelling is reduced or absent (Figure 3.4 G). This region is likely to corresponds to the β and ventral $\alpha 2$ subtypes of tanycytes. At the level of ventral $\alpha 2$ cells GFAP is entirely absent. At the level of the β tanycytes GFAP is seen in the ME, however it appears to be in cells outside the ependymal layer, and not in contact with the CSF. A few scattered cells presumed to be astrocytes are seen in the hypothalamic parenchyma, particularly in the arcuate nucleus.

Nestin:

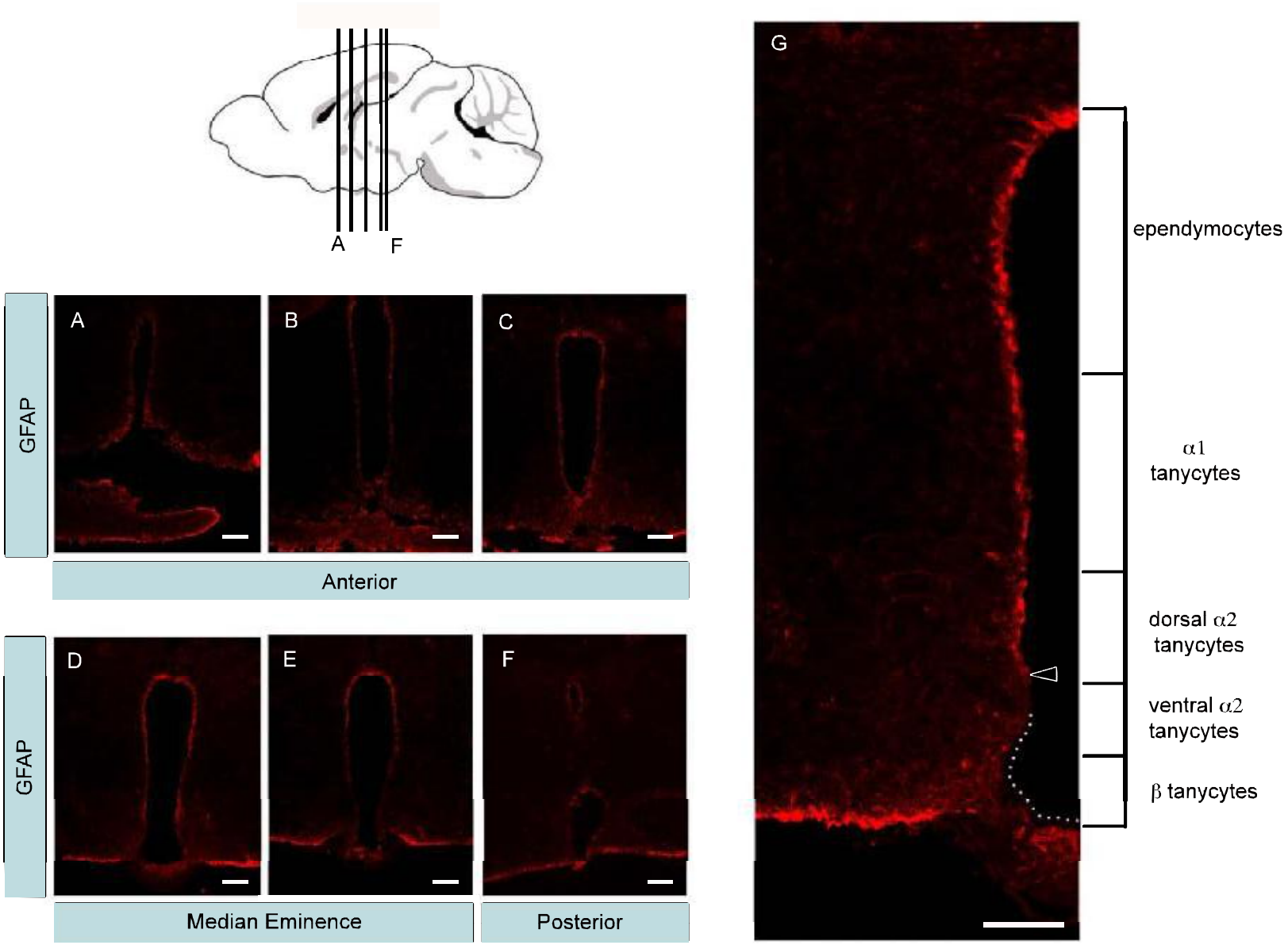
Nestin is a type IV intermediate filament. It cannot form homodimers, but instead acts by binding heterologously to other intermediate filament proteins such as vimentin (reviewed in [61]). It is suggested that nestin plays a role in the coordination of changes in intracellular dynamics, and as such may be required by dividing and migrating cells. Nestin is widely expressed throughout development in various types of mitotic cells, including radial glia, but is generally downregulated as cells become post-mitotic. In the adult brain reactive gliosis due to injury is associated with the upregulation of nestin. It has

Figure 3.4

Expression of GFAP

A-F: GFAP expression along the A/P axis of the hypothalamus. Scale bars = 100μm.

G: High power magnification of D, showing GFAP expression at the level of the median eminence. Approximate boundaries between ependymocytes and tanycyte subtypes are marked. The white line indicates the ventricle boundary where it is not obvious. There is a sharp boundary between $GFAP^+$ ependymal cells dorsally and $GFAP^-$ ependymal cells ventrally (outlined arrowhead). Scale bar = 50μm.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F
GFAP
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α1
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dorsal α2
tanycytes
ventral α2
tanycytes
β tanycytes

also been suggested to be a marker of adult NSCs, and is found in both the SVZ and the SGZ. It has been reported to be present in the ependyma of the third ventricle in rats, in cells presumed to be tanycytes [78],[59]. The same authors noted that nestin levels do not correlate with levels of proliferation, suggesting that nestin may be expressed in stem cells that are more quiescent rather than rapidly proliferating transit amplifying cells. They suggest that the nestin$^+$ cells of the third ventricle differ significantly from the nestin$^+$ cells of the SVZ, both morphologically and in their coexpression of GFAP.

The expression of nestin was examined using an antibody raised in mouse (Figure 3.5). While I could not detect any tanycytic expression of nestin at the level of the ME, a clear population of nestin$^+$ cells was seen more anteriorly, at the level of the SCN. These cells resembled tanycytes in their morphology, having the cell body in the ependymal layer and a single long process, in this case extending towards the SCN (Figure 3.5 A, C and D). Despite this morphological similarity, it seems unlikely that these cells are in fact tanycytes, as their location is considerably further anterior. In the median eminence several apparently nestin$^+$ cells were seen, although none with the telltale long process of tanycytes (Figure 3.5 B, E and F). These cells were found either in the ependymal layer of the floor of the third ventricle, or immediately ventral to this. However, some background labelling was seen whenever mouse antibodies were used in the median eminence (Figure 2.1 A), particularly in the blood vessels common to the area, and so it cannot be certain that these cells are truly expressing nestin.

3.2.3 Developmental markers:

Many of the pathways that are active in development have also been found in adult stem cell systems. As both adult and developmental stem cells undergo very similar processes such as proliferation and differentiation choices, albeit in a very different environment, this is not surprising. Whether or not adult stem cells recapitulate the precise pathways seen in the same area during embryo

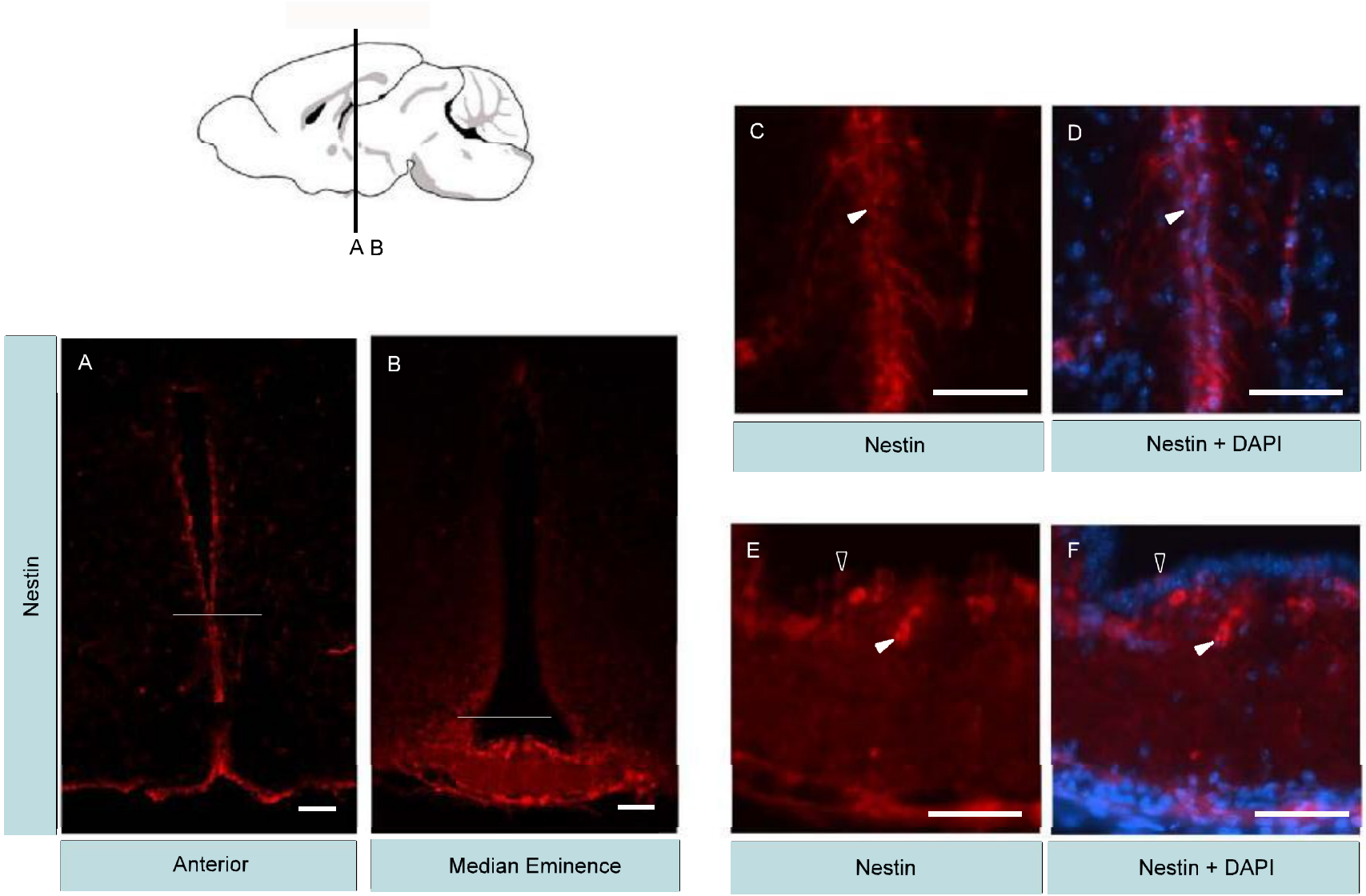
Figure 3.5

Expression of nestin

A, B: Expression of nestin along the A/P axis of the 3 week old mouse hypothalamus. Scale bars = 100μm.

C, D: High power magnification of the area marked in A. Nestin$^+$ cells with a single long process can be clearly seen. Co-labelling with DAPI confirms that the nestin$^+$ cells have their cell body in the ependymal layer of the third ventricle (an example cell is highlighted by the solid arrowhead). Scale bars = 50μm.

E, F: High power magnification of the area marked in B. Occasional apparently nestin$^+$ cells are seen in the ventral ependymal layer (outlined arrowhead), as well as in the ME (solid arrowhead). The nestin antibody may sometimes label blood vessels, and it is not clear if this is what is marked in the ME. Scale bars = 50μm.


A B
Nestin
A
B
C
D
Anterior
Median Eminence
Nestin
Nestin + DAPI
E
F
Nestin
Nestin + DAPI

development is another question, to which there is currently no good answer. However a reasonable hypothesis is that some of the markers seen in the embryonic hypothalamus may be seen in the adult stem cell population, and may give some clues as to how these cells behave and how they are regulated.

Six3:

The Six3 transcription factor is an important early marker of anterior neural tube, and later on is progressively restricted to specific areas. It has been reported to be present in the hypothalamus of both adult mouse and human [79],[80]. It has been suggested to act to maintain NS/PCs by both maintaining an undifferentiated state and shortening the cell cycle to promote proliferation [81].

I examined the expression of Six3 using both an antibody to detect the protein and in situ hybridization to detect mRNA (Figure 3.6). In both cases I saw expression in the same areas. In contrast to Conte et al [79] I did not see any expression in the hippocampus or the medial habenula, however Six3 was clearly present in the reticular thalamic nucleus and the ventral ependymal lining of the third ventricle, at the level of the median eminence (Figure 3.6 C, D). It was also present in some cells of the suprachiasmatic nucleus (Figure 3.6 A). From close examination of the antibody pattern in the ependymal layer it appeared that there were actually two levels of Six3 expression: strong expression in the ventral most portion, and weaker expression adjacent to this (Figure 3.6 F). This was confirmed by the in situ analysis, which displayed two analogous levels of mRNA expression (Figure 3.6 G, H). It is likely that the stronger expression is at the level of the β tanycytes, while the weaker expression is in the dorsal α2 and maybe α1subtypes. It is not clear from this data whether ventral α2 tanycytes belong in the strong or weak expression group. No Six3 expression was detected in ependymocytes. Six3 was also observed in occasional scattered cells around the same region, mainly located in the arcuate nucleus.

Figure 3.6

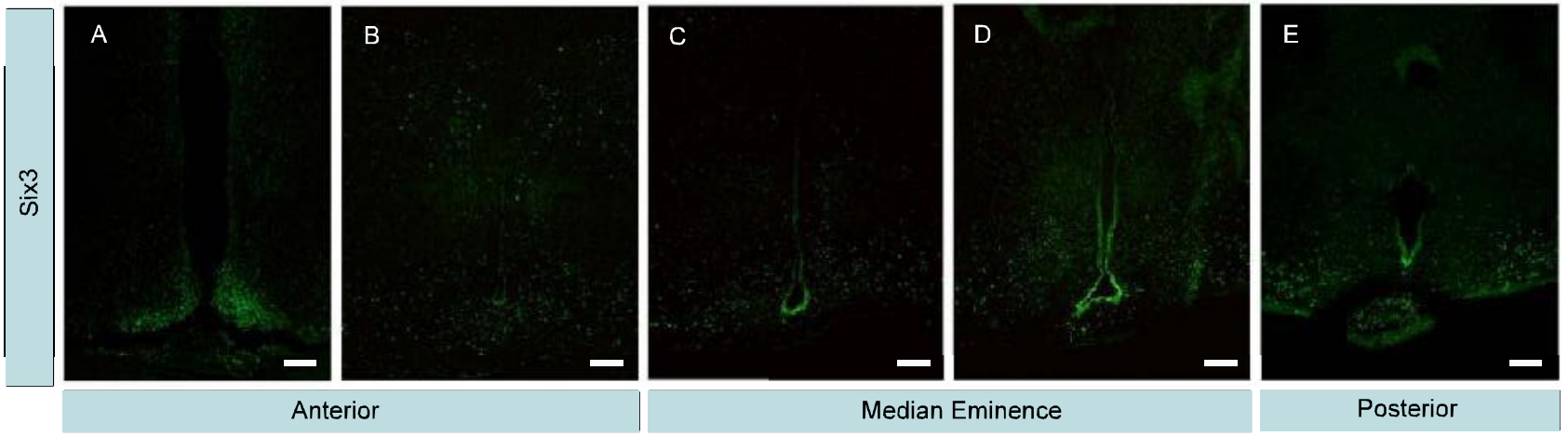
Expression of Six3

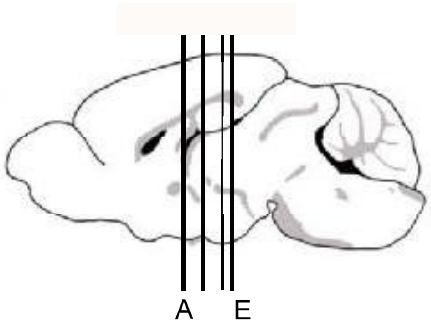
A-E: Six3 expression along the A/P axis of the hypothalamus. Scale bars = 100μm.

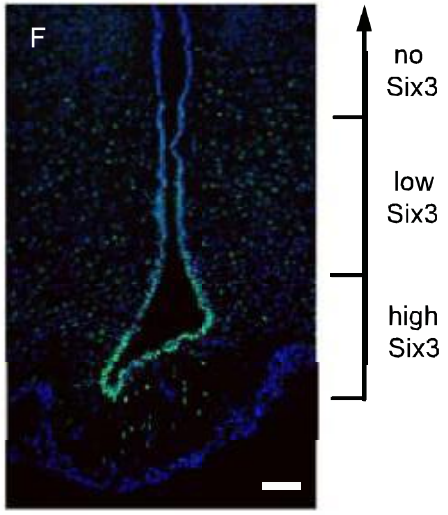
F: High power magnification of the ventral ependymal layer at the level of the median eminence. Three distinct zones of Six3 expression can be observed: the ventral most portion expresses high levels of Six3, while the region just dorsal to this expresses a low level of Six3. Regions located further dorsally again do not express detectable levels of Six3. Six3 can also be seen in some parenchymal cells of the arcuate nucleus. Scale bar = 50μm.

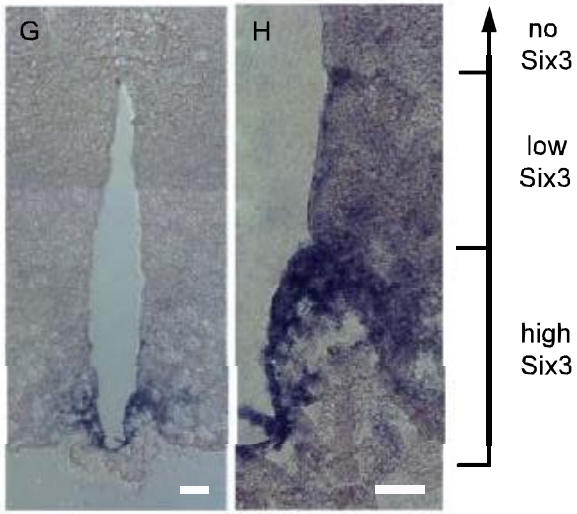
G: *In situ* hybridisation of Six3, demonstrating that the distribution of Six3 mRNA mirrors that of the protein. Scale bar = 100μm.

H: High power magnification of G, confirming the presence of high and low level expression zones for Six3 mRNA. Scale bar = 50μm.


Six3
A
B
C
D
E
Anterior
Median Eminence
Posterior


A
E


F
no Six3
low Six3
high Six3


G
H
no Six3
low Six3
high Six3

Sonic Hedgehog:

Shh is a ventral neural tube morphogen important for the specification of neural tissue, including hypothalamic tissue [5], [82]. More recently, it has also been implicated in the control of adult NSCs. It has been shown to positively regulate proliferation in both the SGZ and SVZ of rodents [50],[49]. Shh has been reported in the ventral third ventricle ependyma of rats, along with the associated molecules Patched and Smoothened, however it has not yet been examined in mice [83].

I found it difficult to use the Shh antibody on adult mouse tissue – in particular the area around the median eminence contains a lot of background labelling (Figure 2.1), making it impossible to ascertain if Shh is really present. As an alternative, I used in situ hybridisation to look for Shh mRNA (Figure 3.7). While specific Shh-expressing cells could not be singled out, it does seem that Shh is present in a diffuse area of the ventral CNS. It appears weaker at the level of the anterior hypothalamus, and stronger in median eminence / posterior regions. In particular it is expressed in the arcuate and ventromedial nuclei (Figure 3.7 D). In general the level of staining seen in the ependymal layer matches that of the surrounding tissue, apart from a few patches of stronger expression.

Nodal:

Nodal is an early signalling molecule important for hypothalamic specification. It has been suggested to act cooperatively with Shh in this role [4]. Its distribution in the adult CNS has not been reported in the literature.

Nodal expression seemed to be limited to the ventral floor of the third ventricle, and was not present more anteriorly (Figure 3.8 A-C). It is highly likely that nodal is marking the β tanycytes, as nodal$^+$ cells also have a cell body located in the ependymal layer and a single long process extending across the median eminence (Figure 3.8 D).

Figure 3.7

Expression of Shh

A-F: ISH showing location of Shh mRNA along the A/P axis of the hypothalamus. The location of the arcuate nucleus (ARC) and ventromedial nucleus (VMH) are marked on D. Scale bars = 100um.

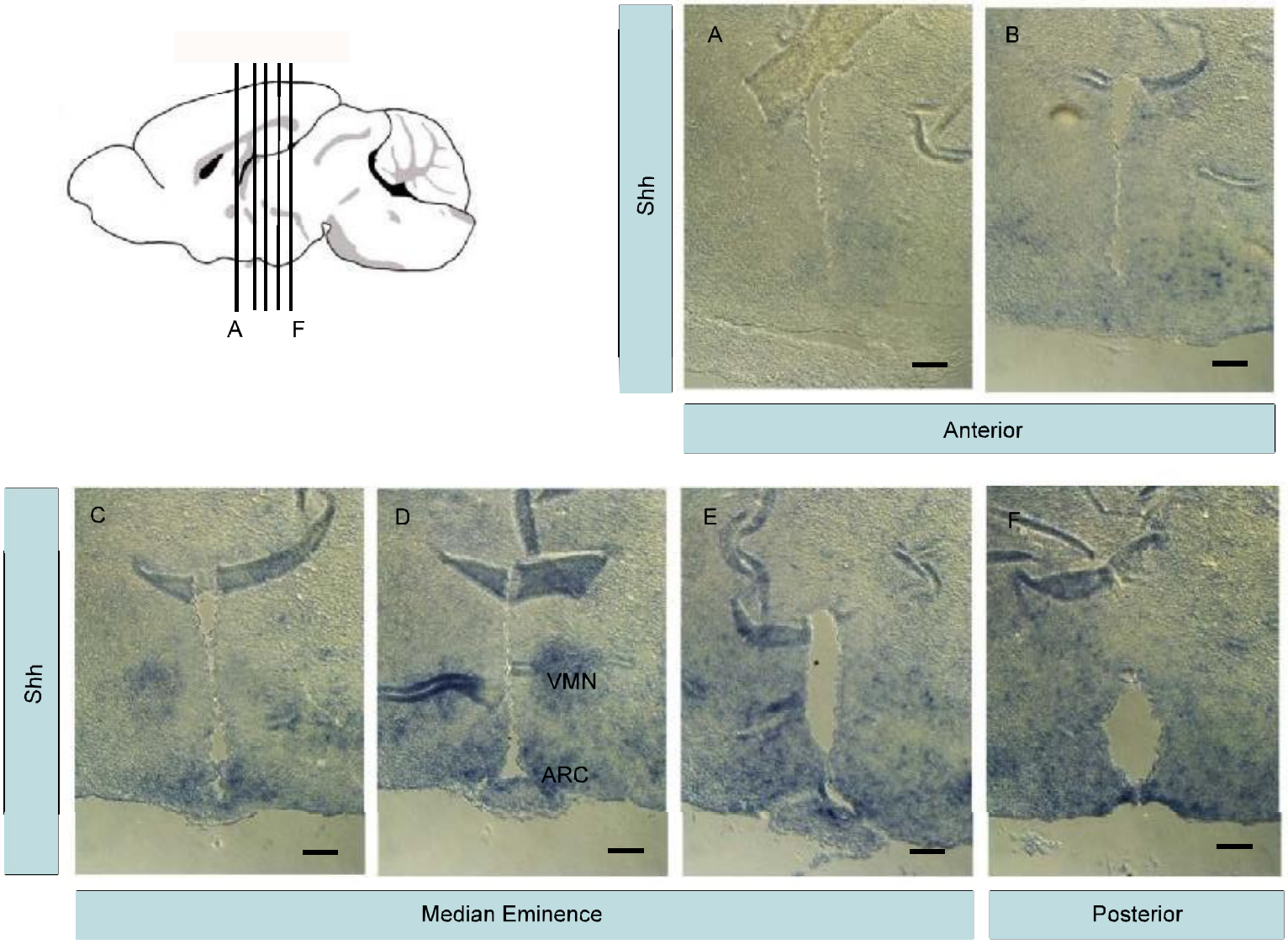
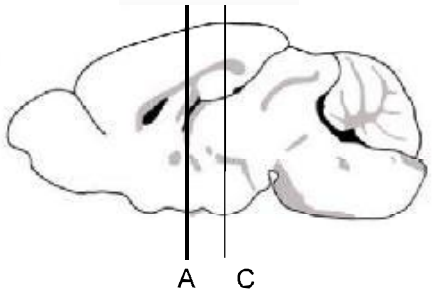

A
F
Shh
A
B
Anterior
Shh
C
D
VMN
ARC
E
F
Median Eminence
Posterior

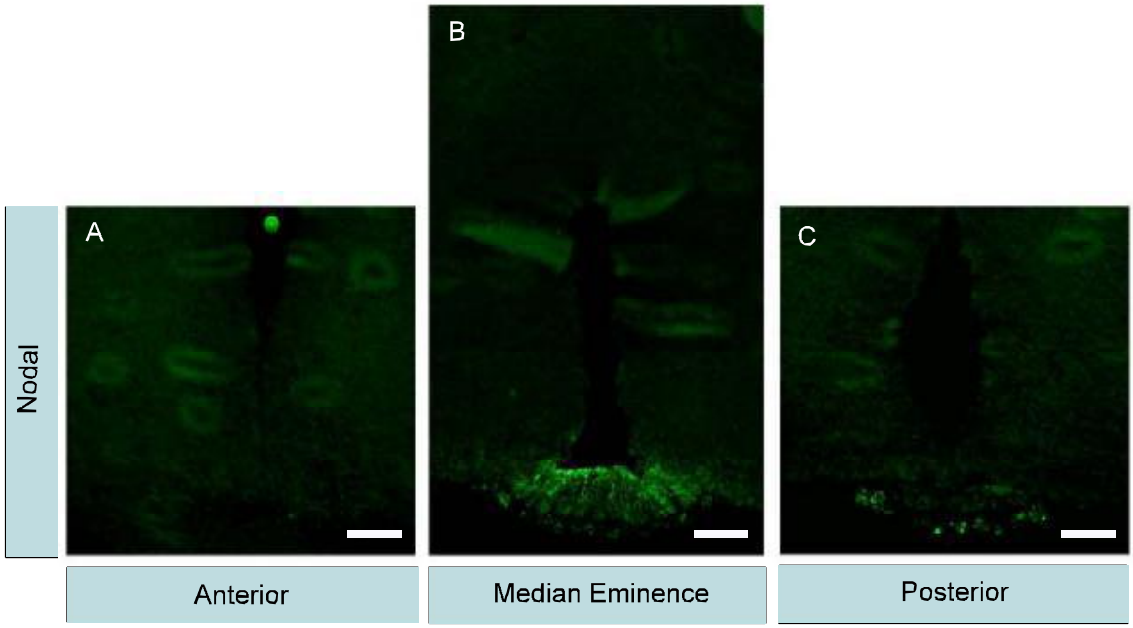
Figure 3.8

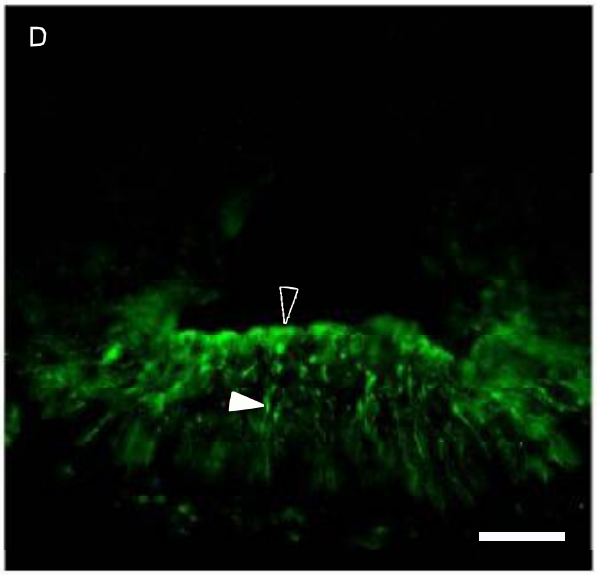
Expression of Nodal

A-C: Expression of nodal along the A/P axis. Scale bars = 100μm.

D: High power magnification of D. Nodal$^+$ cells have a position and morphology which suggestive of β tanycytes, with a cell body in the ventral floor of the third ventricle (outlined arrowhead) and a long process extending into the median eminence (solid arrowhead). Scale bar = 50μm.


A
C


B
A
C
Nodal
Anterior
Median Eminence
Posterior


D

3.2.4 Radial glia / tanycyte markers

As outlined in the introduction, it has been proposed that tanycytes may act as the NSCs in the hypothalamus. I therefore examined the distribution of known tanycytic markers. These could be used to identify the precise location of the tanycytes, as well as to distinguish between some of the subtypes. In addition it enabled me to compare whether the stem cell and developmental markers examined previously colocalised with the tanycytes.

Vimentin:

Vimentin is an intermediate filament widely reported to be present in tanycytes [13]. It is particularly useful as it is strongly expressed in tanycytic processes, and so can be used to determine the target of the process and so the subtype of the tanycyte. Developmentally, vimentin is expressed in radial glia, but downregulated in mature neurons and astrocytes [84].

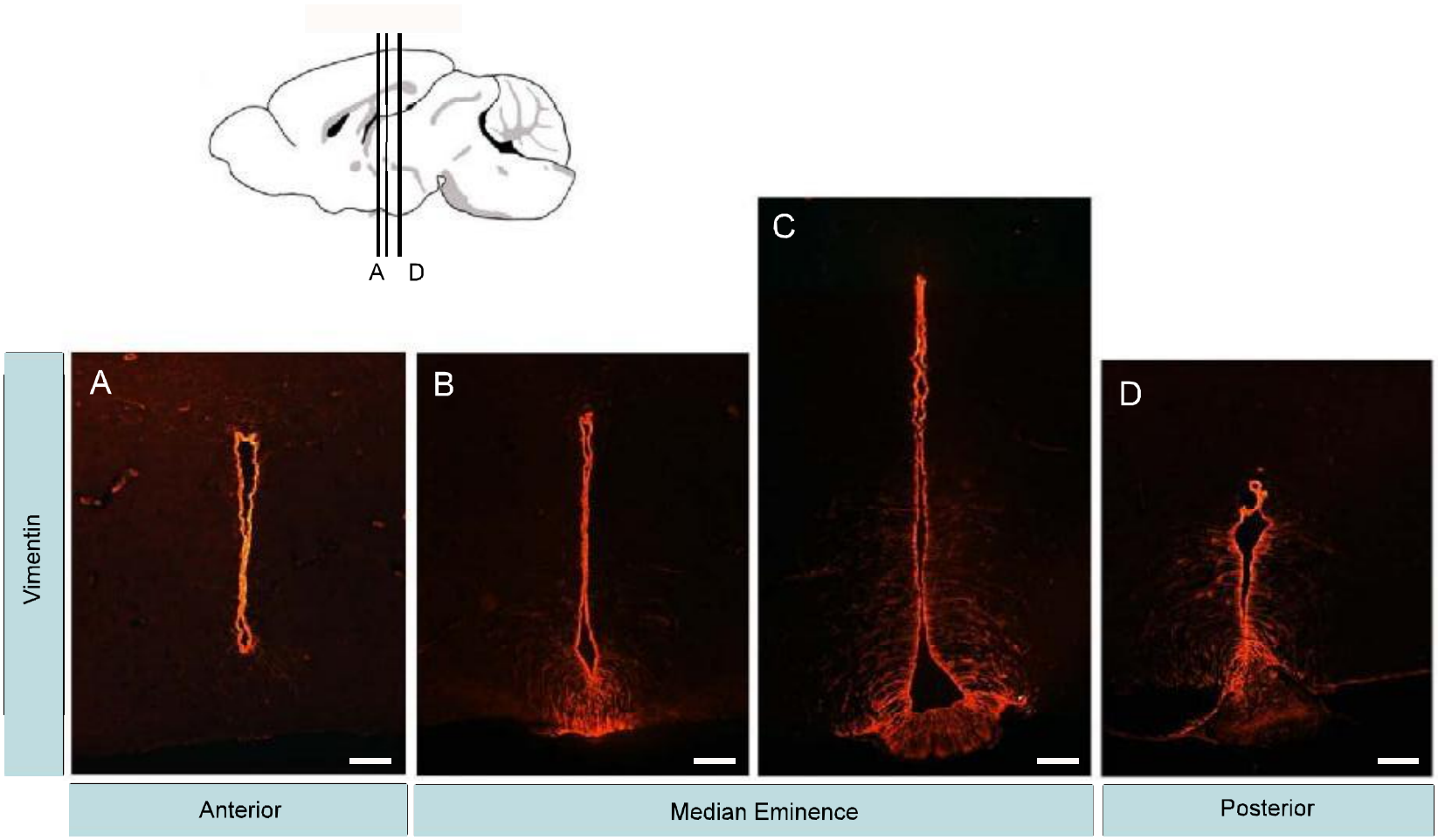
When combined with an antigen retrieval technique, vimentin gave very robust labelling of tanycytes, including the processes (Figure 3.9). Their distribution and morphology was exactly as described in the literature: the cell bodies were clearly located in the ependymal lining of the third ventricle, with a long process extending into the median eminence (β tanycytes) or the parenchyma (α tanycytes) (Figure 3.9 E). Moving from anterior to posterior they were first seen shortly before the median eminence becomes a distinct structure, in the ventral most portion of the ependyma, and extended along all portions of the ventricle posterior to this (Figure 3.9 A-D). By the level of the ME they covered the entire ventral two thirds of the ventricles height. Dorsal to this the ependymocytes were also very strongly labelled, but could be morphologically distinguished by their lack of a vimentin$^+$ process.

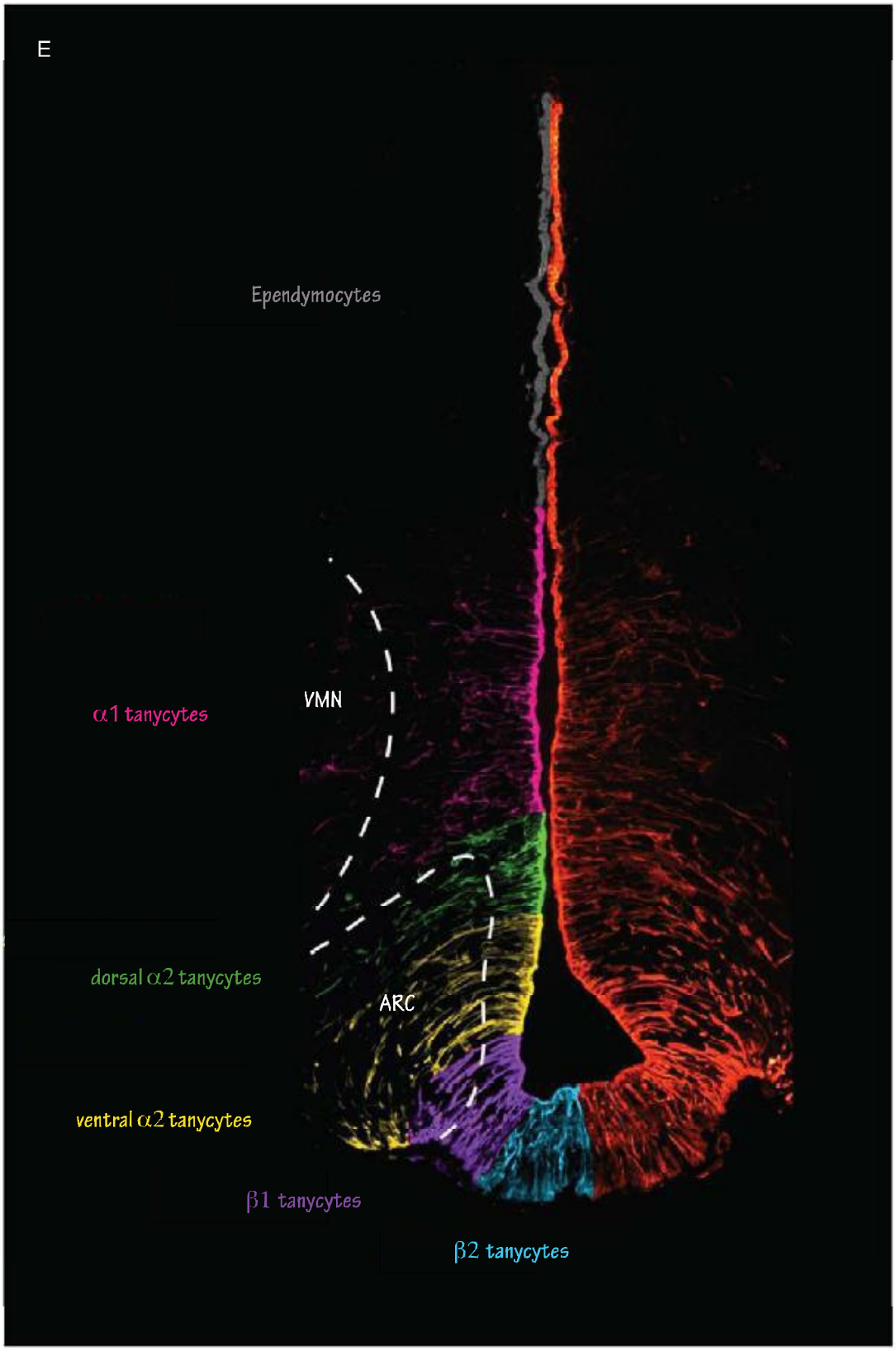
Figure 3.9

Expression of vimentin

A-D: Vimentin expression along the A/P axis of the hypothalamus. Scale bars = 50μm.

E: High power magnification of C, showing detail of vimentin$^+$ processes. As tanycyte subtypes can be distinguished on the basis of process destination, the left side has a false colour overlay to demonstrate the position of the different subtypes (blue = β2 tanycytes, purple = β1, yellow = vα2, green = dα2, pink = α1 and grey = ependymocytes). The approximate positions of the ventromedial nucleus (VMN) and arcuate nucleus (ARC) are also marked.


A
D
Vimentin
A
B
C
D
Anterior
Median Eminence
Posterior


E
Ependymocytes
VMN
α1 tanycytes
dorsal α2 tanycytes
ARC
ventral α2 tanycytes
β1 tanycytes
β2 tanycytes

DARPP-32:

DARPP-32 is a dopamine and cAMP regulated phosphoprotein, which is widely recognized to be present in tanycytes. It is often considered as a marker specifically of β tanycytes [13]. Despite this, several authors report its presence in rat throughout the ventral third of the ventricle, including tanycytes projecting to the arcuate nucleus [19],[85]. In light of this, I wanted to examine its distribution specifically in the mouse.

The antibody I used stained the cell body strongly but only weakly labelled the processes, if at all (Figure 3.10). This is in contrast to previous studies, and did not allow me to determine for certain the projection targets of marked cells. It was seen in three distinct areas of the hypothalamus: the ventral tanycytes (Figure 3.10 (D, E), as expected, and also in some scattered cells of the SCN (Figure 3.10 B). In the tanycytes it appeared to strongly label the β tanycytes, and to be more weakly expressed in the α tanycytes (Figure 3.10 G). It is unclear whether the entire population of α tanycytes expressed DARPP-32, or, as seems more likely, it was confined to the α2 cells alone. Finally, ependymocytes at the dorsal tip of the third ventricle were seen to weakly express DARPP-32 (Figure 3.10 D-F).

BLBP:

Brain lipid binding protein (BLBP) is found in the majority of embryonic radial glial cells [86]. Inhibition of BLBP is reported to block the formation of neurons in culture, and so it may play a role in neural differentiation [87]. In the adult canary, it is reported to be present in hypothalamic tanycytes, as well as some other putative radial glia derivatives and in astrocytes [88].However, it is not clear if it is also expressed in mammalian tanycytes.

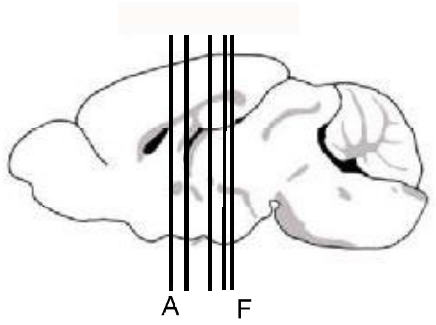
I could not detect BLBP in tanycytes, however it was in numerous scattered cells throughout the hypothalamus (Figure 3.11). These cells were more numerous

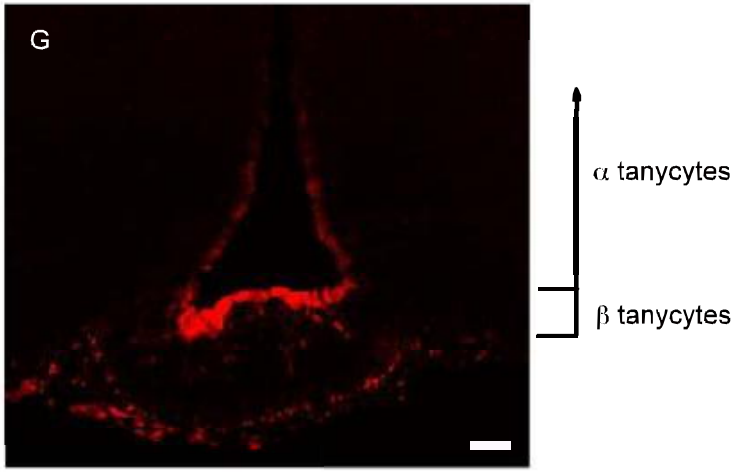
Figure 3.10

Expression of DARPP-32

A-F: Expression of DARPP-32 along the A/P axis. Scale bars = 100μm.

G: Enlargement of D, showing strong DARPP-32 expression at the level of the β tanycytes, and a weaker expression more dorsally at the level of α tanycytes. Scale bar = 50μm.


A
F


G
α tanycytes
β tanycytes

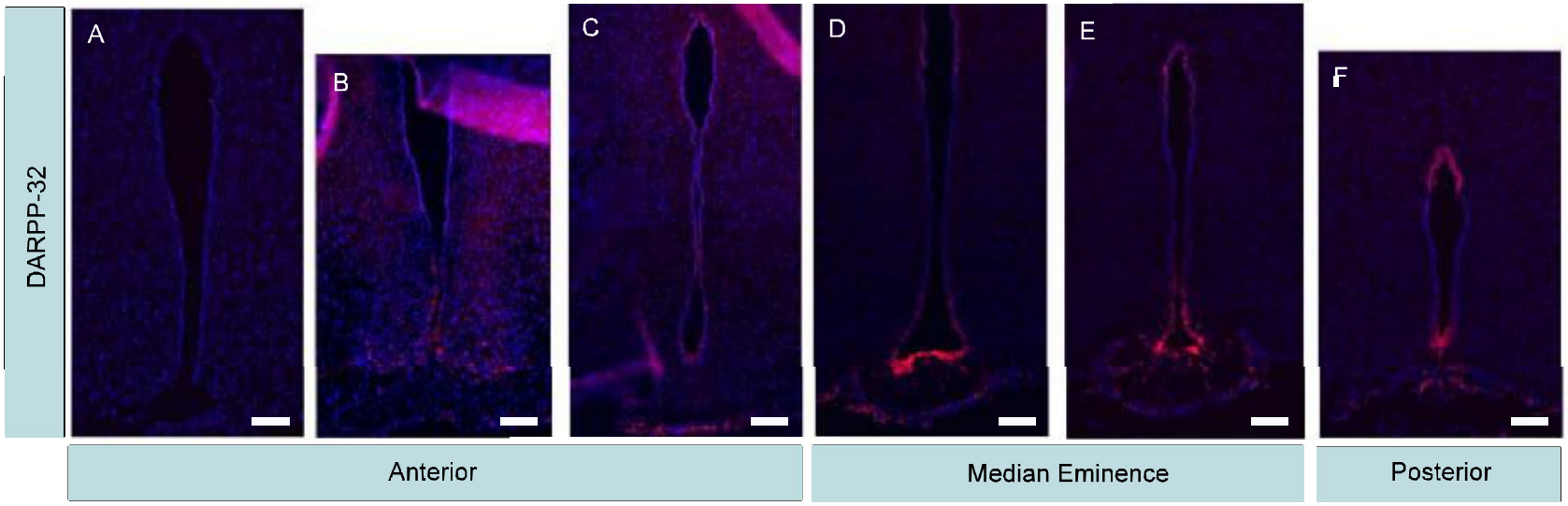
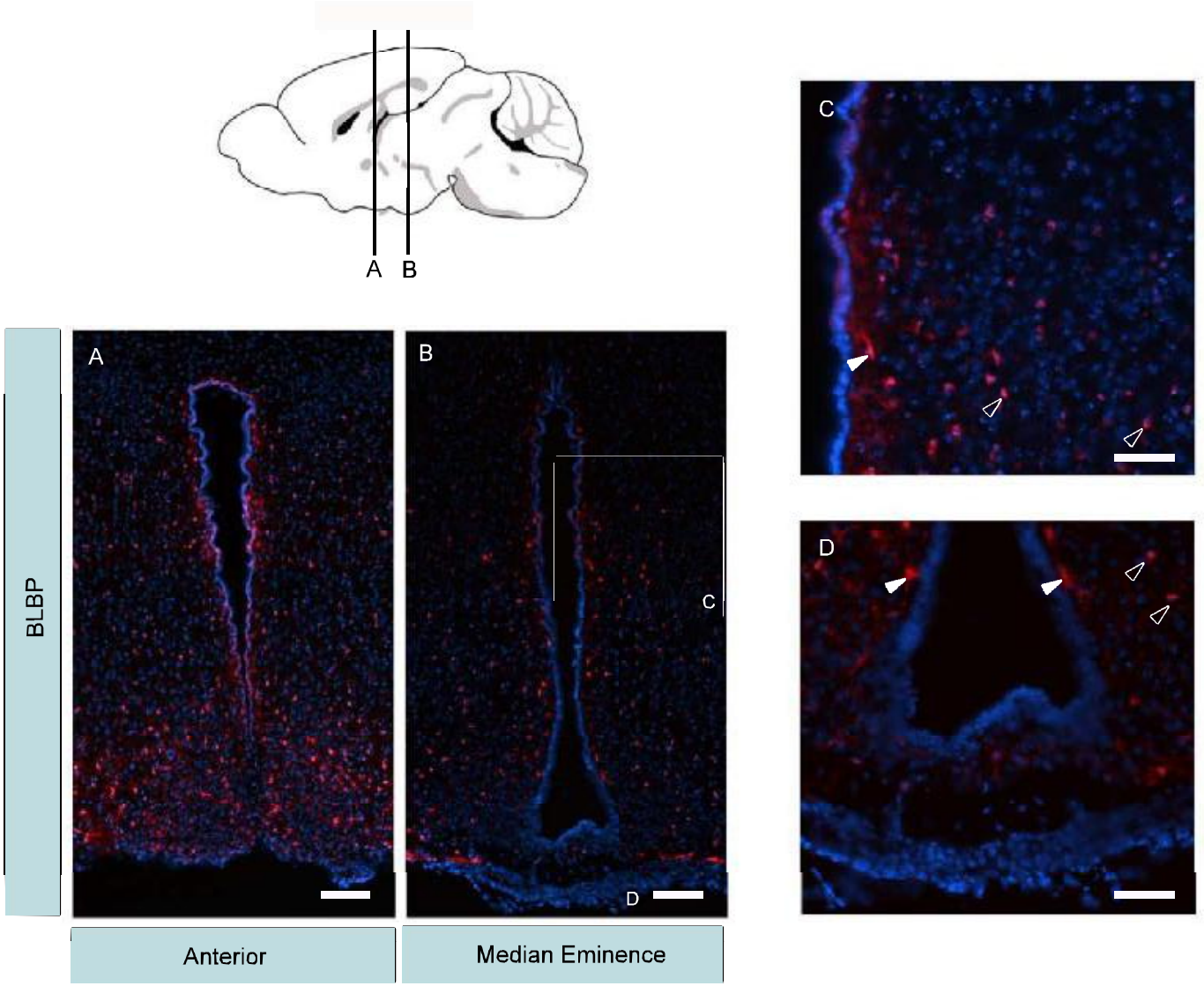

DARPP-32
A
B
C
D
E
F
Anterior
Median Eminence
Posterior

Figure 3.11

Expression of BLBP

A,B: Expression of BLBP in the anterior and ME-level hypothalamus. Scale bars = 100μm.

C,D: High power magnifications of the boxed areas indicated in B. In both ventral (D) and mid/dorsal (C) areas individual $BLBP^+$ cells can be seen scattered throughout the parenchyma. In both locations $BLBP^+$ cells can be found both proximal (solid arrowheads) and distal (outlined arrowheads) to the ventricle, although they are never seen within the ependymal layer cells. Scale bars = 50μm.


A
B
BLBP
A
B
C
D
C
D
Anterior
Median Eminence

close to the ventricle, although ependymal cells themselves were never seen to express BLBP. BLBP$^+$ cells were seen at all A/P levels, and were not concentrated in any particular nuclei.

3.3 Discussion

3.3.1 Markers indicative of NSCs are present in the adult hypothalamus

In this chapter I have demonstrated the presence of a number of markers indicative of a neural cell population with proliferative potetial. Some had been previously observed by other authors, some had been described only for other species, and some are described here for the first time. These markers can be split into three groups, although some markers could easily be included in more than one group:

- Stem cell markers
- Developmental markers
- Radial glia / tanycyte markers

There are an increasing number of proteins being hailed as stem cell markers, including many I have not been able to test for this project. However the markers I have picked – Sox2, GFAP and nestin – are some of the most widely reported and thoroughly tested of the many available. As with all stem cell markers, they are probably not expressed exclusively in NSCs. This applies to GFAP in particular, as it is also strongly expressed in mature astrocytes. All three of these markers, plus Sox1 and Sox3, are expressed in extensive but well-defined regions of the adult mouse hypothalamus. The combination of all these markers together in the same cells or regions provides much stronger evidence than can be suggested through analysis of any single marker. This is backed up by the presence of developmental markers such as Shh and Six3. Indeed in this context these may also be better considered as stem cell markers – Shh has been

implicated in the control of other adult NSC systems, whilst Six3 is thought to influence cell cycle and proliferation.

3.3.2 Marker expression suggests two particular regions may contain NSCs

When all the data is combined together, two regions stand out as expressing an interesting combination of markers that may indicate the presence of NSCs. These areas occur at two separate A/P levels: the first at the level of the SCN (hereafter referred to as the anterior/SCN region) and the second at the level of the ME (hereafter referred to as the ME region).

The Anterior/SCN region:

There are two separate zones of marker expression in this region – the ventral ependymal lining and the SCN (Figure 3.12). The ependymal lining at this point is reported not to contain tanycytes, and is presumed to consist of some form of ependymocyte. My analysis reveals that these cells are nestin^{+}, and possess a single long process that extends ventrolaterally in the direction of the SCN. These cells also express the three SoxB1 transcription factors and GFAP. The SCN itself also expresses some key markers, including SoxB1, Six3, GFAP, DARPP-32 and BLBP. Some of these such as the Sox proteins can be found in cells throughout the nucleus, whilst others are limited to the ventral most portion (Figure 3.12 I-M). The extent to which all these markers overlap in individual cells is unclear, although confocal pictures of Six3 antibody on Sox2-eGFP mouse sections indicates a mix of single- and double – positive cells (Figure 3.12 I, J).

The ME region:

The cells in this region have been more thoroughly characterised in published studies than those more anteriorly, in part because the tanycytes reside here. I confirmed the presence of known tanycyte markers such as vimentin and

Figure 3.12

Markers in the anterior hypothalamus

A: Saggital carton showing the A/P level of this region of the hypothalamus

B: Nissl section and cartoon showing the position of major nuclei and structural features

C-H: Antibody pictures summarising the markers of interest in this area. Scale bars = 100μm.

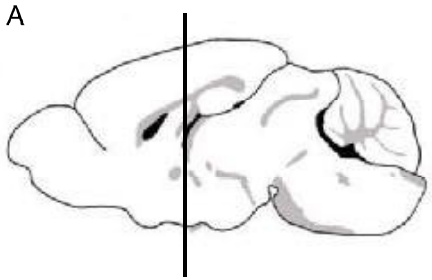
I: High powered confocal magnification of Six3 (red) and Sox2-eGFP (green) colocalisation in the SCN

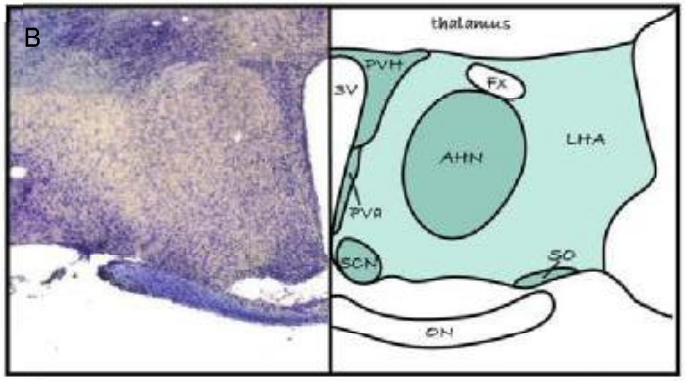
J: Enlargement of J, indicating the presence of $Six3^{+}/Sox2^{-}$ cells (red arrow), $Six3^{-}/Sox2^{+}$ cells (green arrow) and $Six3^{+}/Sox2^{+}$ double positive cells (yellow arrow).

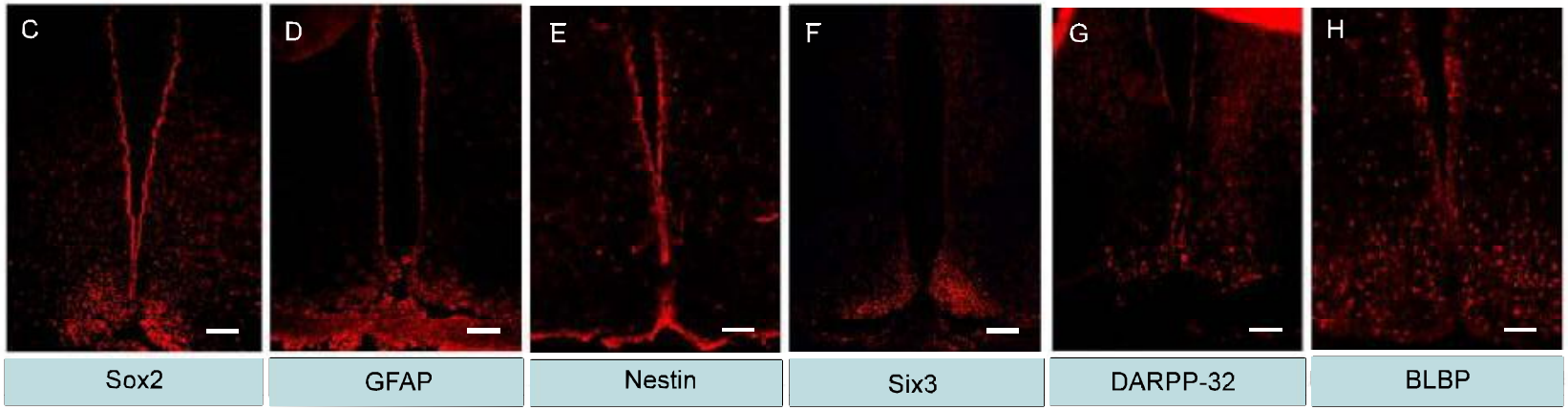
K: High power magnification of Six3 (green) and nestin (red) expression in the SCN

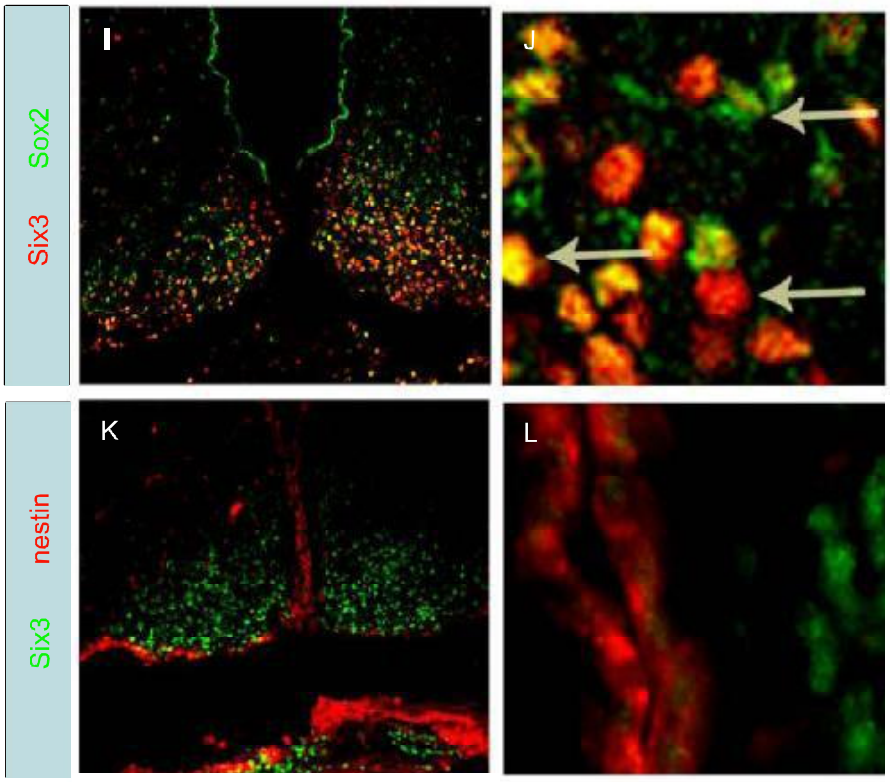
L: Enlargement of K, indicating that there is no colocalisation between Six^{+} and $nestin^{+}$ populations.

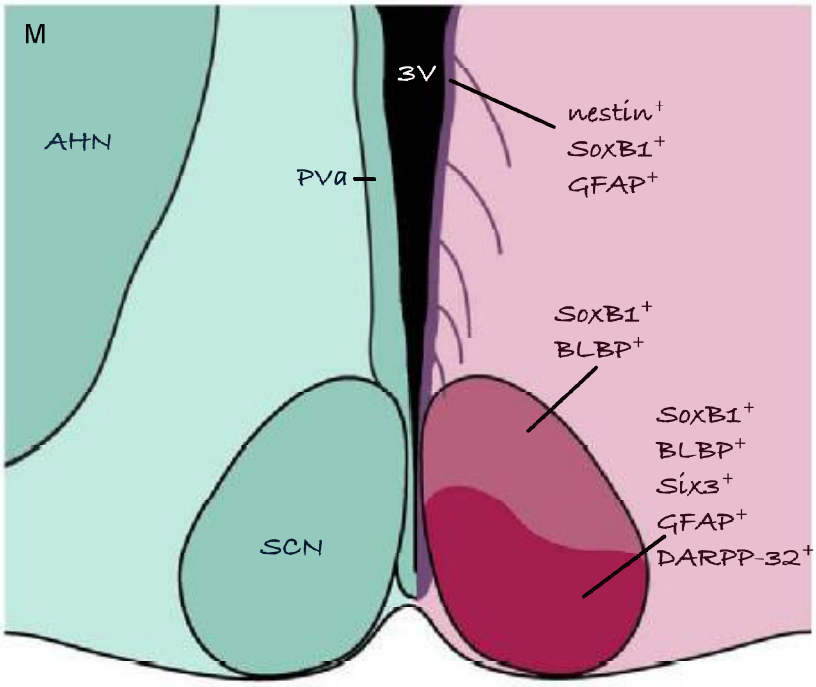
M: Cartoon illustrating the major zones of interesting markers. Markers in the same zones are not necessarily expressed in the same individual cells.


A


B
thalamus
PVH
3V
FX
AHN
LHA
PVa
SCN
SO
ON


C
D
E
F
G
H
Sox2
GFAP
Nestin
Six3
DARPP-32
BLBP


Six3
Sox2
I
J
Six3
nestin
K
L


M
3V
AHN
PVa
nestin+
SoxB1+
GFAP+
SoxB1+
BLBP+
SoxB1+
BLBP+
Six3+
GFAP+
DARPP-32+
SCN

DARPP-32, and provided new evidence to suggest that Six3 and nodal can also be used to identify these cells (Figure 3.13). Six3 appears to be strongly expressed in both β1 and β2 tanycytes, and weakly expressed in α2 tanycytes. Nodal is only seen in the β population. As with other areas of the ependymal layer, SoxB1 proteins seem to be expressed throughout the tanycyte population. As the tanycyte layer is generally only a single cell thick and markers such as SoxB1, Six3, DARPP-32 and nodal were seen in a continuous layer, it is presumed that these proteins are coexpressed in the same set of cells. In addition to tanycytes, the SoxB1 proteins, and Six3 were seen in cells scattered throughout the hypothalamic parenchyma, most commonly in the ME, ARC and VMN. BLBP, a radial glia marker not expressed in tanycytes, was also seen in widely scattered cells. All three of these markers were detected by polyclonal antibody, and therefore it was not possible to ascertain if they colocalise to the same cells.

Previous analyses suggested that the ME might contain NS/PCs. However, my data additionally suggests that the ME may contain a number of different NS/PC populations. Alternatively, it may be that the different areas and marker sets originate from the same NS/PCs at different stages in their progression from stem cell to differentiated cell. Thus, one set could be acting as stem cells, while the other is some sort of intermediate precursor.

3.3.3 Some markers may be unique to the hypothalamic NSC niche

Just as some stem cell markers are likely to be common to all NSC populations, other markers may be unique to the area in which they are found. Here I report the presence of two markers – Six3 and nodal – which have not been previously associated with NSCs. If the cell populations in which they are expressed are confirmed to be stem cells, then these may provide markers specific to hypothalamic stem cells. Neither nodal protein nor Six3 mRNA were observed in the SVZ or the SGZ (data not shown), suggesting that these are not general NSC markers.

Figure 3.13

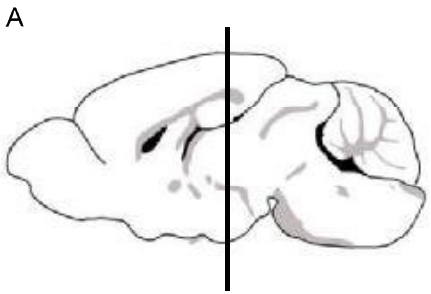
Markers at the level of the ME

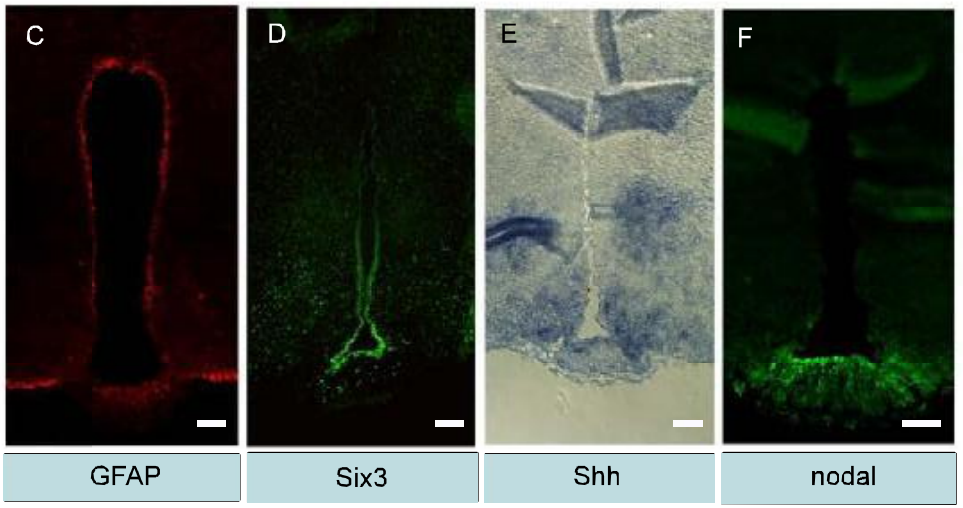
A: Saggital carton showing the A/P level of this region of the hypothalamus

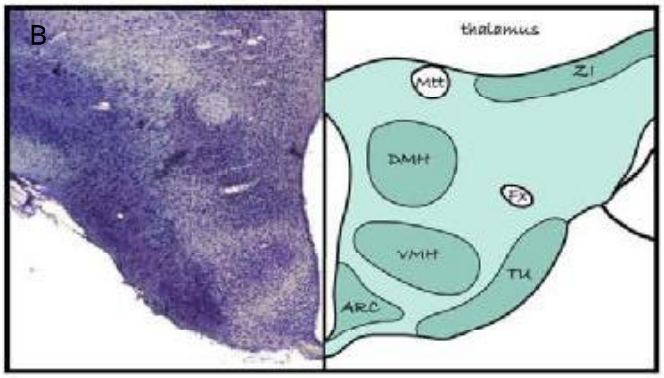
B: Nissl section and cartoon showing the position of major nuclei and structural features

C-J: Antibody / ISH pictures summarising the markers of interest in this area. Scale bars = 100μm.

K: Cartoon illustrating the major zones of interesting markers. Markers expressed in the ependymal layer (tanycytes and ependymocytes) are assumed to all be in the same cells, markers in other areas (ME or scattered cells) are not necessarily coexpressed in the same individual cells.


A


C
D
E
F
GFAP
Six3
Shh
nodal


B
thalamus
MH
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DMH
FX
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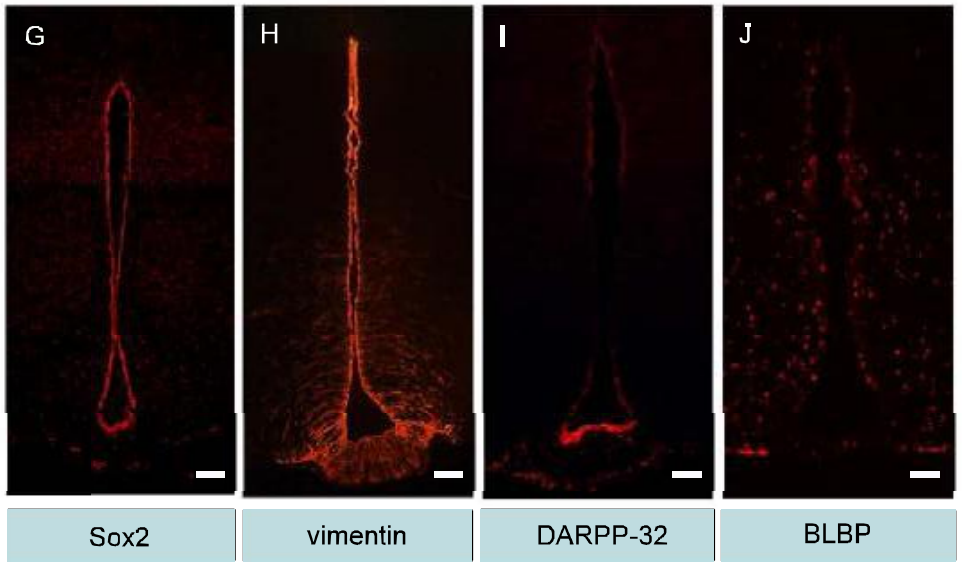
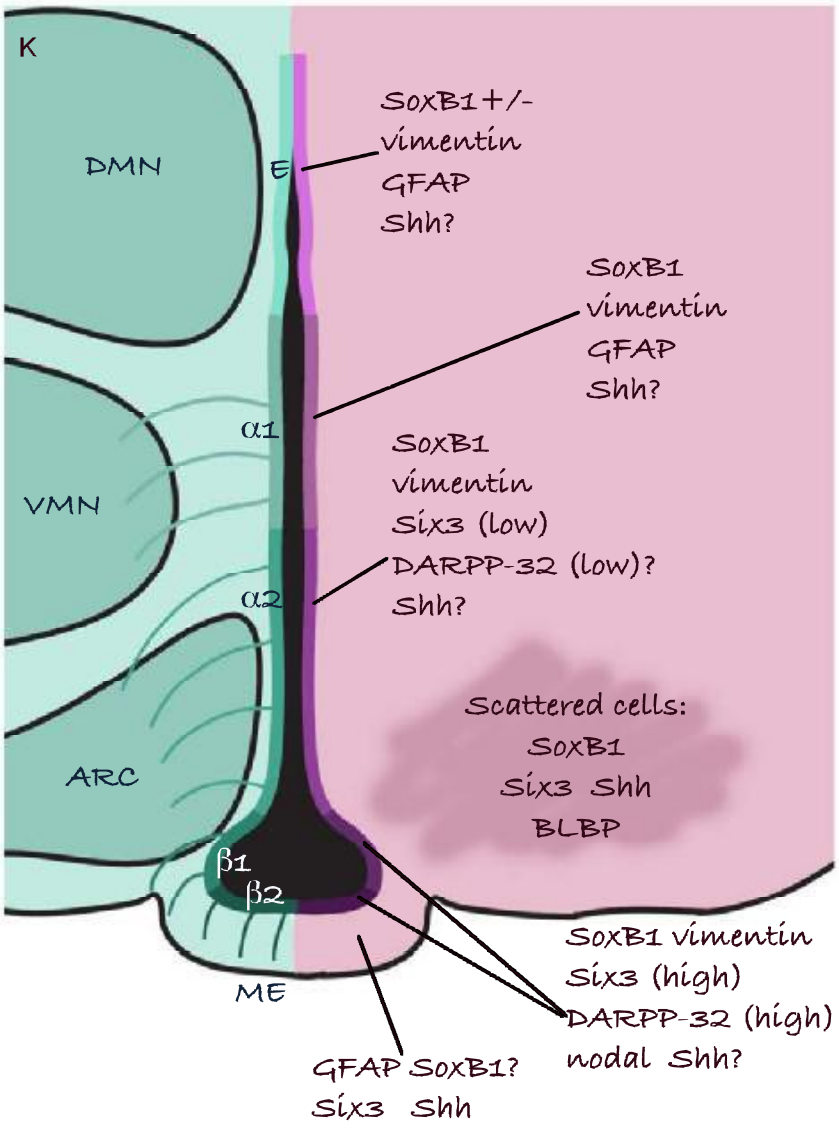
TU
ARC


G
H
I
J
Sox2
vimentin
DARPP-32
BLBP


K
DMN
VMN
ARC
ME
E
α1
α2
β1
β2
SoxB1+/-
vimentin
GFAP
Shh?
SoxB1
vimentin
GFAP
Shh?
SoxB1
vimentin
Six3 (low)
DARPP-32 (low)?
Shh?
Scattered cells:
SoxB1
Six3 Shh
BLBP
SoxB1 vimentin
Six3 (high)
DARPP-32 (high)
nodal Shh?
GFAP SoxB1?
Six3 Shh

Chapter 4

Chapter 4 - Neurospheres

4.1 Neurosphere cultures

There are many in vitro assays designed for the culture and study of neural stem cells, but one of the most useful and commonly employed is the neurosphere system. It was first used in 1992 by Reynolds and Weiss [89], who isolated cells from the adult brain and showed they could, under appropriate conditions, produce both neurons and astrocytes. Variations of this technique have since been used in many studies, and neurospheres have been generated from both adult and embryonic brains, in species as diverse as chicken (C Pearson and S Robins, unpublished), mouse [46], rat [59] and even human [90].

The basic principle behind the neurosphere system is to expand neural stem cells in suspension culture. Cells are dissected out from the tissue of interest, and dissociated to form a single cell suspension. They are then cultured with mitogenic factors to induce proliferation, resulting in floating balls of cells known as neurospheres. Once grown, a variety of experiments can be employed, including passaging the neurospheres to demonstrate self-renewal, and differentiating them to illustrate multipotentiality (clonal neurospheres only – see discussion section 4.6). The standard method used to grow the spheres is to grow them in non-adherent plates, and use a serum-free medium supplemented with N2 and B27. The mitogenic factors used are generally FGF-2 and/or EGF, both at a concentration of 20ng/ml. In order to passage spheres they are dissociated back to a single cell suspension and replated. The theory is that neurospheres may be formed by either true stem cells or early progenitors, and in order to distinguish between the two, the self-renewal capacity must be tested. Only stem cells have the capacity to self renew indefinitely, and therefore to be passaged an infinite amount of times. Progenitor cells on the other hand, will gradually lose the ability to proliferate after a few passages. In reality, the number of passages needed to define a stem as opposed to a

progenitor cell probably varies with the precise conditions, but a generally accepted rule of thumb is that only stem cells will last for over ten passages [32]. To differentiate neurospheres, they are generally plated on an adherent substrate (either as whole spheres, or after dissociation) in a reduced concentration of growth factors. After about a week the cells can be fixed, and antibodies used to determine what cell types are present.

Having previously demonstrated that many stem cell markers are present in the adult mouse hypothalamus, I wanted an in vitro technique that could both confirm the presence of NS/PCs cells and be used to elucidate some aspects of their character. It has already been demonstrated that it is possible to derive neurospheres from mouse hypothalamus, however very little characterisation has been performed on the resulting isolated cells [58]. I aimed to establish a robust and consistent method to generate neurospheres from the hypothalamus, and then to perform a basic characterisation of the resulting spheres in terms of the cell types contained in both undifferentiated and differentiated spheres. In conjunction with the earlier observation that the hypothalamus contains some unique stem cell markers I hypothesised that hypothalamic spheres may express some unique regional markers, and may maintain some regional character throughout culturing. To this end I aimed additionally to generate neurospheres from both the hypothalamus and from other regions of the CNS, and to ascertain whether markers could differentiate between them.

4.2 Hypothalamic Neurosphere Results

4.2.1 Neurospheres can be derived from adult mouse hypothalamus

To investigate whether I could derive neurospheres from adult mouse hypothalamus I used a neurosphere protocol modified from the Morrison lab [91]. This protocol detailed the production of neurospheres from mouse SVZ and SGZ, so the main alterations were to the dissection. The main steps in this

process are illustrated in Figure 4.1. The method proved to be reliable, with primary spheres consistently generated in reasonable numbers. One observed difference to many published neurosphere protocols was the length of time taken to grow the spheres to a reasonable size. Most authors agree that spheres from other neural regions grow to full size in about a week, whereas in my cultures nothing was visible after 7 days, tiny spheres could be seen from 9-10 days, and spheres only reached larger sizes after 14 days. This finding is in agreement with a previous study comparing spheres from the third ventricle with those from other areas [92]. At this timepoint, neurospheres varied widely in size from just a few cells wide to over 400μm in diameter. However it was always possible to distinguish the live spheres from any debris in the well, as even the tiniest neurospheres appeared to be surrounded by a smooth phase-dark membrane. Primary neurospheres could be passaged, and produced secondary neurospheres with reasonable efficiency. A single primary sphere was capable of producing many secondary spheres (data not shown), confirming that significant levels of proliferation were occurring, as opposed to reaggregation of dissociated tissue.

Both primary and secondary neurospheres could be differentiated, and similar results were seen irrespective of the passage number. Spheres were placed on an adherent substrate of poly-D-lysine and fibronectin, and cultured for a week. During this time they were exposed to reduced levels of growth factors in the medium – no EGF, and just 10ng/ml of FGF-2. It was discovered that the FGF-2 could not be entirely removed, or the cells failed to spread onto the slide. It is not clear whether this is due to a reduction in proliferation, or a failure to detach from the sphere and migrate. After a week, cells from the neurosphere spread out to form a circular culture no more than a few cells deep. The spread was up to 4mm across, and the remains of the original sphere were usually still visible in the centre. The spheres clearly contained a mixture of cell morphologies, and gave the impression of different cells types leaving the sphere at different timepoints, and so varying in the distance travelled. The outermost cells seen were very large, and may have formed a large flat sheet on

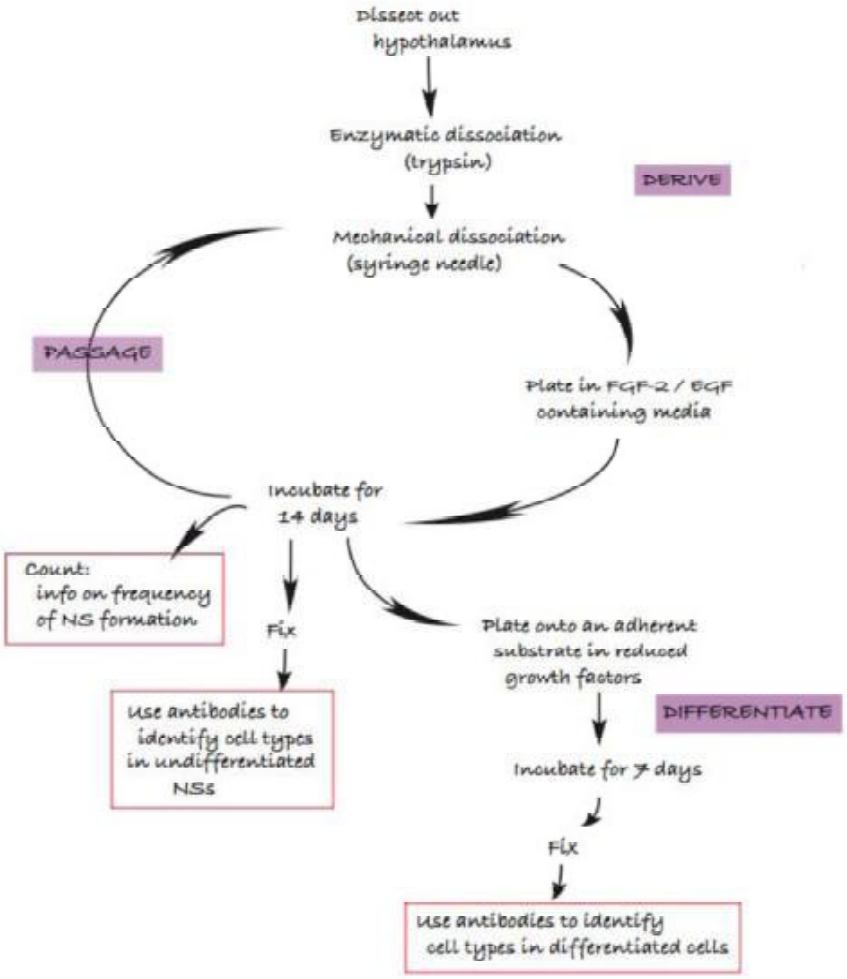


Figure 4.1

Summary of methods used to derive, passage and differentiate neurospheres

Red boxes indicate the main types of information that these experiments can provide.

which the other cell types resided. Cells closer in were less distinctive, and long thin processes assumed to be neural axons and dendrites were mainly seen closer to the centre. This layering effect could also be the result of separate types of progenitor / mature cell migrating at different speeds.

4.2.2 Cell types in undifferentiated hypothalamic neurospheres

In order to investigate the cell types produced in undifferentiated spheres, both primary and secondary neurospheres were sectioned and labelled with antibodies. As with the adult CNS sections in the previous chapter, I examined a combination of stem cell markers, developmental markers and radial glia / tanycyte markers.

Stem Cell Markers:

Undifferentiated neurospheres expressed a wide range of stem cell markers. All three SoxB1 proteins were robustly labelled (Figure 4.2 A, B), and appeared to be expressed in virtually every cell. Cell counts on Sox2 labelled sections revealed an average of 95.2 ± 1.6% of nuclei to express Sox2. Expression, however, was not uniformly high. Sox1+3 (identified with one antibody recognizing both types) seemed to be brightest at the outer edges of the sphere. GFAP labelled a smaller population of cells, often around the outside of the sphere (Figure 4.2 C). However, as GFAP is commonly used as a marker of both immature stem cells and mature astrocytes it is impossible to say which cell type it indicates in this context. Nestin is also expressed throughout the sphere, as well as in a bright band around the outer membrane (Figure 4.2 D). As nestin is a cytoskeletal protein, it was not possible to determine the proportion of cells in which it was expressed.

Radial Glia / Tanycyte markers:

Several radial glia markers were strongly expressed in the spheres. Vimentin levels varied, but seemed evenly distributed throughout the sphere (Figure 4.2 E). DARPP-32 expression varied, and ranged from no expression to very strong

within neurospheres grown at the same time. However, within each sphere it was remarkably consistent. The example shown in Figure 4.2 F shows a medium level of expression. BLBP was one of the most striking antibodies, with very strong expression always seen throughout the entire sphere, plus the outer membrane (Figure 4.2 G). RC2 is another well-characterised radial glia marker. Although I did not detect it on tanycytes in vivo, it is expressed in some hypothalamic neurosphere cells (Figure 4.2 H).

Developmental markers

In chapter 3, I demonstrated that the anterior developmental marker Six3 can still be found in restricted regions of the adult hypothalamus, specifically in the ventral tanycytes (Figure 3.6). Antibody labelling experiments suggest that it is also expressed in hypothalamic neurospheres (Figure 4.2 I), in a subset of cells within each sphere (see also section 4.3.5). This suggests that markers traditionally associated with the developing hypothalamus may be retained into adulthood in NS/PCs. In the early embryonic hypothalamus there are defined zones of marker expression [82]. Nkx2.1 is expressed at ventral levels, with Nkx2.2 seen more dorsally. Dorsal to this is a $Pax6^+$ domain. When these markers were tested in neurospheres, Nkx2.1 and Nkx2.2 were both found to be present (Figure 4.2 J, K), while Pax6 could not be detected (Figure 4.2 L). Both Nkx2.1 and Nkx2.2 were found in a small subset of cells, always located towards the outer edge of the neurosphere. None of these three markers could be detected in sections of adult hypothalamus (data not shown).

Cell Cycle markers:

Ki67 is a nuclear marker expressed during all active stages of the cell cycle (G1, S, G2 and mitosis) but absent in the resting phase (G0), making it a good marker of proliferating cells [93]. $Ki67^+$ cells can be clearly observed in undifferentiated spheres (Figure 4.2 M). Cell counts show some variation between spheres, but indicate that an average of 12.6% (± 3.3%) of cells express Ki67 at any point in time (n=5).

Figure 4.2

Markers in undifferentiated hypothalamic neurospheres

Antibody detection of a range of markers in neurosphere sections. In most cases a single section is pictured; where there was heterogeneity a representative picture was selected.

A-D: Stem cell markers in undifferentiated spheres. D inset is a high magnification confocal picture showing nestin throughout the neurosphere.

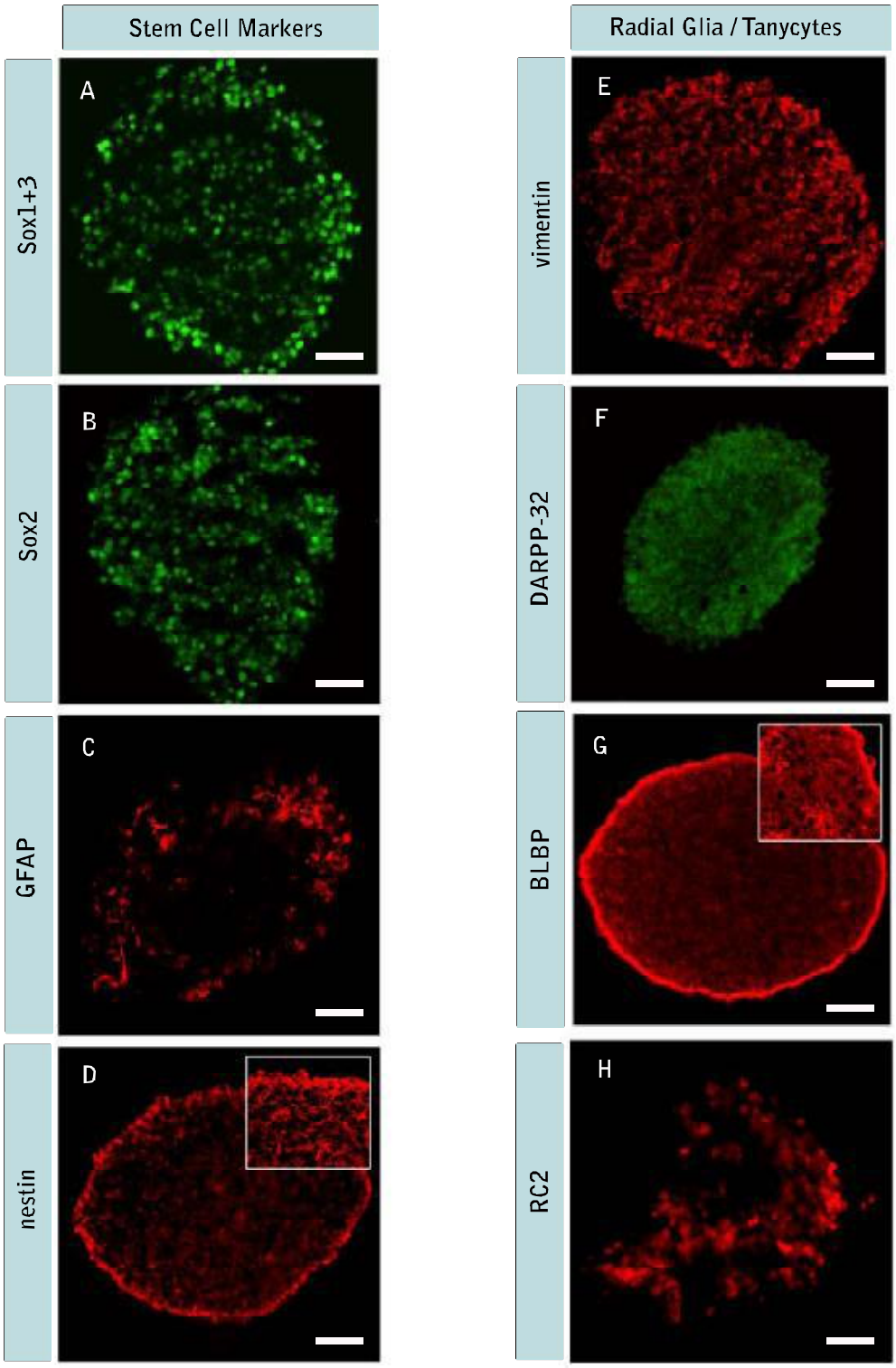
F-H: Radial glia / tanycyte markers in undifferentiated spheres. G inset is a high magnification confocal picture showing BLBP expression throughout the neurosphere.

I-L: Developmental markers in undifferentiated spheres.

M: Cell cycle marker Ki67 in undifferentiated spheres.

N, O: Miscellaneous other markers in undifferentiated spheres.

Scale bars = 50μm.


Stem Cell Markers
Radial Glia / Tanycytes
Sox1+3
A
vimentin
E
Sox2
B
DARPP-32
F
GFAP
C
BLBP
G
nestin
D
RC2
H

Developmental Markers
Cell Cycle Markers
Six3
I
Ki67
DAPI
M
Nkx2.2
DAPI
J
Other Markers
Nkx2.1
DAPI
K
GLAST
N
Pax6
DAPI
L
PSA-NCAM
DAPI
O

Other Markers:

GLAST is a glutamate transporter found only on astrocytic cells. It is also expressed in the majority of radial glia, and has been reported to be present in hypothalamic tanycytes, although I was unable to reproduce this [94]. It is highly expressed throughout the neurospheres (Figure 4.2 N). As GLAST is a cell surface marker it is difficult to quantify the exact proportion of cells in which it is expressed, however it appears to be a large majority. PSA-NCAM is a polysialylated form of NCAM that is generally associated either with development, or with neural plasticity when expressed in the adult. It is reported to be expressed in the known neurogenic zones and also in the ME region of the hypothalamus, however this is known to be a highly plastic and dynamic region and so expression here is not necessarily due to neurogenesis [24]. Its expression was highly variable between different neurospheres, but was generally expressed in just a few cells (Figure 4.2 O).

Undifferentiated neurospheres were also examined for the presence of mature neural cell markers. It was not possible to assess the presence of mature astrocytes, as they are traditionally identified by the presence of GFAP, a marker that is also expressed by NSCs. It is not clear which of these two cell types is indicated by the presence of GFAP in some neurosphere cells (Figure 4.2 C). TuJ1$^+$ immature neurons were only rarely seen in neurospheres, while RIP$^+$ oligodendrocytes were never detected (data not shown).

4.2.3 Cell types in differentiated neurospheres

At 7 days post-differentiation, neurospheres contain a very different set of cell types. Stem cell markers tend to be downregulated, while a majority of cells expresses markers of mature neural cell types. Cells of all three lineages are produced, shown in Figure 4.3. MAP2 is a cytoskeletal protein characteristic of mature neurons, while TuJ1 (also known as βIII tubulin) is found in immature neurons. MAP2$^+$ cells are more numerous than TuJ1$^+$ neurons, and display a distinctly different morphology. TuJ1$^+$ cells are usually bipolar, with very long

processes (Figure 4.3 D). MAP2$^+$ cells can be unipolar, bipolar or multipolar, and either have shorter axons and dendrites or have MAP2 localised only to areas closer to the cell body (Figure 4.3B). GFAP is used here as a marker of astrocytes. There is no way to prove absolutely that it is marking mature astrocytes rather than stem cells in this case; however the morphology of the GFAP$^+$ cells is strongly indicative of astrocytic cells (Figure 4.3 F). Neurospheres contained a high proportion of GFAP$^+$ cells, which tended to radiate out from the original sphere (Figure 4.3 E). Oligodendrocytes were the rarest cell lineage, perhaps because they are likely to take longest to differentiate. They were detected using a RIP antibody, and a few oligodendrocytes were seen in each differentiated sphere, usually very close to the centre (Figure 4.3 G, H).

Cell counts were performed to estimate the relative frequency of the different lineages. Random fields of high magnification pictures were used for the MAP2 and GFAP counts, in order to be able to associate visible antibody labelling with the correct nuclei. As both cell types varied with distance from the centre of the culture, pairs of inner and outer pictures were used for each neurosphere. A minimum of 5 different neurospheres were examined for each antibody. MAP$^+$ neurons were estimated to account for 30.0% ±5.3 of the total cells, while the proportion of GFAP$^+$ astrocytes was 27.8% ±7.2. TuJ1$^+$ immature neurons and RIP$^+$ oligodendrocytes were uncommon enough to be counted on low magnification pictures, and were each estimated to account for <1% of the total cells (TuJ1 = 0.7%, RIP = 0.8%). All four of these cell types were predominantly found nearer to the centre of the culture. Differentiated cultures had a much greater density of cell bodies nearer the centre, and so it seems likely that the third of cells that remained unlabelled were scattered throughout the culture despite being most obvious at the outer edges. Some of these cells labelled with nestin, however beyond that their identity was not determined.

Differentiated neurospheres were also examined for the presence of various neural subtypes. Choline acetyl transferase (ChAT), a marker of cholinergic neurons, was absent (Figure 4.4 G), however some cells were positive for

Figure 4.3

Neural lineage markers in differentiated neurospheres

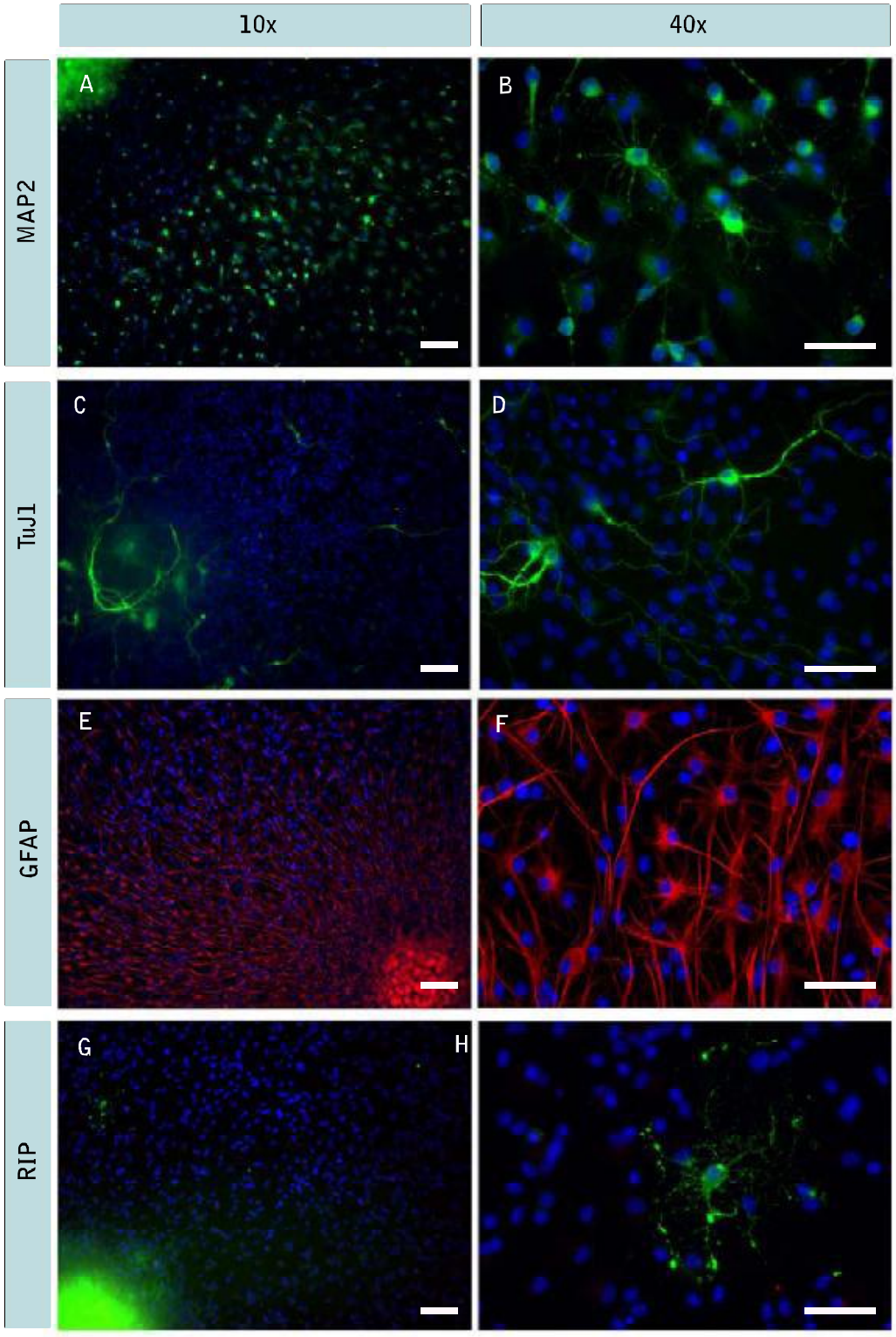
A, B: Expression of MAP2, a marker for mature neurons, was seen in numerous cells.

C, D: TuJ1, a marker of immature neurons, was seen in only a few cells of each culture.

E, F: GFAP, which is expressed in both stem cells and mature astrocytes, is seen in many cells. The stellate shape of the cells suggests that these are likely to be astrocytes.

G, H: RIP is a marker for the oligodendrocyte lineage. A few RIP^+ cells displaying characteristic oligodendrocyte morphology were seen in each culture.

A,C,E,G: Scale bars = 100μm.
B,D,F,H: Scale bars = 50μm.


10x
40x
MAP2
TuJ1
GFAP
RIP
A
B
C
D
E
F
G
H

tyrosine hydroxylase (TH) indicating the presence of dopaminergic neurons (Figure 4.4 F). Antibodies for NPY, POMC and AVP also all gave negative results, despite being cell types found in the hypothalamus (data not shown). Growth hormone releasing hormone (GHRH) is specific a subset of hypothalamic neurons, and this could be detected in some differentiated cells (Figure 4.4 A-E). It labelled cells of varying morphology, including both multipolar and bipolar cells. All $GHRH^+$ cells were also $MAP2^+$, confirming that they are neurons. However only a subset of $MAP2^+$ cells also expressed GHRH, demonstrating that other neuronal types must also be present. $GHRH^+$ cells were also noted to express the transcription factor Nkx2.2, although it is not clear if these are the same $Nkx2.2^+$ cells observed in undifferentiated neurospheres.

Stem cell markers were examined in differentiated spheres, as logically, these would be expected to be downregulated as more mature cell types appear. Surprisingly, Sox1, 2 and 3 were all still present in some cells (Figure 4.4 J-L). Even more unexpected was the observation that Sox2 was most strongly expressed in $MAP2^+$ neurons (Figure 4.4 M). It is not clear how this can be accounted for, except that it may be that Sox2 is not as specific for undifferentiated cells as commonly believed. Alternatively, it may take longer to be downregulated in neurons than other cell types. Additionally, some $nestin^+$ cells of indeterminate morphology were present (Figure 4.4 N), as well as $Ki67^+$ proliferating cells (Figure 4.4 I).

4.3 Alternative Neurosphere Media:

At the time I was performing this work, it was noted by D McNay (personal communication) that tanycytes are sensitive to insulin levels, and that conventional medium supplements contain a high level of insulin. Accordingly, he devised an alternative, low-insulin media formulation, and found it dramatically improved the growth of hypothalamic neurospheres. Having tested

Figure 4.4

Other markers in differentiated neurospheres

A-E: Expression of growth hormone releasing hormone (GHRH) in differentiated neurospheres. Three main morphologies of $GHRH^+$ cells were observed: multipolar cells with GHRH throughout the cell (A), multipolar cells where it was expressed only in the cell processes (B) and bipolar cells (C). The neuronal character of these cells was confirmed by MAP2 colocalisation (D), and they were also observed to express the transcription factor Nkx2.2 (E). Scale bars = 20μm (A-C) or 50μm (D, E).

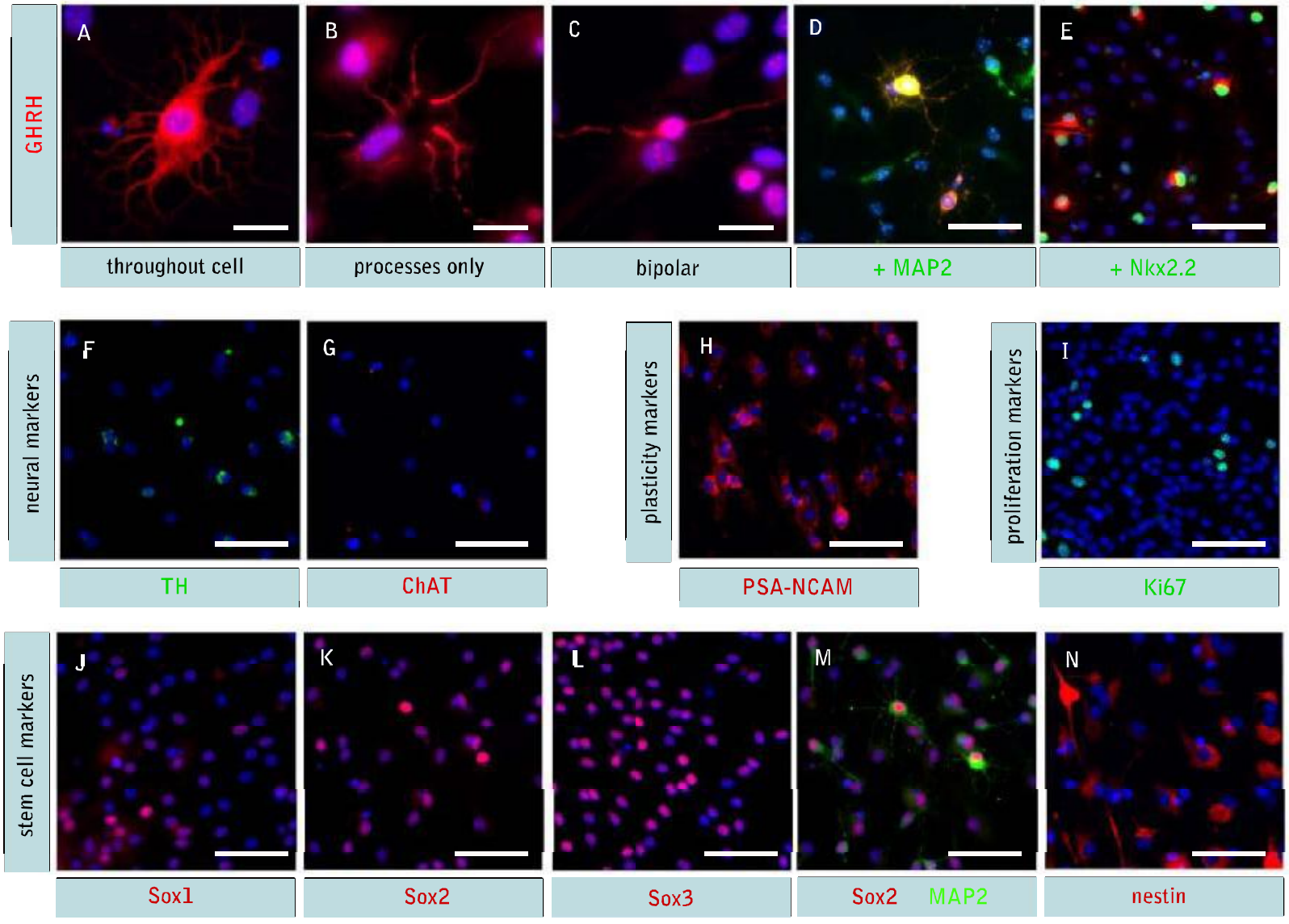
F: A few TH^+ dopaminergic cells may be present. Scale bars = 50μm.

G: No $ChAT^+$ cholinergic cells were observed. Scale bars = 50μm.

H: PSA-NCAM expression varied between cultures, but was often observed on many cells. Scale bars = 50μm.

I: Ki67 immunoreactivity demonstrates that some cells are still proliferating. Scale bars = 50μm.

J-M: Expression of stem cell markers. Sox1 (J), Sox2 (K), and Sox3 (L) were all still expressed in many differentiated cells. It is not possible to be sure whether they are in all nuclei at greatly varying levels, or whether cultures contain a mix of positive and negative cells. The highest levels of Sox2 expression were generally seen in $MAP2^+$ neurons (M). $Nestin^+$ cells were also observed (N). Scale bars = 50μm.


GHRH
A
B
C
D
E
throughout cell
processes only
bipolar
+ MAP2
+ Nkx2.2
neural markers
F
G
TH
ChAT
plasticity markers
H
PSA-NCAM
proliferation markers
I
Ki67
stem cell markers
J
K
L
M
N
Sox1
Sox2
Sox3
Sox2 MAP2
nestin

his new formulation, I also found it to be much superior to the conventional medium, in terms of both speed of growth and passagability of the neurospheres. The basic difference was in the formulation of the N2 supplement, which conventionally contains a high level of insulin. This was removed, and replaced with a low (physiological) level of insulin-like growth factor 1 (IGF-1) to ensure that insulin receptors would still be stimulated, but not overloaded to the point of desensitization. Although B27 supplement contains a little insulin, it was not found to be at a level that would inhibit sphere growth, and so this supplement remained unchanged. In addition, heparin was added to this media formulation, in order to aid FGF signalling.

Some of the previous experiments (growth kinetics, plus a variety of antibody markers in both undifferentiated and differentiated neurospheres) were repeated in the new medium in order to check the two methods were compatible. The following table summarises the main similarities and differences:

	Standard Media	Low Insulin Media
Time to form neurospheres	14 days	7-10 days
Max no. of passages	2	8+
Max size	400uM+	400uM+
Grown in FGF only	few, 1 passage only	many, 1 passage only
Grown in EGF only	few / none	few, 5+ passages
Differentiation: FGF required?	yes	yes
Differentiation: time	7 days	6 days (or less?)
Differentiation: neural lineages?	all 3	all 3

Table 4.1: Comparison of standard and low-insulin media

In terms of protein expression, most markers in both undifferentiated and differentiated spheres appear to be identical. The only exception to this seems to be the number of $GFAP^+$ cells seen leaving the neurosphere in differentiated

cultures, which was reduced in low-insulin cultured spheres (data not shown). $GFAP^+$ cells were still observed in the remnants of the original sphere, but fewer were present in the surrounding area.

In conclusion, low-insulin medium is a good alternative to the standard formulation. It produces spheres faster, and greatly increases the number of passages that can be achieved. It also seemed to achieve these things in a more consistent manner than the standard medium. Marker analysis would tend to suggest that the same basic cells are being cultured in each case, and so conclusions drawn from spheres cultured in one type of media should also hold true for those cultured in the other. With this in mind, I decided to conduct future experiments in low-insulin media.

4.4 Regional Identity of Neurospheres

There is much debate about the extent to which neurospheres derived from different regions differ to each other, and how long these differences can be maintained under in vitro conditions. The question is really what ultimately causes the observation that in vivo, cells from different neurogenic niches behave differently, for example in their migration patterns and final cell types. It may be an intrinsic difference in the stem cells themselves, or a result of the surrounding niche and other environmental factors. The argument for intrinsic difference is made by Klein et al, who compared neurospheres cultured from the forebrain with those from the cerebellum [95]. Neurospheres were transplanted back into early postnatal CNS, either into the region from which they were originally derived (homotopic transplantation) or the alternative region (heterotopic transplantation). Both cell types behaved similarly to in vivo stem cells when homotopically transplanted, undergoing tangential migration in the case of forebrain cells, or become granule layer neurons or Purkinje cells in the cerebellum. In contrast, heterotopic transplantations resulted in limited migration and few neurons, with the majority of cells expressing GFAP. This

suggests that intrinsic differences between stem cells isolated from the two areas determined their competence to respond to the niche in which they were transplanted.

Alternatively, it has been suggested that adult neurospheres from different regions converge to one indistinguishable cell type after culture, which would imply that external influences are dominant to intrinsic ones [58]. The authors cultured neurospheres from the hypothalamus and the hippocampus, and showed they could generate neuroendocrine cell types normally only found in the hypothalamus from both sets of neurospheres. However, this study was conceived using the assumption that the hypothalamus is a non-neurogenic region and so only progenitor cells could ever be derived from that region, and mounting evidence now suggests this is not the case. In reality, it demonstrates that neurosphere cells from different regions have the potential to form cells that would not normally be required in that region in vivo, but does not preclude the possibility that some regional character is maintained.

Although arguments have been made for maintenance of regional character in neurospheres, no suggestions have been made as to what the intrinsic regulators of character may be. They may rely on the expression of specific proteins, or on epigenetic mechanisms. As I have previously identified proteins that may be uniquely expressed in the hypothalamic neurogenic niche (see chapter 3 - Six3 and nodal) I wanted to examine their expression in neurospheres. I decided to compare hypothalamic neurospheres with two other regions – SVZ and spinal cord (SC). In addition I wanted to compare the growth kinetics of spheres from the different regions, as this may also indicate differences in their component cell types.

4.5 Regional Neurosphere Results

4.5.1 Growth kinetics

Neurospheres were successfully generated from three regions of the CNS: the hypothalamus, the SVZ of the lateral ventricle and the central canal of the spinal cord. Although it was possible to grow all three neurosphere types in standard medium, final experiments were conducted in low-insulin media. The number and size of neurospheres were assessed after 10 days for each passage. Each experiment typically lasted to the third passage before being stopped due to low numbers of SVZ and SC neurospheres. As SVZ neurospheres are normally considered to be infinitely passagable, this is assumed to be due to my conditions having being optimised for hypothalamic cells. The results are summarised in Figure 4.5. After the initial derivation, SVZ cultures were always seen to contain a much higher proportion of very large spheres than other regions (Figure 4.5 A). In contrast, SC neurospheres were typically very small, with only a small number over 50μm diameter (Figure 4.5A). This trend was continued after passaging, although it became less pronounced, particularly with respect to the difference between hypothalamic and SVZ spheres (Figure 4.5 B). For the neurosphere counts, only spheres over 50μm in diameter were counted, as is general practice in most neurosphere publications. Generally much higher numbers of hypothalamic neurospheres were produced compared to the other regions, which may again be due to the precise culture conditions (Figure 4.5 I-K).

4.5.2 Markers in undifferentiated and differentiated neurospheres

To investigate whether neurospheres from different regions were made up of similar cell types, I examined a number of different markers. Markers such as Sox2 and vimentin were expressed in a similar pattern in all neurospheres examined (Figure 4.6 A-F), as were Sox1+3, nestin and GFAP (data not shown). All spheres also contained $Ki67^{+}$ proliferating cells (Figure 4.6 G-I). Nkx6.1 is a

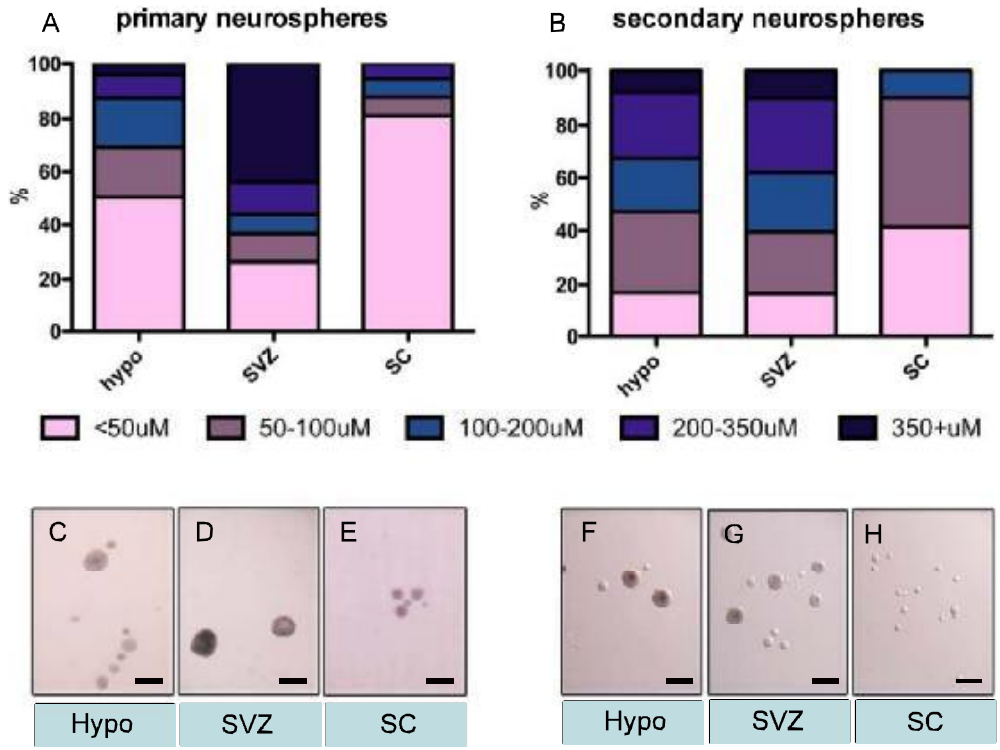
Figure 4.5

Growth kinetics of neurospheres from different regions

A, C-E: Relative sizes of primary neurospheres from the hypothalamus, SVZ and SC. A minimum of 50 neurospheres larger than 50μm diameter were analysed, and the percentage in each size range calculated (A). C-E show representative pictures of the spheres. Scale bars = 500μm.

B, F-H: Relative sizes of secondary neurospheres (calculated as for primary spheres). Scale bars = 500μm.

I-K: Numbers of neurospheres per well derived from each region, for primary (I), secondary (J) and tertiary (K) neurospheres. All cultures were derived from the same mouse at the same time, and plated at 16,700 cells/mL in at least 24 wells (of a 24 well plate) per region.


A
primary neurospheres
B
secondary neurospheres
%
100
80
60
40
20
0
hypo
SVZ
SC
<50uM
50-100uM
100-200uM
200-350uM
350+uM
C
D
E
F
G
H
Hypo
SVZ
SC
I
primary neurospheres
J
secondary neurospheres
NS per well
40
30
20
10
0
K
tertiary neurospheres
150
100
50

transcription factor expressed in the spinal cord during development. It was found to be strongly expressed in a majority of SC neurosphere cells (Figure 4.6 L), but never in hypothalamic or SVZ spheres (Figure 4.6 J,K). This suggests that Nkx6.1 can be used as a specific marker of spinal NS/PCs, and may confer those cells with some unique characteristics. Nodal could not be detected in neurospheres from any of the regions (data not shown). No differences were seen between differentiated neurospheres from alternate regions. All 3 regions produced astrocytes and oligodendrocytes, in addition to neurons. The $MAP2^+$ neurons were found to express Sox2, as seen previously in hypothalamic cultures (Figure 4.6 M-O). Somewhat surprisingly, all 3 cultures also produced $Nkx2.2^+/GHRH^+$ positive neurons (Figure 4.6 P-R), which lends support to the idea that the stem cells can produce neuronal types not required for the region in which they reside.

4.5.3 Six3 as a hypothalamic regional marker?

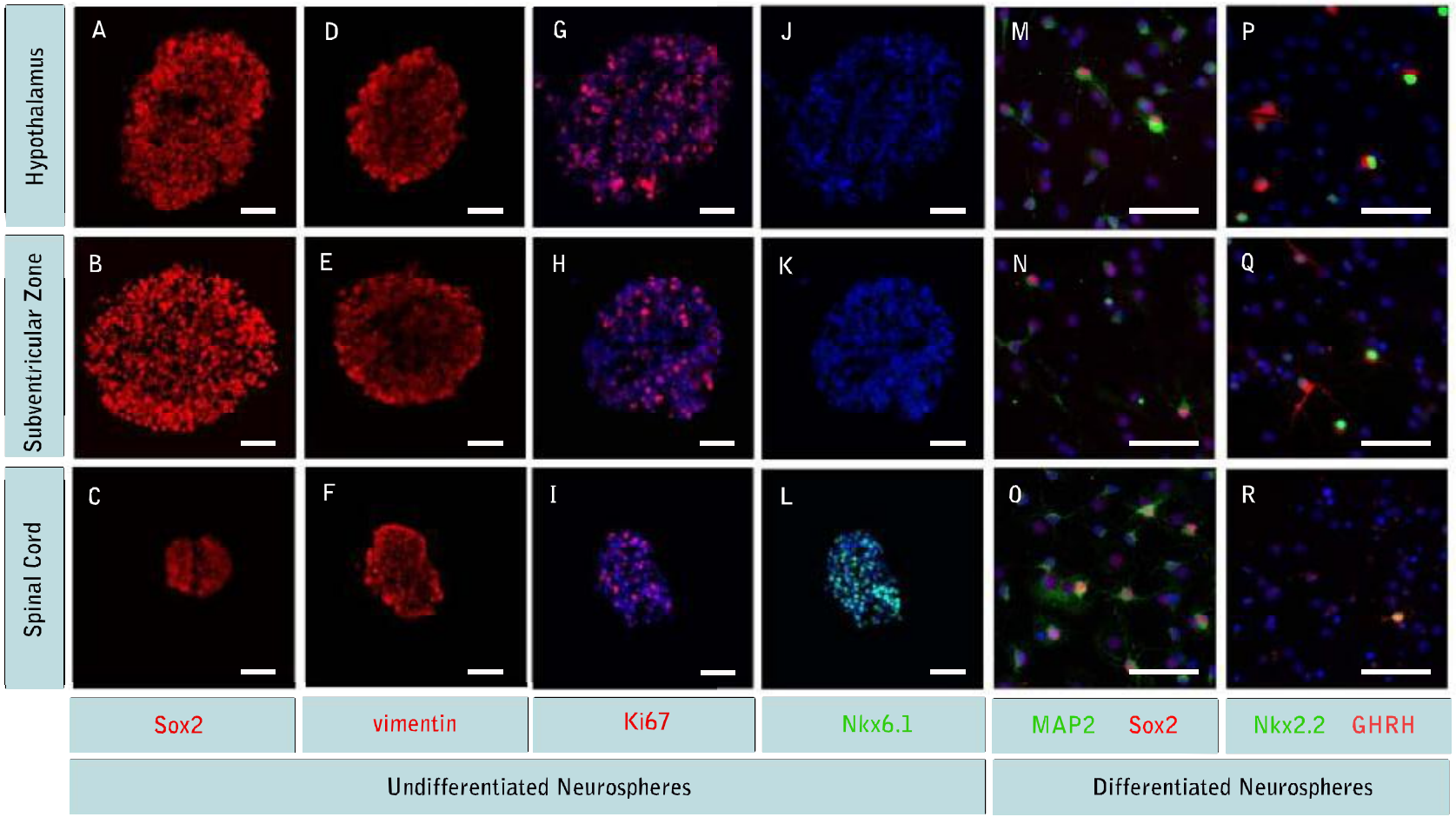
Given the restriction of Six3 expression to specific regions of the hypothalamus, as noted in chapter 3 (Figure 3.6), I wanted to investigate whether Six3 could act as a regional marker for hypothalamic neurospheres. The impression given by antibody labelling was that only neurospheres from the hypothalamus (and not from the SVZ or SC) expressed Six3 (data not shown). Unfortunately Six3 antibody labelling has high background when used on neurospheres, and so I performed in situ hybridizations for Six3 mRNA on secondary neurosphere sections to confirm this impression. Six3 mRNA was variable in neurospheres from all three regions (Figure 4.7). In situ is not an ideal technique for quantifying relative levels of expression, as variation is often seen even within the same slide. Despite this, some very clear differences could be seen in the strength of staining between neurospheres from different regions, of a large enough magnitude that they are unlikely to be artefacts. Hypothalamic sections (Figure 4.7 C-E) were seen to have the strongest staining, as well as the highest proportion of sections with at least some positive cells (11 out of 12 sections).

Figure 4.6

Markers in undifferentiated and differentiated neurospheres from different regions

A-L: Markers in undifferentiated secondary neurospheres derived from the hypothalamus (A, D, G, J), SVZ (B, E, H, K) and SC (C, F, I, L). Sox2 (A-C), vimentin (D-F), Ki67 (G-I) are expressed in all spheres, Nkx6.1 is expressed only in SC spheres (J-L). Scale bars = 50μm.

M-R: Markers in differentiated neurospheres. $Sox2^+/MAP2^+$ (M-O) and $GHRH^+/Nkx2.2^+$ (P-R) double positive neurons are seen in cultures from all three regions. Scale bars = 50μm.


Hypothalamus
Subventricular Zone
Spinal Cord
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
Sox2
vimentin
Ki67
Nkx6.1
MAP2 Sox2
Nkx2.2 GHRH
Undifferentiated Neurospheres
Differentiated Neurospheres

Subventricular spheres (Figure 4.7 H-J) also varied between sections, but had more totally negative sections (4 out of 18) and no very strongly stained ones. Only a few sections of spinal spheres could be obtained, but they generally seemed to contain a very low level of Six3 mRNA (Figure 4.7 M-O).
Given the surprising result than non-hypothalamic neurospheres could also contain Six3, I examined the regions from which the spheres were derived in more detail by ISH. No trace of Six3 mRNA could be found in the spinal cord, including the central canal (Figure 4.7 L), which has been shown to be the sphere-forming region [96]. The situation in the SVZ was less clear – the majority of sections appeared to be negative, but few areas with apparent weak expression were noted (Figure 4.7 G, arrow). In summary, it appears that Six3 expression in spheres correlates with expression in the region of origin. It is found more often, and at higher levels, in spheres from the hypothalamus, relative to other CNS areas.

4.6 Discussion

In general, hypothalamic neurospheres derived in either standard or low-insulin media behaved in a similar manner to other neurospheres described in the literature. Although they were slow-growing, they reached similar proportions in the end, and when grown in low-insulin media they could be passaged extensively. The markers expressed in undifferentiated spheres were generally consistent with the spheres containing mainly stem or progenitor cells, with a preponderance of 'stem markers' such as SoxB1 and nestin, and only a relative minority of cells expressing TuJ1 or GFAP (which may also indicate stem cells as opposed to mature astrocytes in this case – see section 4.2.2). No RIP^+ oligodendrocytes were ever seen in undifferentiated spheres.

Differentiated spheres also behaved much as predicted, with a large number of cells becoming either $MAP2^+$ neurons or $GFAP^+$ astrocytes. A few RIP^+ oligodendrocytes were also always observed. Despite displaying evidence for

Figure 4.7

Six3 as a specific regional marker?

A-B: Six3 mRNA expression in the adult hypothalamus at low (A: Scale bar = 100μm) and high (B: Scale bar = 50μ m) magnification.

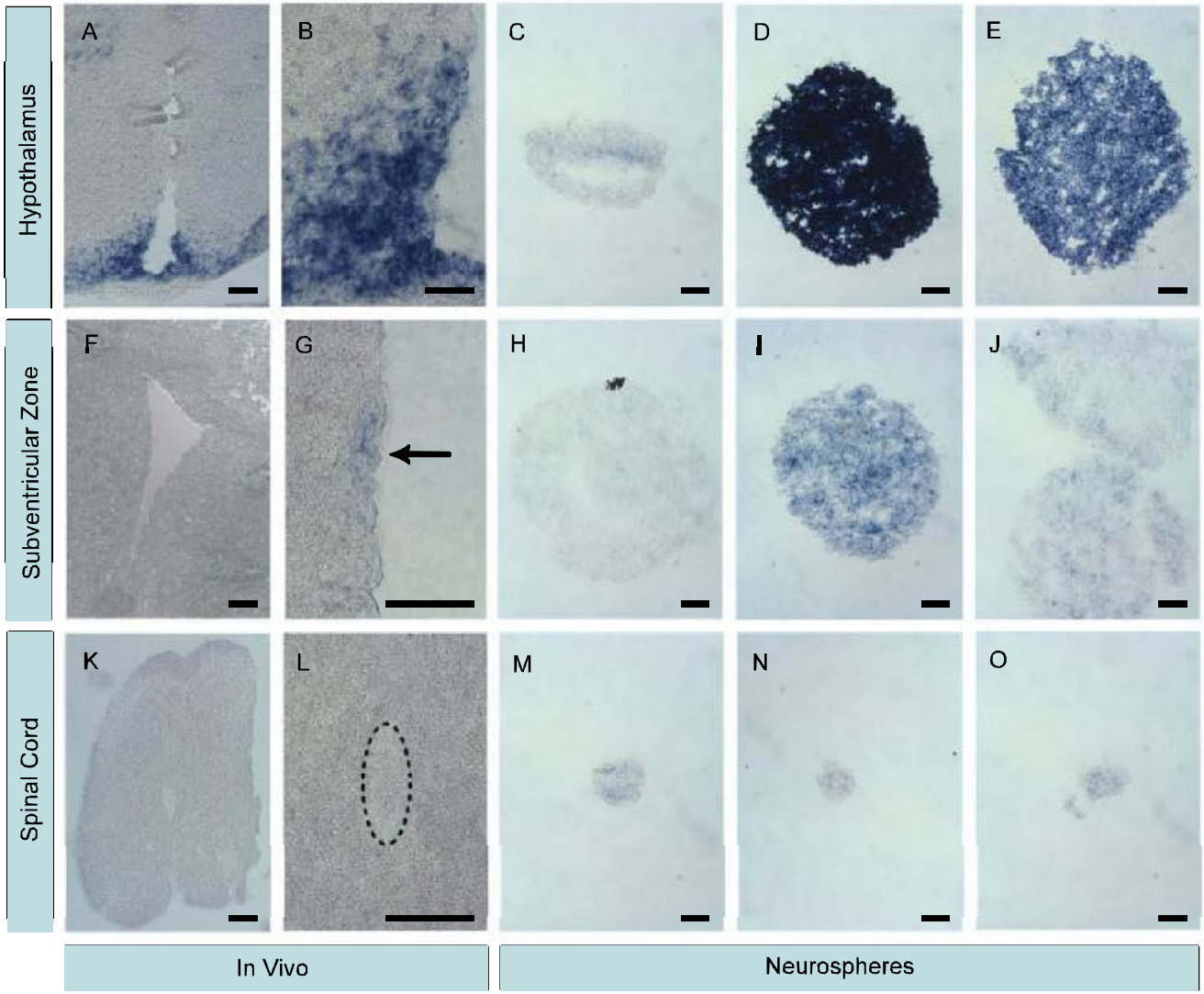
C-E: Three representative sections of secondary hypothalamic neurospheres, showing various levels of Six3 expression. Scale bars = 50μm.

F-G: Six3 mRNA expression in the adult SVZ at low (F: Scale bar = 100μm) and high (G: Scale bar = 50μm) magnification. Although the SVZ initially appears to be Six3 negative, small patches of possible Six3 expression could be seen at high magnification (G, arrow).

H-J: Three representative sections of secondary subventricular neurospheres, showing various levels of Six3 expression. Expression was generally weaker than for hypothalamic spheres. Scale bars = 50μm.

K-L: Six3 mRNA is not seen in the adult spinal cord at low (K: Scale bar = 100μm) and high (L: Scale bar = 50μ m) magnification. Note that the central canal (highlighted by black dashed line in L) is clearly negative for Six3.

M-O: Three representative sections of secondary spinal cord neurospheres, showing various levels of Six3 expression. Six3 mRNA expression was always weak or negative. Scale bars = 50μm.


Hypothalamus
Subventricular Zone
Spinal Cord
A
B
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D
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I
J
K
L
M
N
O
In Vivo
Neurospheres

all three neural lineages, this does not necessarily confirm that I am isolating multipotent progenitors as the spheres used are not proven to be clonal. It is possible that suspended cells or small neurospheres join together during culture, meaning that the different lineages arise from different original progenitors. Although the experiment has already been done for rat hypothalamic neurospheres [59], given more time, it would be useful to prove that mouse spheres can also be derived and differentiated clonally. The most thorough method of doing this is to plate only a single cell per well, although this is quite impractical for producing the large number of spheres required for further experiments. An easier but less rigorous alternative is to test whether the plating density used tends to produce clonal spheres. This can be done by co-culturing two populations of differentially-labelled cells, for example cells from two different fluorescent-reporter mice. If the resulting neurospheres always consist purely of one cell type then it is unlikely that spheres are commonly merging together. If the neurospheres contain mixed populations then they are definitely formed from at least two original cells. Although I have not performed this experiment myself, many authors refer to plating at 'clonal density', for example Brazel et al suggest that a clonal density for adult spheres is 20,000 cells/mL, higher than my plating density of 16,700 cells/mL [46]. More recently (after I had established my neurosphere protocol), several papers have cast doubt on whether these conditions are truly clonal [97],[98]. Coles-Takabe et al, performed a thorough analysis of the density required for clonal sphere formation, and suggested that a top limit of 10,000 cells/mL for primary (SVZ) cultures and 1000 cells/mL for passaged cultures were the critical densities [98]. As I make no claims that my neurospheres are proven to be multipotent, it is not of great importance whether they are actually clonal or not. Nevertheless, it would be interesting to culture spheres at the densities mentioned above, and perform triple-labelling immunohistochemistry for the three neural lineages to confirm or deny the fact.

It was interesting to examine which neuronal cell types were produced by differentiated neurospheres. Although it initially seemed as though the presence

of GHRH$^+$ neurons indicated a preference for uniquely hypothalamic stem cell types (Figure 4.4 A-E), the fact that these cells could also be derived from SVZ and spinal cord spheres (Figure 4.6 P-R) merely suggests that all neural stem cells have a wider repertoire in vitro than the cells they are responsible for producing in vivo. This is supported by the fact that other hypothalamic neural subtypes, some of which have been observed to be produced in vivo [64], were not identified in differentiated neurospheres. This includes ChAT, AVP, NPY and POMC (Figure 4.4 G / data not shown). It is clear from this data that that the in vivo environment plays a large role in directing the fate of differentiating cells, as would be expected. In vitro studies such as this can only be used to draw conclusions about the potential to produce particular cell types in the context of the artificial conditions used.

An unexpected but interesting finding was that MAP2$^+$ neurons continue to express Sox2 in differentiated neurospheres, not only in hypothalamic spheres but also those derived from the SVZ or spinal cord. There are several possible explanations for this. Sox2 is known to be expressed in neural precursors as well as stem cells, and it may be that although the neurons are sufficiently mature to express MAP2, they have not yet fully differentiated and lost Sox2 expression. It would be interesting to see if longer culture periods could produce MAP2$^+$/Sox2$^-$ cells. Alternatively, Ferri et al have shown that Sox2 can be expressed in some specific subsets of differentiated neurons in the adult mouse brain [47]. They found Sox2 in numerous neurons of the thalamus and striatum, as well as occasional cortical pyramidal cells. Knockdown of this expression led to neurodegenerative effects, suggesting that Sox2 plays a role in the maintenance of some mature neurons.

In general, my comparisons of hypothalamic neurospheres with those from the SVZ and spinal cord support the idea that NSCs vary between regions due to intrinsic differences between stem cells. It also seems that at least some in vivo markers are maintained in neurospheres (summarised in Figure 4.8), and this may prove useful for ascertaining the role of those markers in NS/PCs. Although

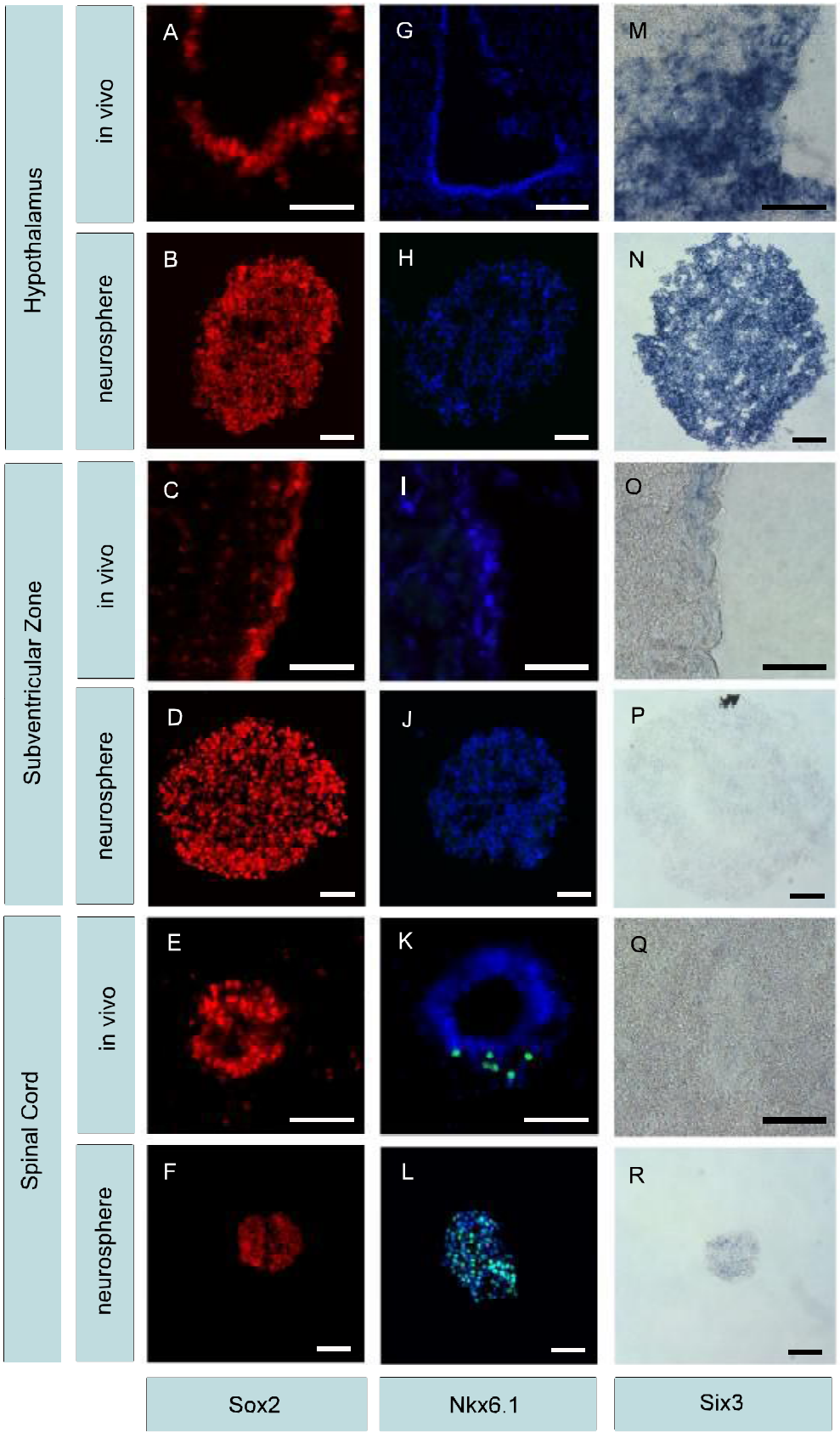
Figure 4.8

Correlation of in vivo and in vitro markers

A-F: Expression of Sox2 in vitro and in neurospheres. Sox2 is expressed in the hypothalamus (A) the SVZ (C) and the SC (E), plus neurospheres cultured from all those regions (B, D, F respectively). Scale bars = 50μm.

G-L: Nkx6.1 is present in both the central canal of the spinal cord (K) and neurospheres and spinal cord derived secondary neurospheres (L). It is not seen in either the hypothalamus (G) or the SVZ (I), or spheres derived from those regions (H, J). Scale bars = 50μm.

M-R: Six3 is strongly expressed in both the median eminence / arcuate region of the hypothalamus (M) and some hypothalamic neurospheres (N). It is occasionally weakly expressed in the SVZ (O), and weakly expressed in some subventricular spheres (P). It also appears to be weakly expressed in spinal neurospheres (R), despite not being detected in the spinal cord itself (Q). Scale bars = 50μm.


Hypothalamus
Subventricular Zone
Spinal Cord
in vivo
neurosphere
in vivo
neurosphere
in vivo
neurosphere
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
Sox2
Nkx6.1
Six3

Six3 did not prove to be an absolute marker for hypothalamic NS/PCs, the level of expression did seem to vary by region, and correlate with the in vivo expression. It may be that it is maintained in spheres generated from $Six3^+$ positive cells, although the presence of $Six3^-$ spheres suggests that it is not essential to stem cell proliferation. The role (if any) of Six3 in neurospheres has yet to be determined, but in the embryonic system it has been implicated in preventing differentiation and encouraging proliferation by shortening the length of the cell cycle [81]. It is hoped that our laboratory may start work on producing a Six3-GFP mouse in the future, and this would prove a useful tool for answering the questions that I have raised about Six3 in adult NS/PCs. The transcription factor Nkx6.1 showed a very clear regional difference, with strong expression in all spinal cord neurospheres, and none in hypothalamic or subventricular cultures. This correlated perfectly with its in vivo expression, where it could be seen in a few subependymally-located cells near the central canal of the spinal cord, but was undetectable in other areas (Figure 4.7 G-L). Together with other work from the lab demonstrating that application of EGF to spinal explants increases the number of $Nkx6.1^+$ cells (K. Chinnaiya, unpublished), this suggests that Nkx6.1 may prove to be a unique marker for spinal progenitor cells that is maintained in in vitro conditions.

Chapter 5

Chapter 5 – Neurosphere Subdissections

5.1 Introduction

As described in the introduction, and extended in my work in chapter 4, neurosphere-forming NS/PCs do exist in the hypothalamus. However no study has yet attempted to define the major site(s) of proliferation. My observations that the marker profile of both ependymal and parenchymal cells in the hypothalamus varies along the A/P and D/V axes (chapter 3), coupled with the observation that neurospheres derived from dissections of whole hypothalamus showed some variation (chapter 4), raises the possibility that:

- Hypothalamic stem cells may be located in only discrete parts of the hypothalamus
- Different populations of hypothalamic NS/PCs may exist

In order to begin to examine these hypotheses, I subdissected the hypothalamus to discover which regions were capable of forming neurospheres.

5.2 Results

5.2.1 Defining the subdissections

I wanted a subdissection protocol that would be entirely unbiased, and make no assumptions about where the neurosphere-forming cells might be. I also wanted to be able to distinguish between the two regions expressing stem cell markers identified in chapter 3 (Figures 3.12 and 3.13). I therefore decided to divide the hypothalamus into 12 separate parts, using three A/P divisions, each with three D/V subdissections along the midline plus a more lateral dissection (Figure 5.3 A+B). The brain was first cut into three coronal slices corresponding to the three

A/P levels (Figure 5.3 A), and then four small subdissections were removed from each slice (Figure 5.3 B). The A/P subdivisions were designed to give an anterior region corresponding to the level of the suprachiasmatic nucleus (termed A), a mid region at the level of the median eminence (termed ME) and a more posterior region (termed P). The D/V subdivisions were always dissected relative to the position of the third ventricle, with the ventral (V) dissection taking the ventral-most part (plus the ME at the relevant level), the dorsal (D) dissection taking the dorsal most part, and the mid (M) dissection taking a region approximately half way between the two. The V/M/D dissections always included a portion of the ependymal lining of the third ventricle, whereas the lateral (L) dissection was taken from a similar D/V level to the mid dissection, but always too far laterally to contain ependyma.

In order to confirm that that the subdissections matched the anatomical regions I intended, a number of control experiments were performed (Figures 5.1 and 5.2). The subdissections were taken, and instead of being cultured as neurospheres were fixed immediately, sectioned, and labelled with antibodies. The antibodies included a number of the markers used in chapter 3, as well as neuropeptide transmitters found only in defined regions of the hypothalamus. Comparing the subdissections with the marker pattern seen in vivo allowed me to pinpoint precisely where each piece came from, as well as confirm that the subdissections were consistent over a number of experiments (n=4).

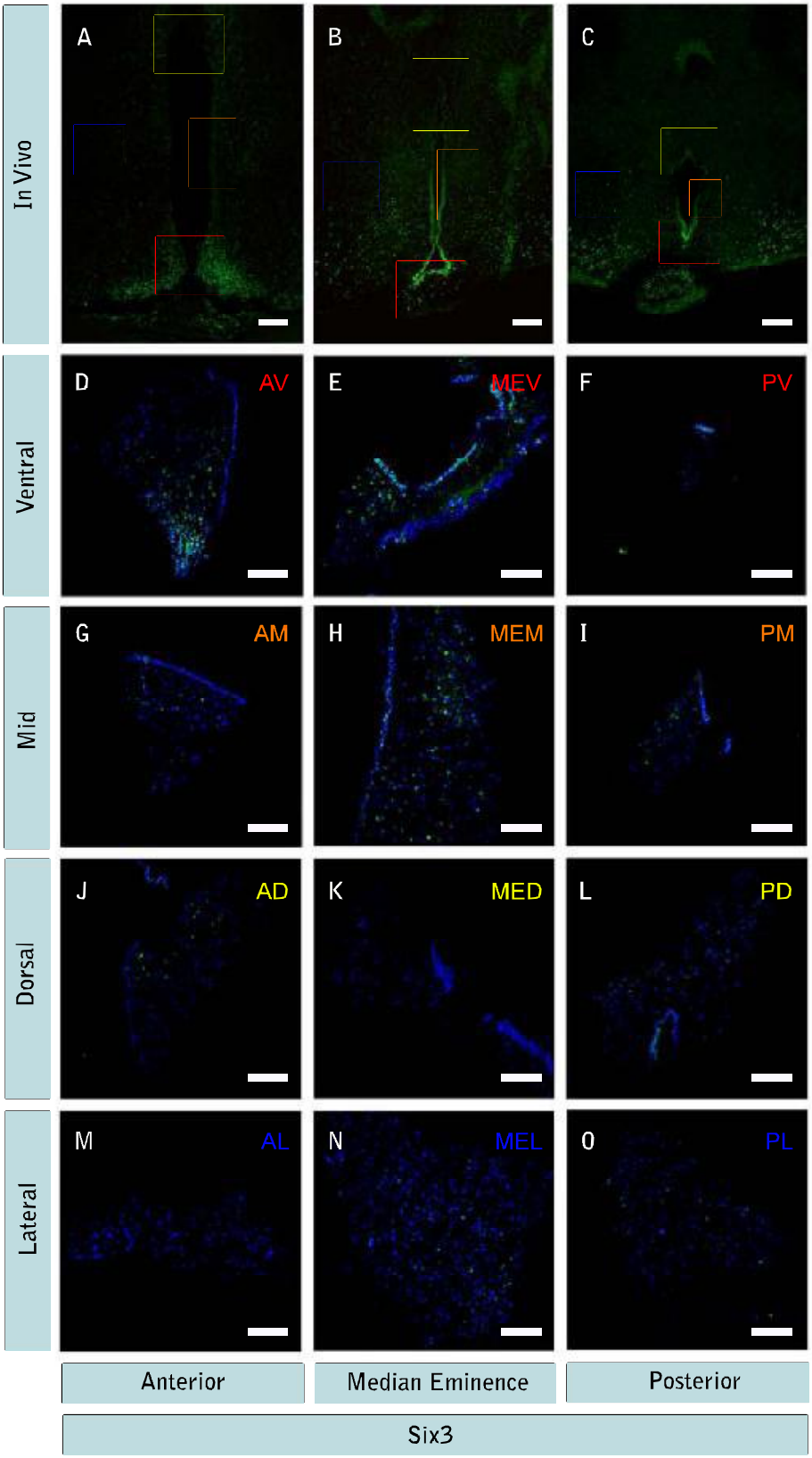
The pattern of Six3 expression can be seen in Figure 5.1. In addition, the subdissection has been counterstained with DAPI, and both this and the morphology of the dissections give many clues about their origins. The ependymal lining is clearly visible as a dense layer of $DAPI^+$ nuclei. It is present on all nine of the ventricular subdissections (D-L), but never on the lateral ones (M-O). The ME is also clearly visible on the MEV subdissections, with a thin layer of ependymal cells, the ME parenchyma containing very few (and often elongated) nuclei, and then a thick outer layer of cell bodies (E). When the pattern of Six3 expression in the subdissections is compared with the in vivo

Figure 5.1

Location of subdissections using Six3 expression

A-C: Expression of Six3 at the three A/P levels: anterior (A), ME (B) and posterior (C). The coloured boxes show the predicted positions of each subdissection: red = ventral, orange = mid, yellow = dorsal and blue = lateral. Scale bars = 100μm.

D-O: Six3 expression in subdissections from each of the 12 areas. Scale bars = 100μm.


In Vivo
Ventral
Mid
Dorsal
Lateral
A
B
C
D
AV
E
MEV
F
PV
G
AM
H
MEM
I
PM
J
AD
K
MED
L
PD
M
AL
N
MEL
O
PL
Anterior
Median Eminence
Posterior
Six3

expression there is a clear match. The predicted position of each subdivision is shown in Figure 5.1 A-C, marked by coloured boxes (V=red, M=orange, D=yellow, L=blue). As expected, the dorsal (J-L) and lateral (M-O) subdissections are largely negative for Six3, barring the occasional parenchymal cell and faint expression in the ependyma of the PD region (L, also seen in vivo: C yellow box). The MEM (H) and PM (I) regions both have a few more scattered $Six3^+$ cells, and weak ependymal expression, whilst MEV (E) and PV (F) have strong ependymal expression, consistent with stronger Six3 expression most ventrally. The AV subdissection has no ependymal expression, but a strong patch of parenchymal expression clearly corresponding to the suprachiasmatic nucleus (subdissection: D, in vivo: A, red box).

In addition to Six3, I used neuropeptide markers of specific neuronal subtypes to verify the location of subdissections (Figure 5.2). Cells expressing neuropeptide Y (NPY) are located in the ventral arcuate nucleus [11]. Their location was examined both by antibodies and by using a NPY-GFP mouse. The antibody clearly labelled both cell bodies and axons (projected to extensive areas of the hypothalamus, data not shown) while the mouse knock-in expressed GFP in only the cell bodies (Figure 5.2 A-F). The arcuate nucleus could be clearly seen from the ventral surface of the brain (A) as well as on ME slices (B). It was in MEV subdissections (C) but not more dorsal or lateral pieces (D-F). The position of the anterior slice at the level of the suprachiasmatic nucleus was confirmed using antibodies for arginine vasopressin (AVP). In the hypothalamus, AVP^+ neurons are found only in anterior areas, in the suprachiasmatic nucleus (SCN) ventrally and the paraventricular nucleus (PVH) more dorsally [99], (Figure 5.2 G). In accordance with this, only AV and AD subdissections contained AVP^+ cells (H-K). AVP^+ cell bodies were not seen in other regions either in vivo or in subdissections (data not shown).

In addition to the analyses highlighted above (Figures 5.1 and 5.2), similar analyses were performed using other antibodies. These include Sox2, Sox1+3, TH, BLBP, and GFAP (data not shown). This data fully supported the examples

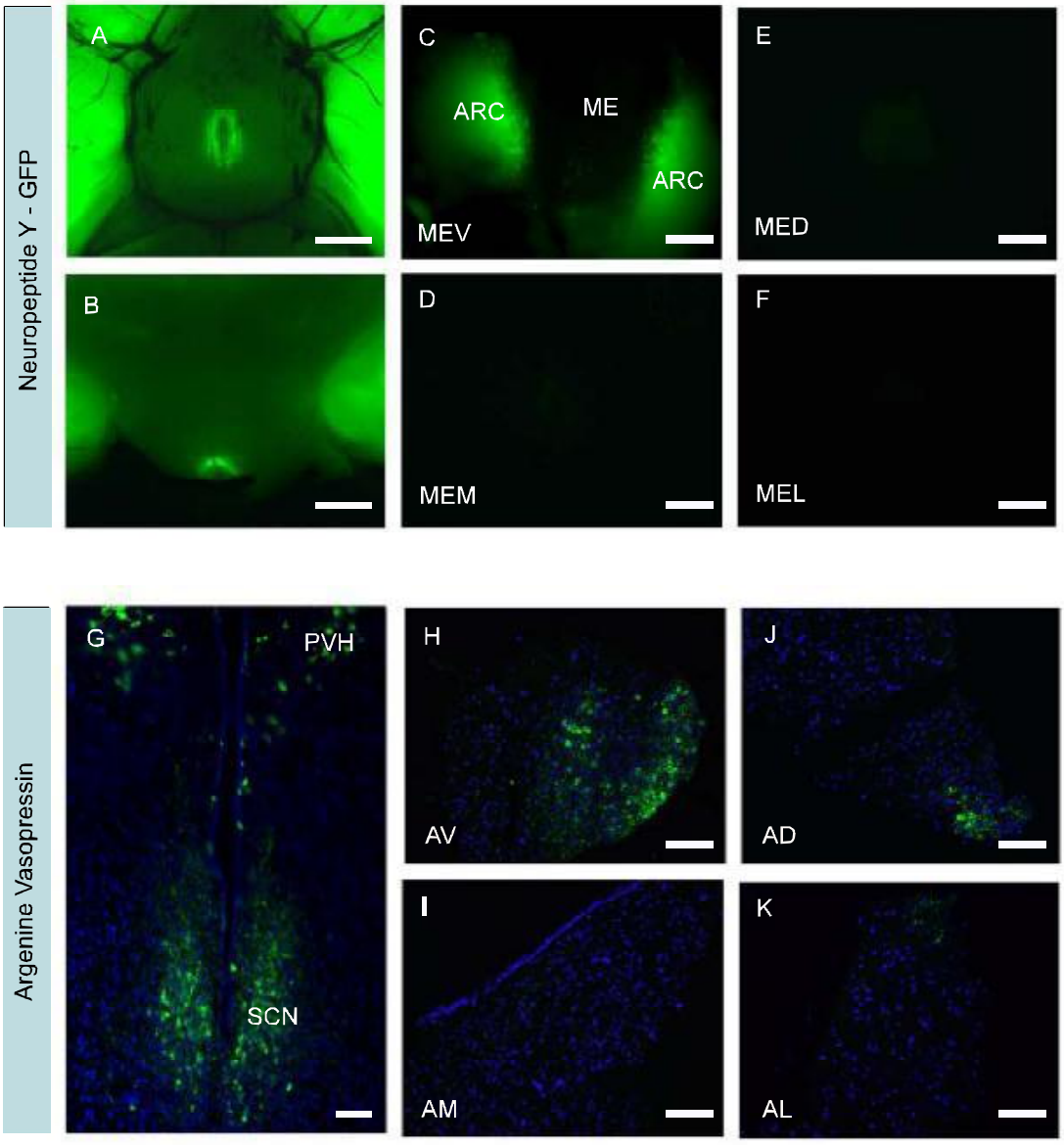


Figure 5.2

Location of subdissections using neuropeptide markers

A-F: Expression of NPY in ME subdissections, using an NPY-GFP mouse. NPY expression in the ARC can be clearly seen both from the ventral surface of the brain (A) and on slices (B). It can be seen in the MEV subdissection as two GFP^+ regions seperated by the GFP^- ME (C), but is not present in MEM (D), MED (E) or MEL (F). Scale bars = 500μM (A, B) or 100μM (C-F).

G-K: Expression of AVP in subdissections. Using antibodies, AVP can be seen in the SCN and PVH in vivo (G). It is present in a large area of the AV subdissection (H), and a small area in AD (J). AM (I) and AL are negative (K). Scale bars = 100μM.

shown. In conclusion, I am confident that the subdissections correspond accurately to the regions intended, and that subdissections performed on different mice do not vary significantly from each other.

5.2.2 Neurosphere subdissections in standard media

Preliminary subdissection results were obtained from neurospheres grown in standard media. The hypothalami from two mouse brains were subdissected into 12 regions as described above (Figure 5.3 A and B). The equivalent subdissections from the two mice were pooled together, dissociated, and cultured in at least three wells of a 6-well plate. They were plated at a slightly reduced density of 10,000 cells/mL, as only small numbers of cells could be obtained from each subdissection. 14 days later, the number of neurospheres in each well was counted. Four experiments were performed. In separate experiments the absolute numbers of neurospheres varied quite widely, however numbers between repeated wells were consistent. Moreover, the overall pattern observed was consistent in both biological and technical replicates.

Figure 5.3 C shows the numbers of primary neurospheres generated in a single experiment. The huge majority were derived from the MEV (mean of 289.0 NS per well ± 22.0), with smaller numbers from MEM (30.0±4.7) and PD (12.5±2.5). In this experiment a few spheres were also generated from AV (8.0±2.0) although this was not repeated in subsequent experiments. These four regions were then passaged at a density of one neurosphere per well in 24-well plates to produce secondary spheres (5.3 D-H). Although the total sphere counts were similar between MEV, MEM and MED (with only a single secondary sphere produced from AV) it was clear from looking at the spheres that very significant differences existed in the size of spheres. Intriguingly, MEV produced tiny neurospheres, whilst MEM and PD produced a mix of large and small spheres. To quantify this, neurospheres were recounted, this time discriminating between small (<100μm approximately) and large (>100μm) diameter spheres.

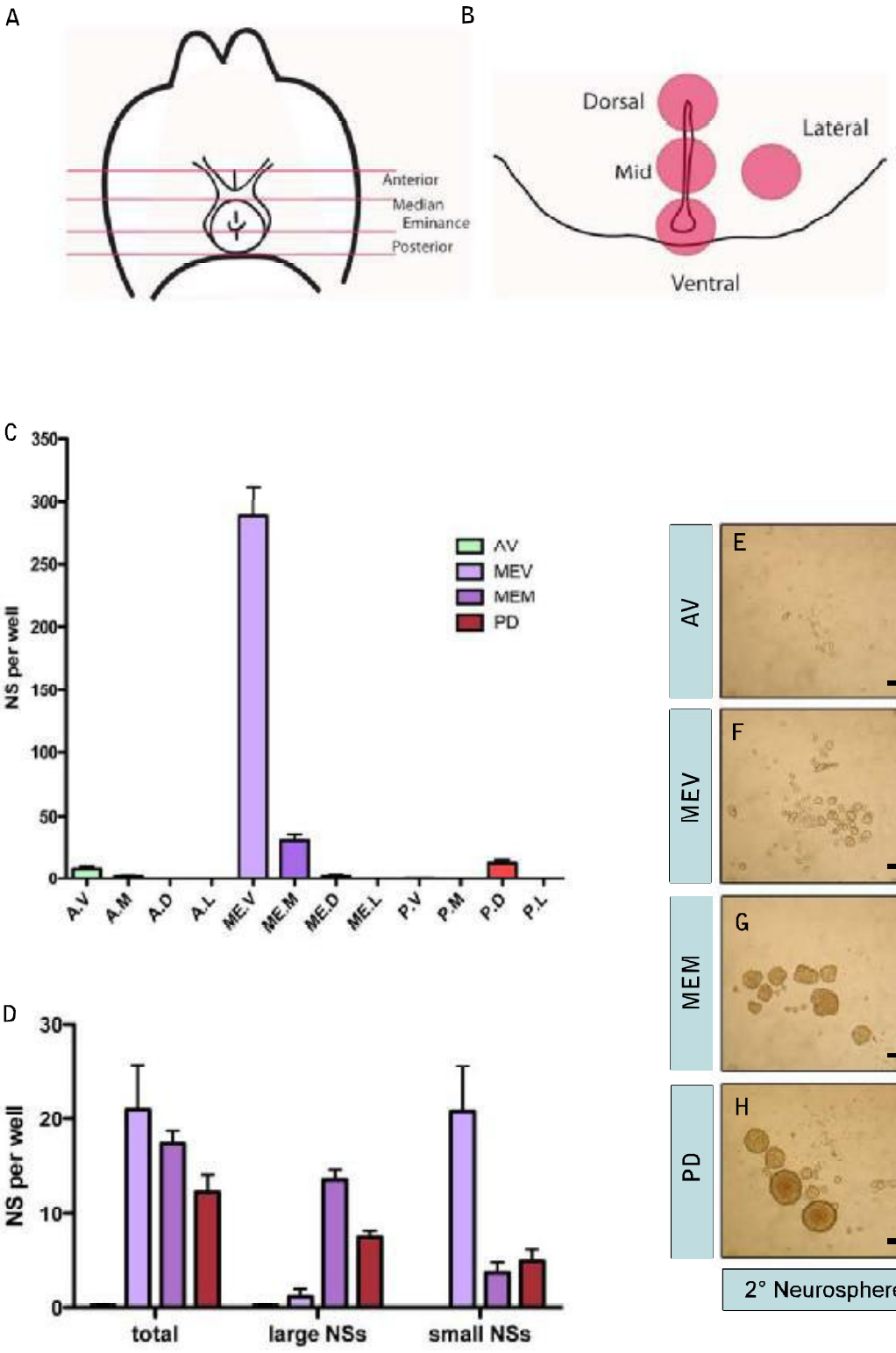
Figure 5.3

Neurosphere subdissections in standard media

A, B: Cartoon illustrating how the hypothalamus is subdivided. Three A/P levels are generated by making thick coronal slices (A). Each of these is then used to dissect four V/D pieces of tissue (B) using the position of the third ventricle as a guide.

C: Generation of primary neursopheres from subdissected tissue. Only neurospheres over 50μm diameter were counted. AV, MEV, MEM and PD produced enough neurospheres to passage.

D-H: Generation of secondary neurospheres. Although the initial counts were similar (D, total), when spheres were divided into large (>100μm) and small (<100μm) the differences between the subdissections became clearer. MEV produced only small spheres (F), while MEV(G) and PD(H) produced large ones. E-H show representative pictures of the secondary neurospheres. Scale bars = 500μm.


A
Anterior
Median Eminance
Posterior
B
Dorsal
Lateral
Mid
Ventral
C
NS per well
350
300
250
200
150
100
50
0
AV
MEV
MEM
PD
A.V
A.M
A.D
A.L
ME.V
ME.M
ME.D
ME.L
P.V
P.M
P.D
P.L
D
NS per well
30
20
10
0
total
large NSs
small NSs
E
F
G
H
AV
MEV
MEM
PD
2° Neurospheres

This confirmed that MEV secondary neurospheres were almost exclusively small (in fact, below the threshold at which many authors begin to count neurospheres), while MEM and PD produced a majority of large neurospheres.

5.2.3 Subdissections in low insulin media

These preliminary experiments in standard media raised the interesting point that the number of neurospheres initially generated by a region did not necessarily correlate with their passageability, ie the ability to form secondary spheres of a comparable size to primary ones. One explanation for this could be that both true stem cells and early progenitors may be able to produce spheres, but only stem cells can maintain this after passaging. Alternatively, it may be that different populations of stem cells exist, and some are more receptive to the culture conditions (particularly the growth factors used) than others. Whatever the reason, it is clear that there may be D/V differences in neurosphere-forming cells that only become apparent after passaging. There is limited scope for studying this in the standard neurosphere medium, as spheres are not maintained for more than a few passages. I therefore repeated the subdissection experiments in low-insulin media, which maintains neurospheres through many more passages. This time spheres were initially plated at 10,000 cells/mL in 24-well plates, before being passaged and replated at 16,700 cell/mL every 10 days.

The neurospheres grew well in low-insulin media (see section 4.3 and table 4.1 for a general comparison of standard and low-insulin media), and could be kept for at least five passages at the plating density used. Neurosphere counts are summarised in Figure 5.4, and sizes were also assessed and are shown in figure 5.5. There was an overall trend for sphere size to decline gradually with passaging from secondary spheres onwards. Although the proportion of large spheres was less for primary than secondary cultures, this was generally the result of high numbers of tiny neurospheres rather than low numbers of large spheres. It seemed that spheres could be derived from a slightly wider area

using this medium, in particular the production of spheres from MED, which was not seen in standard medium (Figure 5.4). Other than that, the overall pattern of neurosphere-forming cells was identical, with almost no spheres from the anterior region, and none ever produced from lateral tissue. Posterior tissue gave mixed results that varied between experiments: some neurospheres were always formed, but the D/V domain they came from tended to vary (data not shown). Initially the MEV domain gave rise to both the highest number (Figure 5.4) and biggest (Figure 5.5 A) neurospheres. After two passages (tertiary spheres) this declined, with the spheres produced being both smaller and less numerous. By passage 5, no new MEV neurospheres were produced. In contrast, the neurospheres produced from the MEM and MED were initially much smaller than MEV spheres (Figure 5.5A), and found in lesser numbers. After the first passage both of these regions produced more large spheres than MEV (Figure 5.5 B) although the total sphere counts were still lower. By the time they had gone through three passages they had both overtaken MEV for number of spheres produced.

Both undifferentiated and differentiated neurospheres from proliferative regions were analysed with antibodies, to see if different regions varied in their character or differentiation potential. Undifferentiated spheres were analysed with the same markers as for the general characterisation described in section 4.4.2 (using spheres grown in standard medium). No obvious differences could be observed, although relatively low numbers of spheres were obtained, and so statistical analysis of variable markers over many spheres was not possible. Differentiated spheres were also labelled, this time for markers of more mature cells (using spheres cultured in low-insulin media). All three lineages were seen in spheres from each region (although with very low GFAP levels as was characteristic of spheres differentiated in the low-insulin media). All regions could also produce $GHRH^+$ neurons, and contained similar levels of $Ki67^+$ proliferating cells (data not shown).

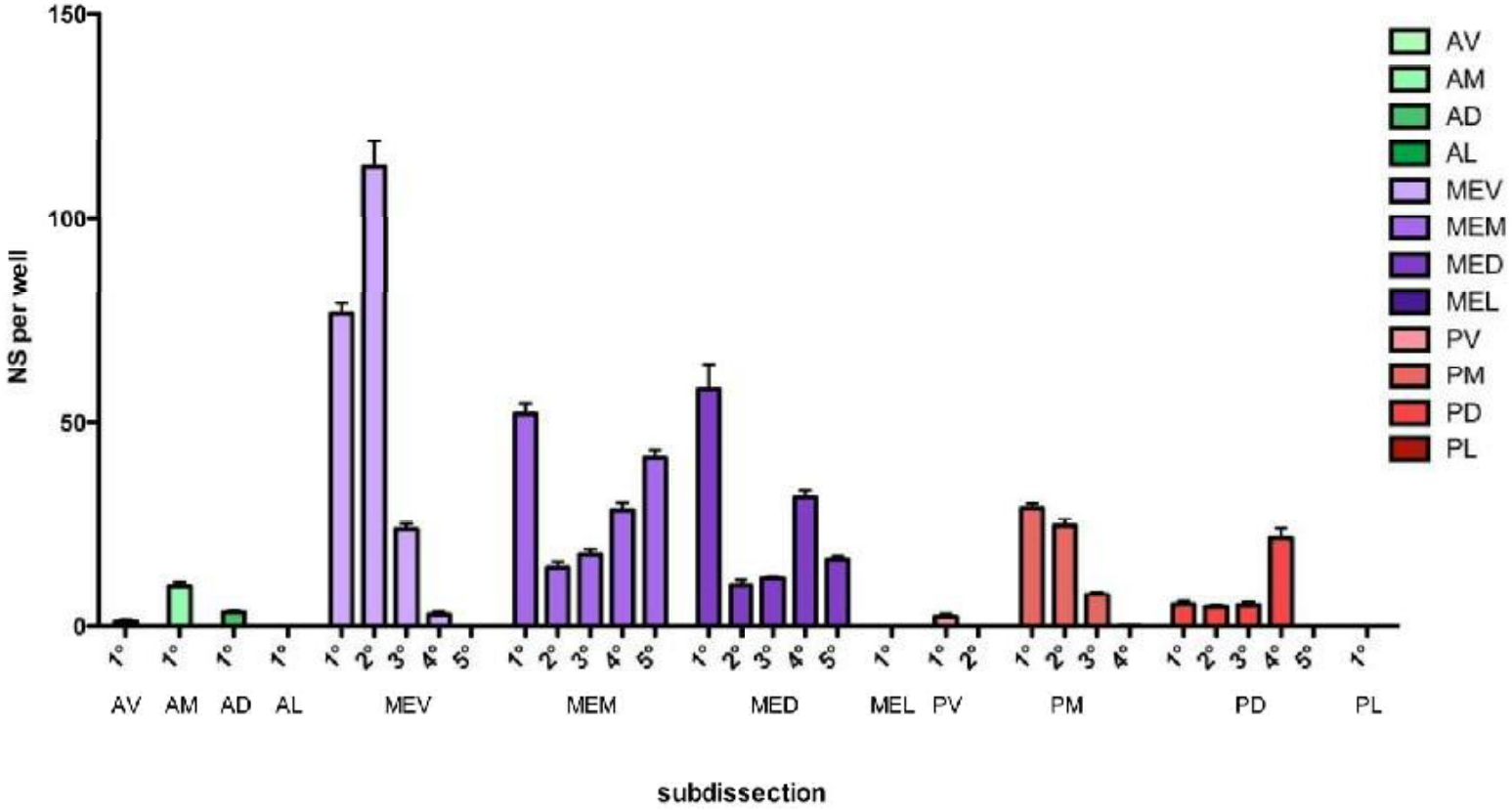


Figure 5.4

Subdissections in low-insulin media

The 12 subdissections were kept for 5 passages. Where there were not sufficient neurospheres formed to passage them, a result is not shown (all labelled passages with no bar are therefore passages that were plated but produced no spheres).

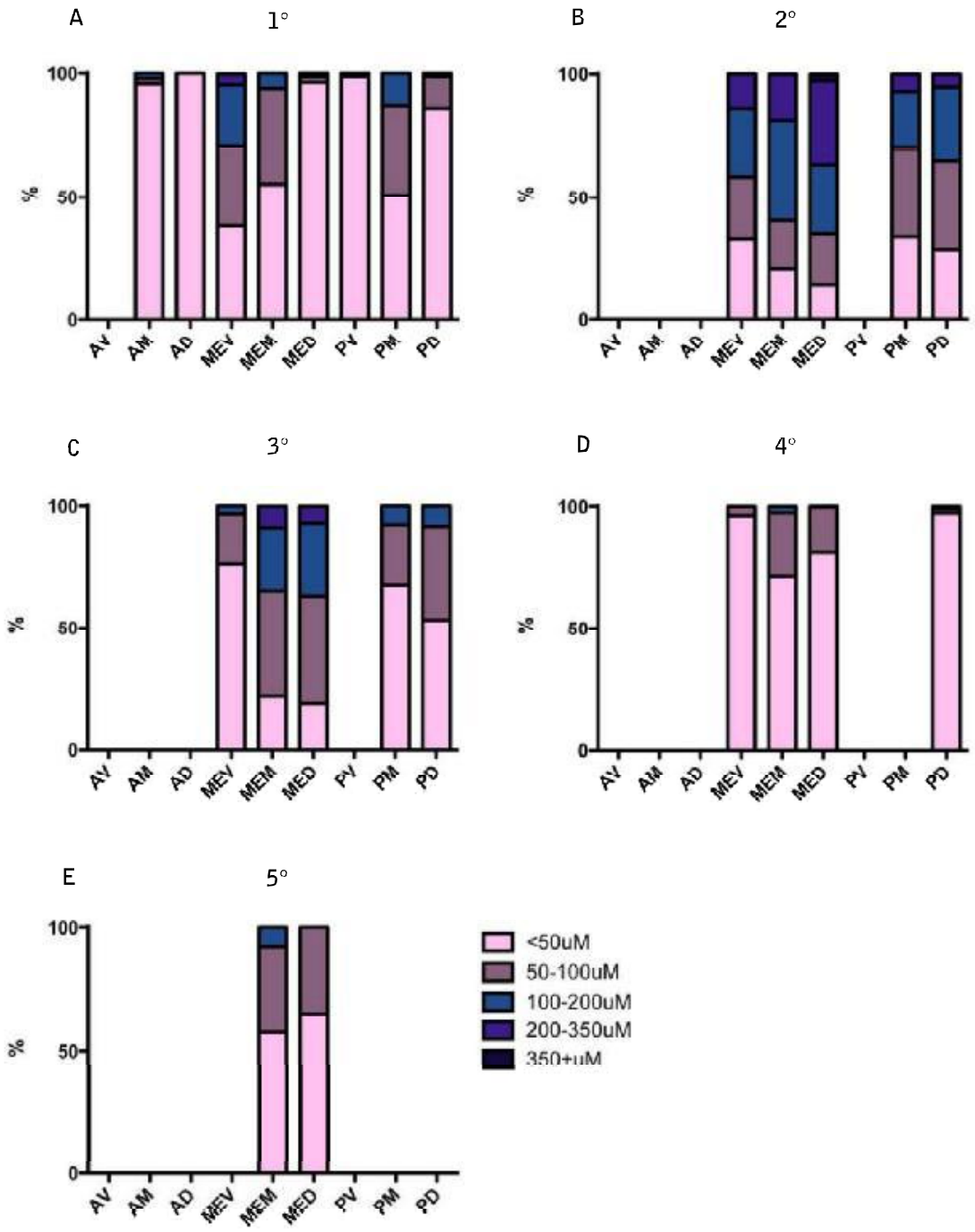


Figure 5.5

Sizes in neurosphere subdissections

Neurosphere diameters were measured using a graticule, and the number of neurospheres in each size category recorded. Not all wells were counted, but once a well had been started every sphere was included. At least 50 spheres were counted for each region, up to 300 if sufficient spheres were available.

5.2.4 α vs. β subdissections

It is clear from the experiments above that the ventricular area at the level of the median eminence is the major source of neurospheres in the hypothalamus. This is also the precise region known to contain tanycytes, which have been hypothesized to act as a neural stem cell (see introductory sections 1.3.5 and 1.6.4). My data strongly supports this hypothesis, which raises another important question: which subtypes of tanycytes give rise to the neurospheres? Given that my data suggests D/V differences in neurosphere behaviour, it seems likely that the separate subtypes may have different proliferative potential.

As discussed in the introduction, tanycytes can be divided into 4 subtypes, known in ventral to dorsal order as β2, β1, α2 and α1. In addition, α2 tanycytes can be divided into ventral and dorsal subtypes on the basis on GFAP expression. In the original subdissections, α1 tanycytes would have been found in the MEM subdissections, and probably the MED region to a lesser extent. Both β1 and β2 are contained in the MEV region. The case is less clear concerning α2 tanycytes; it is possible that vα2 would be included with MEV, and dα2 in MEM. As experiments involving in vivo FGF infusion implicate vα2 as an important proliferative region (discussed in detail in chapter 6), it is vital to be able to distinguish this region from the β tanycytes. To start, I attempted to distinguish α tanycytes from β from ependymocytes, with the intention to look at finer subdivisions later if the initial experiments were successful. Figure 5.6 A and B compare the basic subdissections with the α/β subdissection. The α/β subdissection requires much more precision, as even a few tanycytes included in the wrong dissection could render the results meaningless. It is complicated by being unable to leave a large gap between subdissections, as this could result in a lack of vα2 tanycytes in the α subdissections. In addition, the position of α and β tanycytes at more posterior levels is not clearly defined in the literature. In order to test the subdissections, undissociated pieces of tissue were fixed, sectioned and stained. No α-specific markers were available, so putative β-specific markers were used, with the presence of β-negative ependymal lining

used to define the correct α dissection. Of the proposed β markers – DARPP-32, Six3 and Nodal – only DARPP-32 consistently gave clear labelling in dissected tissue (Figure 5.6 D-F). However I consistently saw strongly DARPP-32$^+$ cells in the α subdissections (Figure 5.6 C, arrow), meaning I was unable to confirm the accuracy of my dissection. This occurred no matter how much care was taken to exclude the ME from the α region, making me question whether DARPP-32 is truly a marker only for β tanycytes. As there have been occasional reports of its expression in tanycytes projecting to the arcuate nucleus (a feature of α2 cells) it may be that the problem lies in the specificity of subtype markers rather than in the dissection.

Despite these questions over the accuracy of the subdivisions, a few preliminary experiments on α/β spheres were undertaken. Generally more spheres were generated from α than β regions, with a few from ependymocytes (E) as well in some cases (Figure 5.6 K). It is possible that occasional α1 tanycytes exist quite dorsally interspersed with ependymocytes, and that these may give rise to E spheres. When α and E spheres were labelled with DARPP-32 expression was variable, but more common in α cultures. As both α and E spheres are unlikely to be derived from DARPP-32$^+$ regions, it seems unlikely that it can be used as a specific marker of spheres derived from ventral DARPP-32$^+$ stem cells.

5.3 Discussion

My results in this chapter clearly define the neurosphere-forming region of the hypothalamus. Despite the interesting combination of stem cell markers observed in chapter 3 (Figure 3.12), the anterior hypothalamus does not produce neurospheres, and so is unlikely to contain a stem cell population. Lateral tissue from any A/P level is also non-sphere forming. As BrdU$^+$ cells have been observed in these regions by various authors, it supports the theory that these may be migrating progenitors, incapable of the extensive self-renewal required for sphere formation. Both the ME region and more posterior areas do

Figure 5.6

α vs β subdissections

A, B: Cartoons comparing the standard subdissection (A) with the α/β subdissection (B) at the level of the ME.

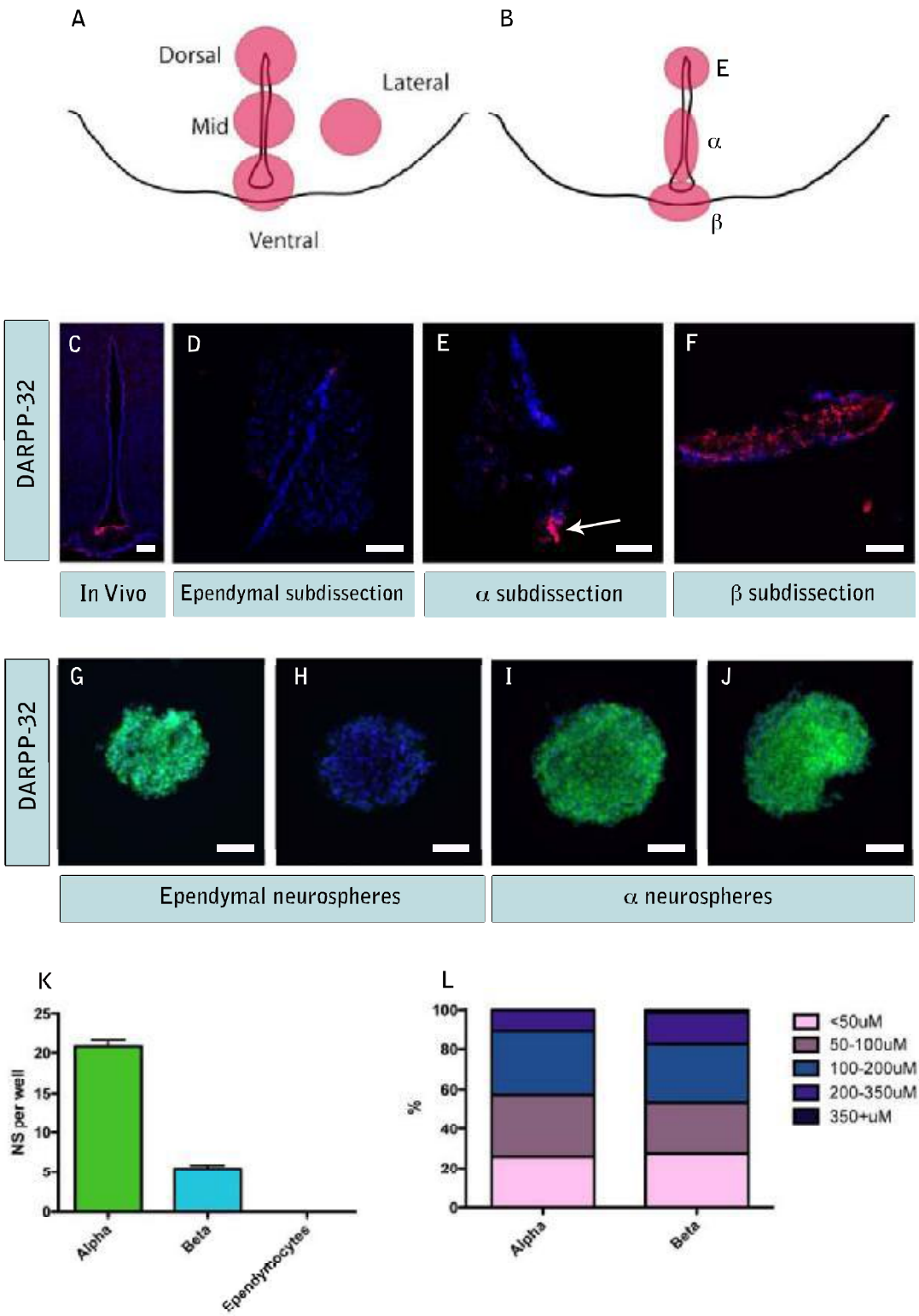
C: DARPP-32 expression in vivo seems to be limited to β tanycytes, but is possibly also expressed in vα2. Scale bar = 100μm.

D-F: DARPP-32 expression in α/β subdissections. It is clearly visible in the β region (F), but also present in a few ependymal cells of the α dissection (E, arrow). Scale bars = 100μm.

G-H: DARPP-32 expression in representative neurospheres derived from E (G, H) and α (I, J). All pictures were taken using the same exposure times, allowing a rough comparison of expression levels. Scale bars = 50μm.

K: Primary neurosphere counts in a single experiment.

L: Primary neurosphere sizes in a single experiment.


A
Dorsal
Lateral
Mid
Ventral
B
E
α
β
DARPP-32
C
D
E
F
In Vivo
Ependymal subdissection
α subdissection
β subdissection
DARPP-32
G
H
I
J
Ependymal neurospheres
α neurospheres
K
NS per well
25
20
15
10
5
0
Alpha
Beta
Ependymocytes
L
%
100
80
60
40
20
0
Alpha
Beta
<50uM
50-100uM
100-200uM
200-350uM
350+uM

form neurospheres. The precise pattern is most easily studied in the ME region, where the third ventricle is longer and therefore D/V distinctions are more obvious. The pattern of neurosphere-forming regions bears a striking resemblance to the location of hypothalamic tanycytes, lending support to the theory that they act as the original stem cell in the hypothalamus. In addition, the D/V pattern of tanycyte subtypes may offer an explanation to the apparent D/V differences in character seen in neurospheres. In both standard and low-insulin media, the MEV was initially seen to be the most proliferative area, with lower sphere production more dorsally. However, after passaging (one passage for standard media, two to three for low-insulin) it became clear that ventral spheres could not regenerate as effectively as more dorsal ones. The most likely explanation for this is that the true stem cell population resides in mid/dorsal regions of the third ventricle, while more ventrally only early progenitors are found. This points to α tanycytes as the most likely candidate to act as a stem cell. Unfortunately, further experiments to test this theory were hampered by the technical difficulties encountered in making truly accurate subdissections. This work is ongoing, and it may be that better definition of the marker changes at the α/β boundary allow it to progress in the future.

Chapter 6

Chapter 6 – FGFs and Hypothalamic Stem / Progenitor Cells

6.1 Introduction

My work described in previous chapters provides strong evidence that stem / progenitor cells resides in the adult mouse hypothalamus. Such cells need control mechanisms to regulate how they proliferate, migrate and differentiate. In this chapter, I will investigate whether fibroblast growth factors (FGFs) are likely to play a role in regulating the behaviour of these cells in the hypothalamus.

6.1.1 FGFs:

The FGF family of growth factors play a significant role in many diverse biological functions. Their influence during development is particularly well understood, however they are also involved in many adult homeostatic processes. FGFs have a diverse range of effects on target cells, including effects on proliferation, migration, differentiation, and fate choice (reviewed in [100]). In both humans and mice there are at least 22 members of the FGF family. To date, four FGFs have been identified in zebrafish, and seven in chickens. There is a high level of cross-species conservation, with up to 90% amino acid sequence homology. FGF-like genes have even been found in viruses, reflecting their ancient evolutionary origin. With regard to their structure, FGFs range in size from 17 to 34kDa, and all contain an internal core region of 34 highly conserved amino acids. The two prototypic FGFs are FGF-1 and FGF-2, also known respectively as acidic FGF and basic FGF. These two FGFs have the core domain arranged as 12 antiparallel β-strands [100].

6.1.2 FGF Receptors:

FGF ligands signal by binding to FGF receptor tyrosine kinases (FGFR) on the cell surface. There are four distinct FGFRs encoded by separate genes.

Alternative splicing of the extracellular domain results in multiple splice variants of each FGFR. Due to a high level of homology both within the FGF and the FGFR families there is a highly overlapping pattern of receptor binding, with most FGFs able to bind to all four receptors. However the relative affinities of each FGF ligand for different receptors varies, and the different receptor isoforms also show differential binding affinities. Heparin sulphate proteoglycans are also required for FGF-FGFR binding. All four FGFRs are transmembrane tyrosine kinase receptors, and all share a number of structural features. They contain three extracellular Ig-like domains (IgI, II and III), plus an acidic domain and an intracellular tyrosine kinase domain. The IgIII domain exists as up to three splice variants, known as IgIIIa, IgIIIb and IgIIIc. FGFR1 and 2 can exist as all three variants, FGFR3 is limited to the IIIb and IIIc forms, and FGFR4 is only found as IIIc. These splice variants can radically alter the affinity of each receptor to the different FGF ligands. The intracellular C-terminal domain contains seven phosphorylatable tyrosine residues, although they are not all thought to be necessary for receptor signalling.

6.1.3 Role of heparin sulphate proteoglycans:

Heparin sulphate proteoglycan (HSPG) is a highly complex protein, containing glycosaminoglycan side chains. These consist of negatively charged disaccharide repeats. FGF signalling requires the dimerisation of FGF-FGFR complexes, and it is thought that HSPGs facilitate this by either binding monomeric FGF into repeating units for presentation to the FGFR (reviewed in [101]), or by connecting individual FGF-FGFR complexes together into dimers [100]. Dimerisation can be between two FGFRs of the same (homodimerisation) or different (heterodimerisation) types.

6.1.4 Intracellular signalling cascades:

There are at least three intracellular signalling cascades that can be activated by FGFR signalling (Figure 6.1). It is thought that the different FGFRs all act via the same signalling pathways, but that separate subtypes have different tyrosine kinase activities, and so drive the pathways with varying force.

Ras/MAP kinase pathway: This is a ubiquitous pathway, which has been observed to respond to FGF in all cell types tested (reviewed in [102]). FGFR binds the docking protein FGF receptor substrate 2 (FRS2), which then assembles a signalling complex consisting of GAB1, Grb2 and the tyrosine phosphatase Shp2. This then activates the RAS/MAP kinase pathway. The MAP kinases involved include the ERK kinases, p38 and JNK kinases. These go on to regulate a variety of transcription factors, and so alter cell behaviour.

PI3 kinase/AKT/PKB pathway: This pathway is also based on the recruitment of the FRS signalling complex, and leads to the tyrosine phosphorylation of GAB1. This then recruits PI3K, leading to the activation of AKT. AKT acts on a number of pro-apoptotic target molecules.

PLCγ pathway: FGFR can bind and phosphorylate phospholipase Cγ (PLCγ) via its SH2 domain, leading to PLCγ activation. Activated PLCγ can then cleave phosphatidylinositol 4,5 bisphosphate into inositiol 1,4,5 trisphosphate (IP3) and diacylglycerol (DAG). IP3 causes the release of calcium from intracellular stores in the endoplasmic reticulum, while DAG activates protein kinase C (PKC) [100].

The very diverse range of functions of FGFs reflects in part their ability to engage a number of downstream intracellular pathways. Whilst it is over-simplistic to suggest that each pathway mediates a different function of FGF, it is possible to make some generalizations about the effects of each. For example, the PI-3/AKT pathway is generally thought to be anti-apoptotic, and so may mediate many of the survival effects of FGFs [102]. Different downstream components of the same general signalling pathway may also mediate different responses, and it has been suggested that within the Ras/MAPK pathway migration may be influenced primarily via the MAPK p38, while another MAPK known as ERK is likely to be responsible for proliferative effects [103]. It should be pointed out that these are merely generalisations, and many contradictory

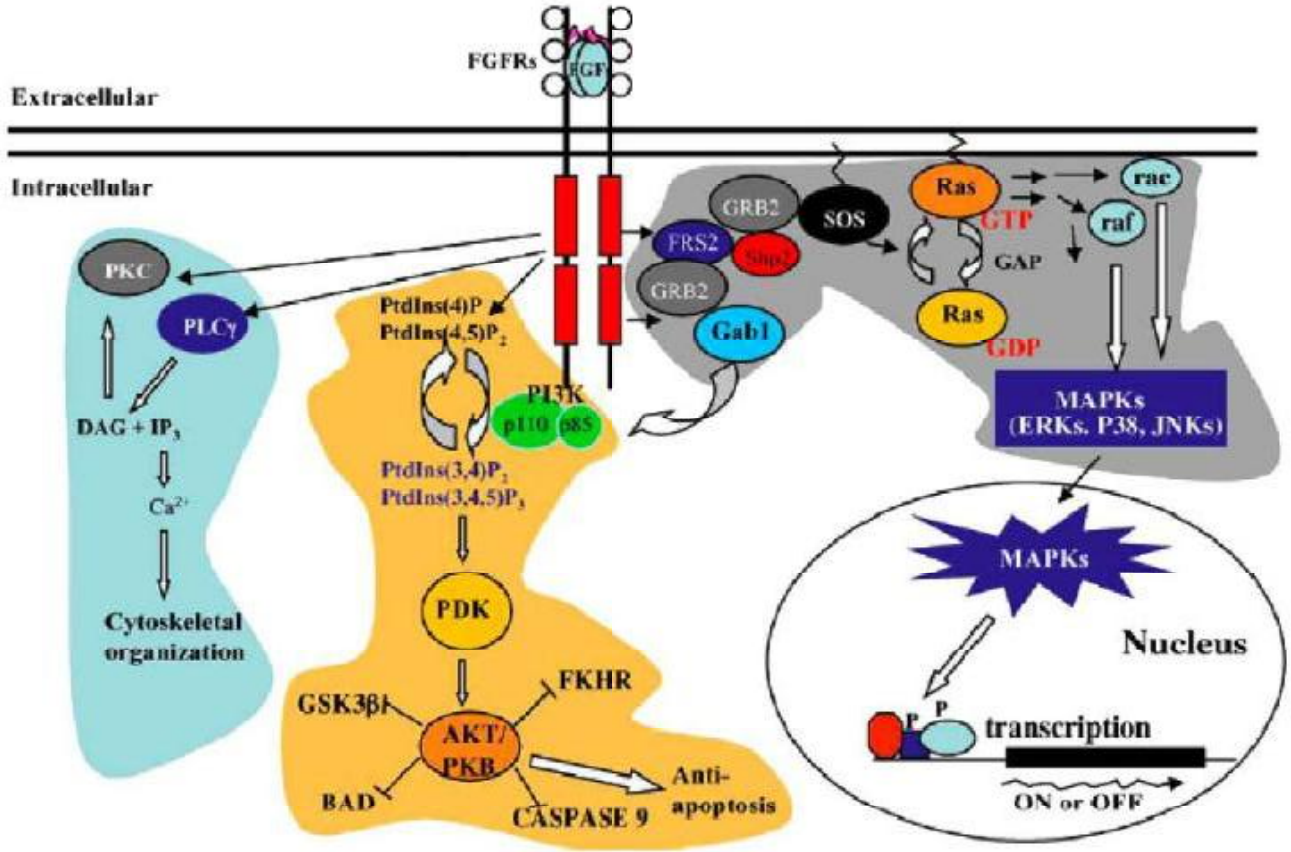


Figure 6.1

FGF signal transduction pathways.

This cartoon summarises the three main downstream pathways of the FGFR: the PLCγ pathway (blue), the PI3K/AKT/PKB pathway (yellow) and the Ras/MAPK pathway (grey).

Taken from Dailey et al [102].

results can be found. In reality, the effect of the different downstream pathways is likely to be highly cell-type specific, influenced by such factors as cell competence and interactions with other signalling pathways.

6.1.5 FGFs in the adult CNS:

Various FGFs are expressed in the adult CNS, the best studied being FGF-1 and FGF-2. An interesting difference between these two is that FGF-1 expression is generally limited only to neurons, whilst FGF-2 is also found in glia [100]. Both are found in a wide range of neural structures, although they are not ubiquitous. FGFR1,2 and 3 are also found in the adult CNS, although FGFR4 has only a very limited expression. FGFR-1 is mainly found in neurons, while FGFR-2 and 3 are primarily on glia.

FGFs, in particular FGF-2, are generally thought to have a mitogenic effect on adult neural stem cells. FGF-2 knockout mice have reduced proliferation due to a smaller pool of subventricular NSCs [104]. Infusion of FGF-2 into the lateral ventricle results in increased proliferation of the SVZ, as measured by BrdU incorporation [105]. The overall consensus seems to be that FGF-2 acts to maintain the stem cell population at the expense of more differentiated progenitors, promoting self-renewal rather than differentiation. In vitro, NSCs are routinely kept in FGF-2 containing medium, which appears to be essential for the maintenance of an undifferentiated state [106].

With respect to the hypothalamus, FGF-10 has recently been reported to be present in the ventral ependymal layer [20]. The authors also found expression of the IIIc isoforms of both FGFR1 and 2 in the hypothalamus, but not the IIIb isoforms. As FGF10 normally only signals via the IIIb receptor isoform, the significance of these results is not yet clear.

6.1.6 Hypotheses:

The extensive role of FGFs in mediating stem cell proliferation in other systems, together with the observation that both FGF10 and FGFRs are present in the adult hypothalamus suggests that FGF signalling may play a role in hypothalamic proliferation. I investigated this by testing the following three hypotheses:

1. That various components of the FGF pathway will be present in the adult hypothalamus, together with evidence of active FGF signalling.
2. That exogenous FGFs will be capable of inducing proliferation in hypothalamic NS/PCs in vivo.
3. That hypothalamic neurospheres will retain the components of the FGF signalling pathway, and will be influenced by active FGF signalling.

I investigated this by first researching more thoroughly what is already known about the distribution of FGFs and FGFRs in the hypothalamus, and secondly by investigating the role of FGF signalling both in vitro and in vivo. The in vivo analyses were performed as a collaborative project with D. McNay.

6.2 Results

6.2.1 Database research

It is known that there are at least 22 FGFs and 4 FGFRs in mice, plus receptor splice variants. Few antibodies are available, and it would be a costly and time-consuming process to personally construct probes and perform in situ hybridisations for all of them. As a more efficient option, I therefore first reviewed the known work in this area, using an online database. I chose to use the Allen Institute for Brain Science's Mouse Brain Atlas, which can be found online at http://mouse.brain-map.org [107, 108]. The atlas contains an in situ library of roughly 20 000 genes, performed on sections of 8 week old C67/BL6 mice, the same strain as used for my own research. The library was derived

using automated procedures to achieve the high throughput required by such a large project. Each gene is initially analysed on saggital sections, but the most significant are then repeated using coronal sections. This is important, as the wall of the third ventricle is a difficult structure to analyse in saggital view, and can even be missed entirely in some section series.

Table 6.1 summarises the results of this search. Of the 26 FGF and FGFR genes investigated, four were not included on the database (FGF-3, -11, -19 and -22), usually because the probe generation step had failed. The remaining 22 sets of pictures were examined for both expression in the ependymal lining of the third ventricle, in particular the tanycyte-containing region, and expression in other regions of the hypothalamus. The majority of genes were only available in saggital format, where the third ventricle is much harder to distinguish. In four cases it was not visible in any of the sections. I identified 3 FGFs (FGF-1, -2 and -7) and FGFR1 that appeared to be strongly expressed in non-ependymal regions of the hypothalamus. It is not possible to be sure in which specific cells types they were expressed, although it is generally thought that FGF-1 is expressed by neurons and FGF-2 by glia. Five FGFs (FGF-1, -2, -6, -10 and -18) and FGFR1 were possibly expressed in the ventricular lining (Figure 6.2). FGF1 was available as two different image series, in both saggital and coronal views. Depending on which set of images was examined, it appeared to be either entirely absent (data not shown) or strongly expressed in both the ventricular wall and many other cells scattered throughout the hypothalamus (Figure 6.2 A). This highlights the difficulty of interpreting this kind of data, in particular when trying to distinguish background from a true signal. Comparing the coronal and saggital views also highlighted how much easier it is to be confident of the location of positive signal when viewed coronally. Often only the ventral portion of the ventricular wall would be visible saggitally, and so only a coronal view could confirm whether a gene was expressed throughout the tanycytic zone.

Gene	In atlas?	Coronal / Saggital	Ependymal expression?	Other expression?	Details
FGF1	Yes	Both	Possible	Yes	Scattered cells throughout hypothalamus, possible ventral ependyma
FGF2	Yes	Saggital	Possible	Yes	Scattered cells throughout hypothalamus, possible ventral ependyma
FGF3	No				
FGF4	Yes	Saggital	No	No	
FGF5	Yes	Saggital	No	No	
FGF6	Yes	Saggital	Possible	No	
FGF7	Yes	Saggital	No	Yes	Rare scattered cells throughout hypothalamus
FGF8	Yes	Saggital	No	No	
FGF9	No				
FGF10	Yes	Saggital	Yes	No	Ventral ME epedyma
FGF11	No				
FGF12	Yes	Both	No	No	
FGF13	Yes	Both	No	No	
FGF14	Yes	Saggital	No	No	
FGF15	Yes	Both	No	No	
FGF16	Yes	Saggital	Not seen	No	
FGF17	Yes	Saggital	Not seen	No	
FGF18	Yes	Saggital	Yes	No	Ventral ME ependyma
FGF19	No				
FGF20	Yes	Saggital	Not seen	No	
FGF21	Yes	Saggital	Not seen	No	
FGF22	No				
FGFR1	Yes	Both	Yes	Yes	Beta tanycytes, VMH cells
FGFR2	Yes	Both	No	No	
FGFR3	Yes	Both	No	Yes	Scattered cells throughout hypothalamus
FGFR4	Yes	Both	No	No	

Table 6.1

FGF and FGFR expression in the hypothalamus

The Allen Brain Atlas was searched for information relating to FGFs and their receptors. Saggital sections did not always contain identifiable ependyma – in this case expression is marked as 'not seen'.

Figure 6.2

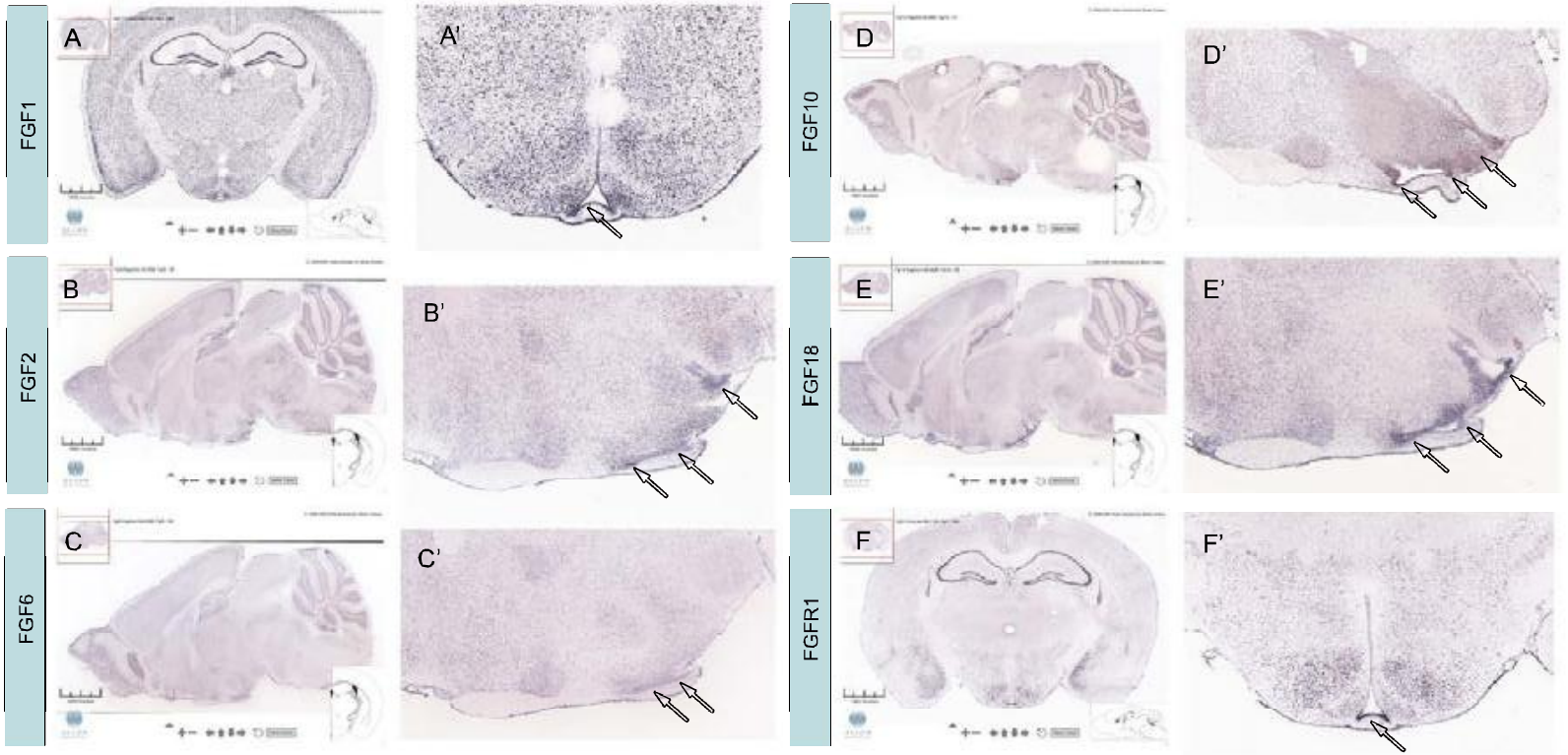
Summary of positive results from the Allen Brain Atlas database search

Summary of the six FGF / FGFR genes judged to be present in the ependymal lining of the third ventricle.

A: FGF1, B: FGF2, C: FGF6, D: FGF10, E: FGF18, F:FGFR1

Black arrows on the enlarged pictures indicate the presence of positive ependymal tissue.

Scale bars indicate approximately 1350μm on coronal sections (A and F) and 1900μm on saggital sections (B-E).


FGF1
A
A'
FGF2
B
B'
FGF6
C
C'
FGF10
D
D'
FGF18
E
E'
FGFR1
F
F'

6.2.2 FGF / FGFR in situ hybridizations

The database search gave me a good starting point for examining the presence of FGFs and their receptors in the adult mouse hypothalamus, but as noted it is not entirely reliable. In addition, it would be helpful to have the data on coronal sections as far as possible, in order to relate any zones of expression to other markers or neurosphere-forming capacity. With this in mind, I gathered probes for the FGF and FGFR genes, focusing on the ones identified in the database search.

FGFR1 could be successfully detected in the hypothalamus (Figure 6.3). As seen in the Allen Database, it was expressed mainly in the ventral ependymal layer at the level of the ME, and in the VMH. This ependymal region corresponds approximately to the location of β tanycytes. More dorsally, regions corresponding to α tanycytes were negative for FGFR1 mRNA. It should be noted that my probes were not designed to differentiate between different isoforms of the same subtype, and so the likely ligands for this receptor cannot be determined.

I also obtained probes for FGF-2, -10 and -18, three of the ligands identified previously. To date, I have not had the opportunity to analyse FGF-1 or -6. No evidence of the presence of FGF-2 was found, although in situ hybridization is more difficult on adult than embryonic tissue, so a negative result should not be considered definitive.

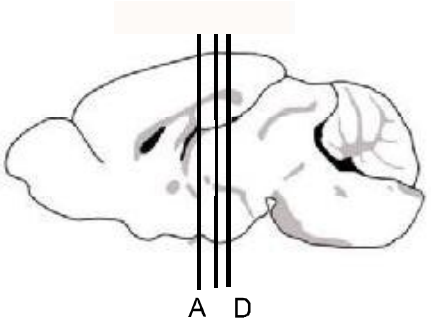
With regard to FGF-10, my in situ results corroborate previous reports [20], and demonstrate that FGF-10 expression in the hypothalamus is restricted to the region of the ventral ependyma at the ME/posterior level (Figure 6.4). From the morphology of the ventricle, I was able to estimate that it is present only in the region corresponding to β1, β2, and ventral α2 tanycytes. As well as being clearly present in tanycytes, it is interesting to note that FGF-10 mRNA is also seen just lateral to the ependymal lining in some places, which may be an indication that it is also expressed in adjacent cells (Figure 6.4 J, K).

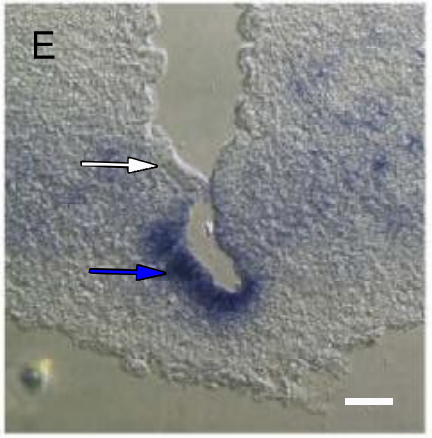
Figure 6.3

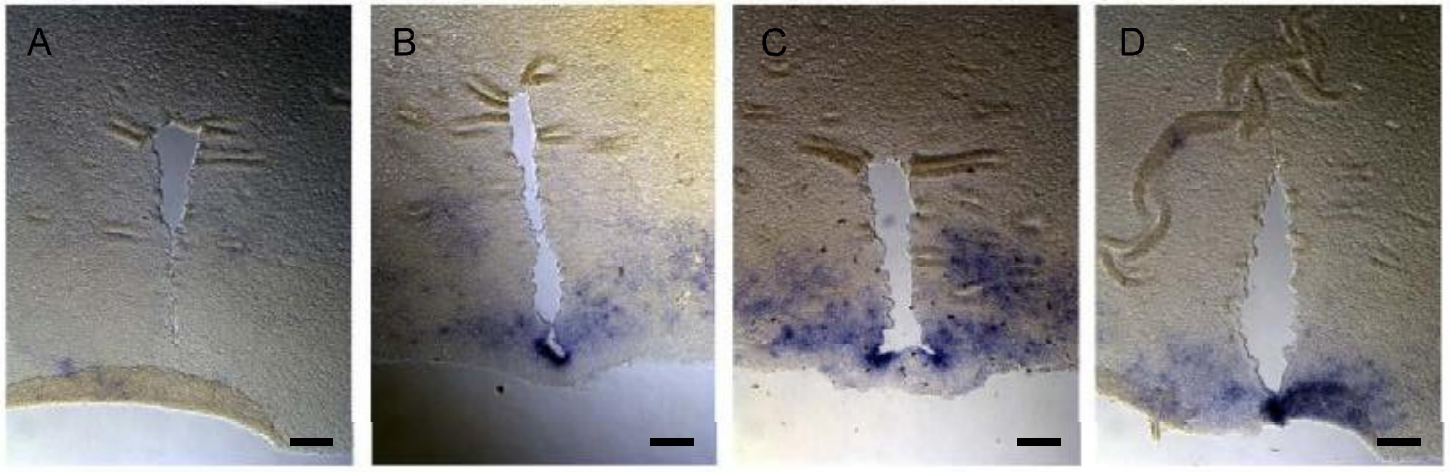
Expression of FGFR1 mRNA in the adult mouse hypothalamus

A-D: Expression of FGFR1 mRNA in the anterior (A), ME (B,C) and posterior (D) hypothalamus. Scale bars = 100μm.

E: High magnification of the section illustrated in B. FGFR1 mRNA is strongly expressed in the ventral-most ependymal layer (blue arrow) where the β tanycytes are found, but not more dorsally in the α tanycyte region (white arrow). Scale bar = 50μm.


A
D


E


A
B
C
D
Anterior
Median Eminance
Posterior

My results clearly demonstrate that FGF-18 is also expressed in the adult mouse hypothalamus, in a highly restricted pattern (Figure 6.5). It is only found within the ependymal lining of the third ventricle at the ME/posterior level, in a restricted D/V zone. Analysis of the morphology of the third ventricle indicates that this region is at the level of ventral $\alpha 2$ tanycytes (Figure 6.5 I). Both the β tanycytes ventrally, and the dorsal $\alpha 2$ / $\alpha 1$ tanycytes dorsally are negative. From high magnification pictures, it is clear that FGF-18 mRNA is present only in tanycytes, and not cells located outside of the ependymal layer. The mRNA consistently displayed an apical subcellular distribution, which may be an indication that the protein expression is also restricted to this domain. FGF-18 was not observed elsewhere in the brain, except for possible low levels in the choroid plexus, and was not seen in either the SVZ or DG (data not shown).

6.2.3 Other evidence of FGF signalling in the hypothalamus

The above analyses demonstrate that FGF ligands and receptors are located in the adult hypothalamus. However, this does not necessarily indicate that all the downstream machinery to translate the FGF signal is also present, or that it can be activated under normal conditions. As stated in the introduction, FGFs have diverse downstream signalling cascades, and it is not feasible to check for the presence of all the relevant factors. It is however possible to use antibodies specific for the activated forms of a downstream effector to give an indication that a pathway is active.

Mitogen activated protein kinases (MAPKs) comprise one of the major FGF signal transduction pathways. They are not exclusively activated by FGFs – many other tyrosine kinase receptors can also activate these pathways, including (but not limited to) those for EGF, CNTF and insulin. There are four main MAPK pathways [109]. Of these, the Jun amino terminal kinases (Jnk1/2/3) and p38 pathways are predominantly activated by stress-induced factors, and are often implicated in the initiation of apoptosis. The less well-characterised Erk5 pathway can be activated by both stress-induced factors and growth factors, and influences a variety of cellular behaviours. However, the main

Figure 6.4

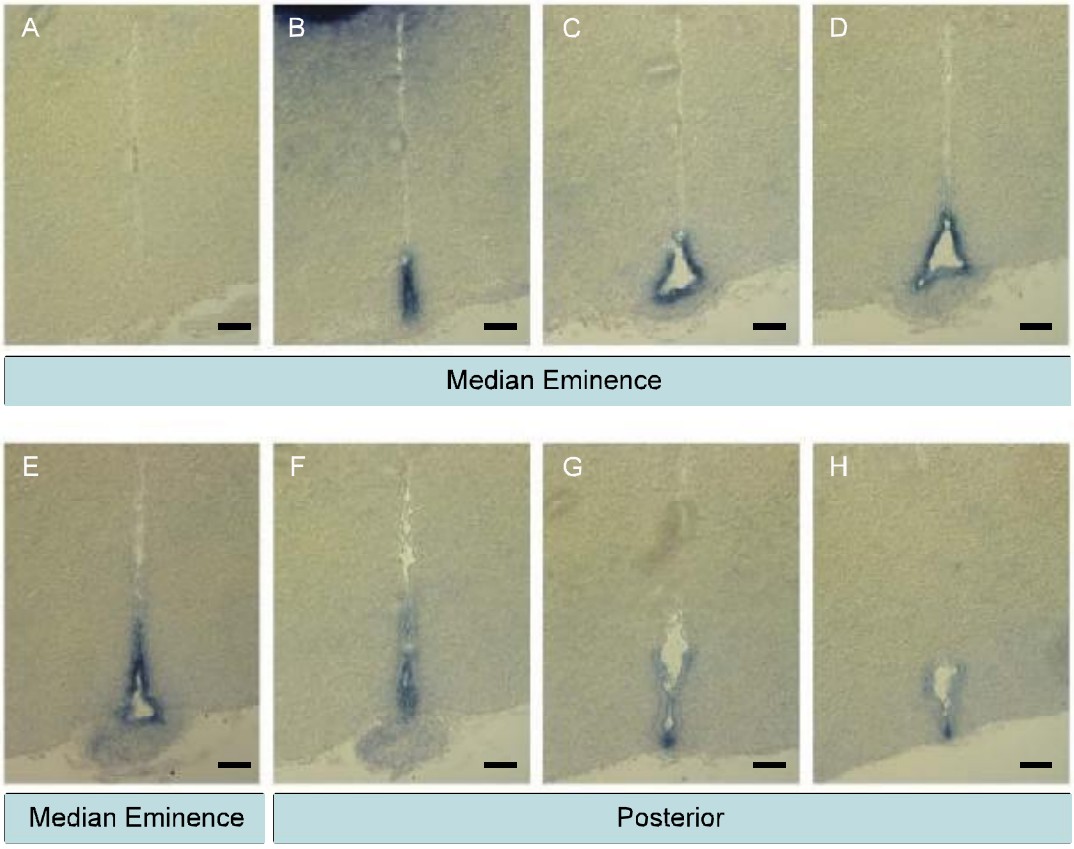
Distribution of FGF10 mRNA in the hypothalamus

A-E: Location of cells expressing FGF-10 in the ME level hypothalamus. FGF-10^{+} cells are seen throughout the ventral third of the ependymal lining of the third ventricle. Scale bars = 100μm.

F-H: Location of cells expressing FGF-10 in the posterior hypothalamus. The pattern is similar to that in the ME. In far posterior sections (G, H) strong expression is seen only in the most ventral cells, with cells in the middle region appearing to have lower levels of mRNA. Scale bars = 100μm.

I: High power magnification of C. The morphology of the ventricle can be used to determine which tanycyte subtypes are expressing FGF-10. Ventral α2 tanycytes, located on the upper lateral recess of the ventricle, and β tanycytes on the ventricle floor, clearly contain FGF-10 mRNA. Dorsal α2 tanycytes do not produce FGF-10. Scale bars = 50μm.

J, K: High power magnifications of FGF-10 containing tanycytes from two different regions. The green dotted lines mark the apical surface of the cells on the right hand side, and the black dotted lines mark their basal surfaces. The mRNA has a primarily apical subcellular distribution in tanycytes (white asterisks), although it is also found at the lateral edges of each cell (arrowheads). In some areas there appears to be expression located just outside the ependymal layer, that may come from separate adjacent cells (black asterisks).


A
B
C
D
Median Eminence
E
F
G
H
Median Eminence
Posterior

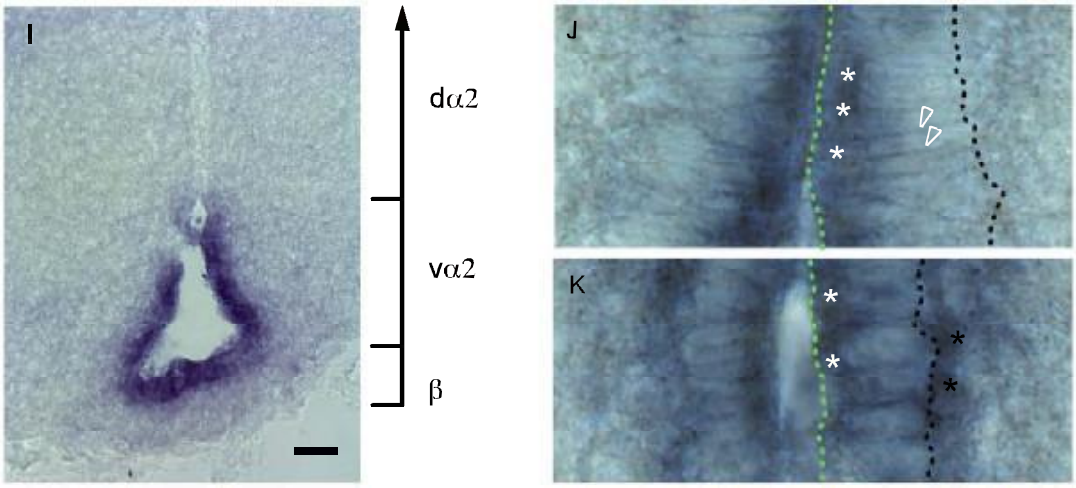

I
dα2
vα2
β
J
K

Figure 6.5

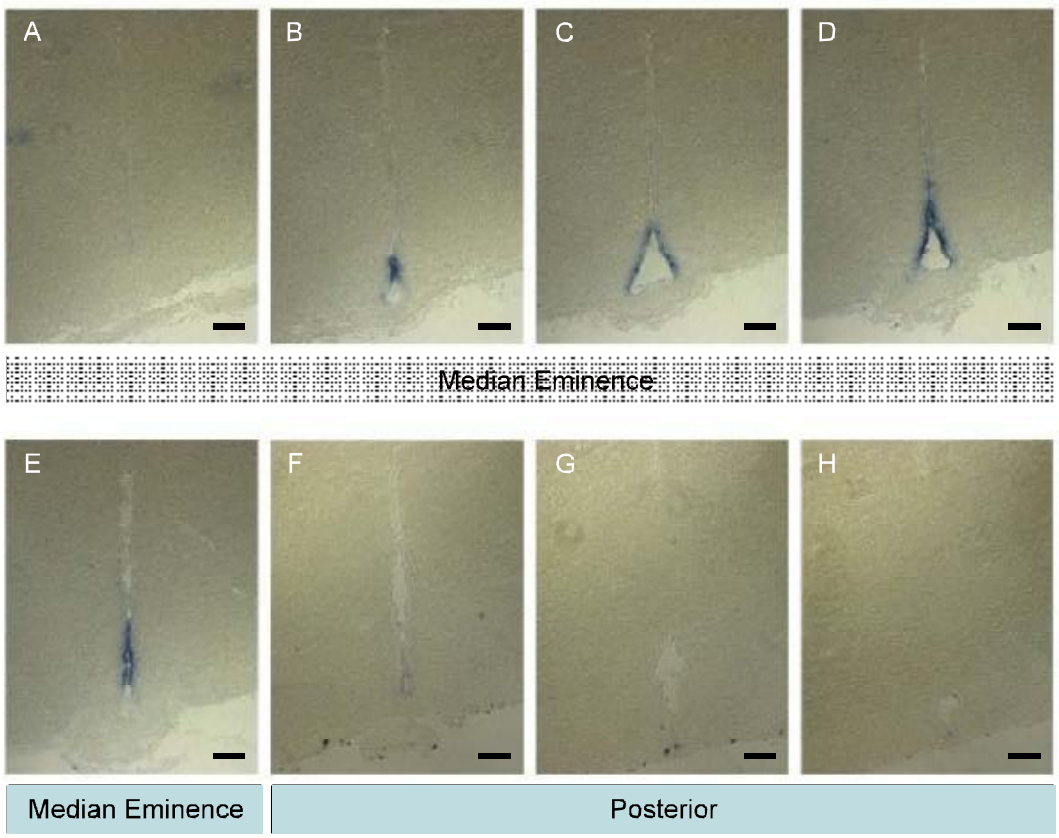
Distribution of FGF18 mRNA in the hypothalamus

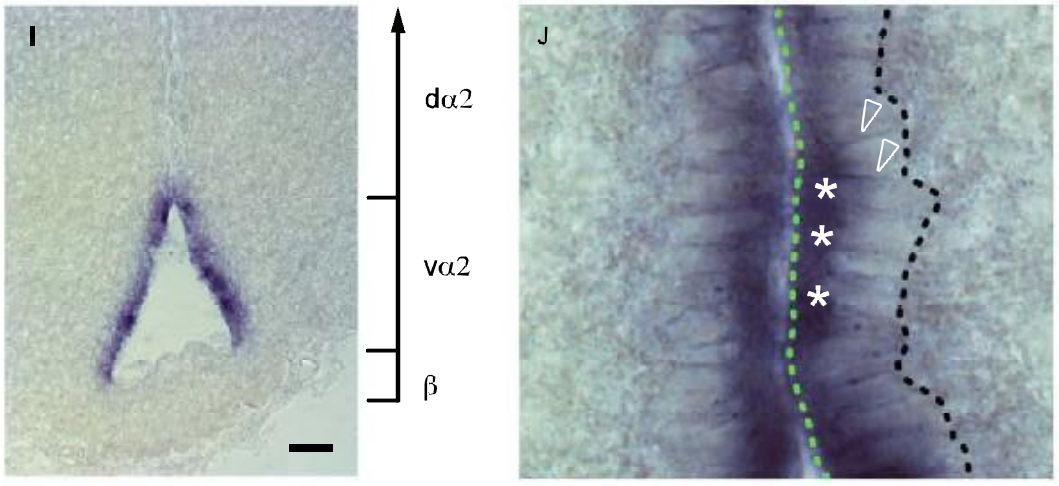
A-E: Location of cells expressing FGF-18 in the ME level hypothalamus. FGF-18^{+} cells are seen only in the ventral third of the ependymal lining of the third ventricle, although cells on the floor of the ventricle are also negative. Scale bars = 100μm.

F-H: Location of cells expressing FGF-18 in the posterior hypothalamus. The pattern is similar to that in the ME, except the signal appears to be weaker. In far posterior sections (G, H) the expression is shifted to the most ventral cells only. Scale bars = 100μm.

I: High power magnification of C. The morphology of the ventricle can be used to determine which tanycyte subtypes are expressing FGF-18. β tanycytes on the ventricle floor do not produce FGF-18, nor do dorsal α2 tanycytes. Ventral α2 tanyctes, located on the upper lateral recess of the ventricle, can be clearly seen to contain FGF-18 mRNA. Scale bars = 50μm.

J: High power magnification of FGF-18 containing tanycytes. The green dotted line marks the apical surface of the cells on the right hand side, and the black dotted line marks their basal surfaces. FGF-18 is only seen within this ependymal layer, and is not present in more lateral cells. The mRNA has a primarily apical subcellular distribution (asterisks), although it is also found at the lateral edges of each cell (arrowheads).


A
B
C
D
Median Eminence
E
F
G
H
Median Eminence
Posterior


I
dα2
vα2
β
J

MAPK pathway known to be activated by growth factor signalling is the Erk1/2 pathway. This pathway is involved in a number of processes relevant to stem/progenitor cells, including proliferation, migration and differentiation. In order to investigate the status of this pathway in vivo, I labelled sections of hypothalamus with an antibody that recognizes only the phosphorylated form of Erk1/2 (pErk1/2), thus acting as a readout for the activation level of the Erk1/2 MAPK kinase pathway.

The results are summarised in Figure 6.6. Cells with a low basal level of pErk1/2 are seen throughout the hypothalamus, particularly in regions near the brain's ventral surface (Figure 6.6 A-G). Stronger expression is seen in the optic chiasm (Figure 6.6 C, D) and the ME (Figure 6.6 E, F), where it is presumed to be the result of expression in axons traversing the region as there are few local cell bodies. Generally speaking, expression in tanycytes seems to be present at a low level, most noticeably in β and vα2 tanycyte populations (Figure 6.6 E-G).

In addition to these broad populations expressing pErk1/2 at a low level, individual cells with much higher expression levels could be detected. Anteriorly, cells presumed to be magnocellular neurons could be identified in the PVH (Figure 6.6 C). In addition, a population of highly pErk1/2^{+} ependymal cells was observed, with processes extending into the hypothalamus (Figure 6.4 A). Although this morphology is strongly reminiscent of tanycytes, these cells are positioned far to the anterior of the tanycyte population, and their identity is not known. Sections located at the level at the ME/posterior level also contained a number of strongly positive cells (Figure 6.6 E-H and indicated by solid arrowheads in I). The majority of these were located within about 200μm of the third ventricle. More significantly for this study, occasional cells displaying increased levels of pErk1/2 are also seen within the tanycyte-containing region of the hypothalamus (Figure 6.6 E-I). In particular, a single cell in the posterior ventricular wall with exceptionally high levels of pErk1/2 was observed (Figure 6.6 G and arrow/arrowheads in I). The cell body could be clearly seen located in the ependymal layer, with a long process extending into the parenchyma.

Figure 6.6

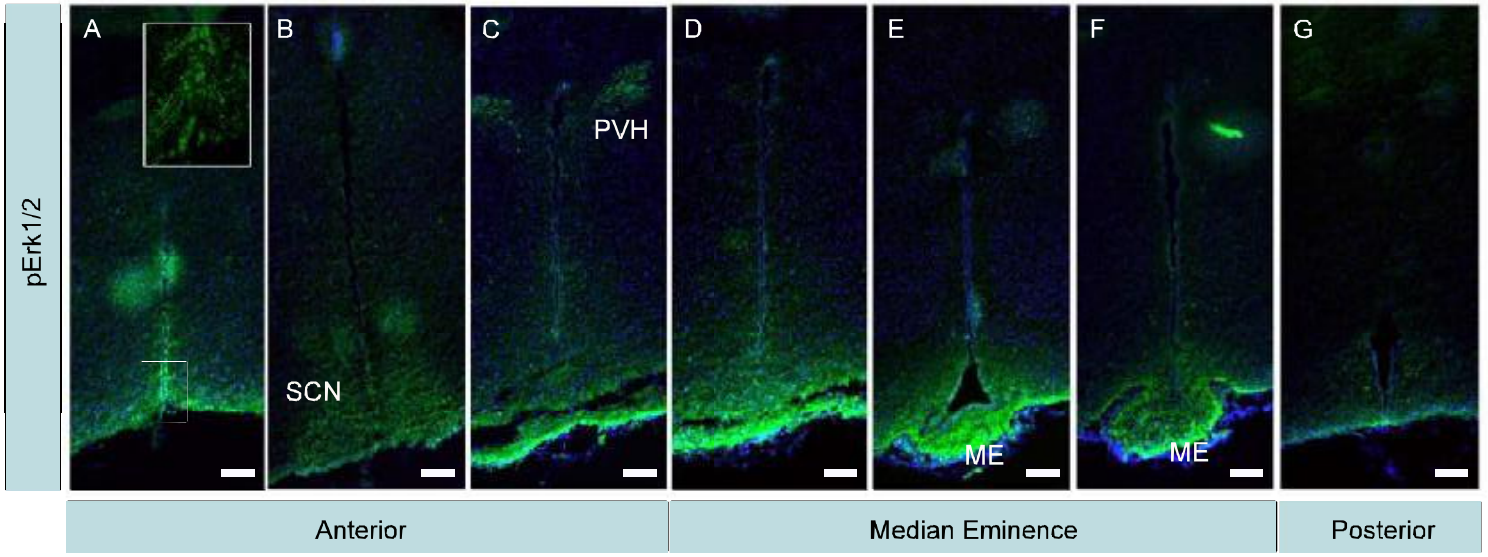
Phosphorylated Erk1/2 MAP Kinase expression in the hypothalamus

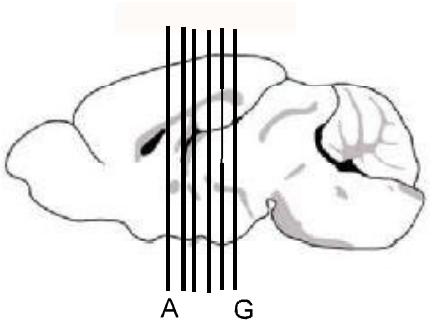
A-C: pErk1/2 in the anterior hypothalamus. Expression was seen in the most anterior ependymal cells (A, and magnified in inset), which had a cell body located in the ependyma and a process extending into the parenchyma (arrowhead). Expression was also in some large cells of the PVH (C). In addition, there appeared to be some diffuse ventral labeling, particularly in the SCN (B). Scale bars = 100μm.

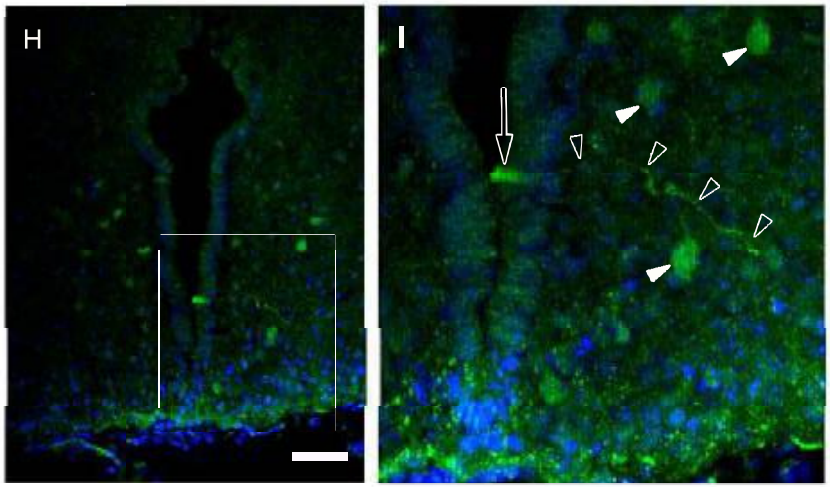
D-G: pErk1/2 in the ME and posterior level hypothalamus. Expression was generally seen in ventral regions, and was particularly strong in the ME (E, F). In addition, stronger pErk1/2 was seen in some individual scattered cells. Scale bars = 100μm.

H: Magnification of section G, using grid-based optical sectioning. The pattern of pErk1/2 could be most clearly seen in posterior regions. pErk1/2$^+$ cells were observed in both the ventricular lining and the parenchyma. Scale bar = 50μm.

I: High power magnification of the white box in G. A highly pErk1/2$^+$ cell can be seen, with its cell body in the ependymal wall (arrow) and a long process extending into the hypothalamus (arrowheads). This morphology combined with its location indicates that the cell is a tanycyte. The surrounding tanycytes appear to be only weakly positive for pErk1/2. Additional pErk1/2$^+$ cells not located in the ventricular wall can also be observed (solid arrowheads).


pErk1/2
A
B
C
D
E
F
G
PVH
SCN
ME
ME
Anterior
Median Eminence
Posterior


A
G


H
I

This morphology together with the location indicates it is a tanycyte, lying within the vα2 territory.

6.2.4 In vivo FGF-2 infusions

In order to assess whether FGFs were capable of promoting proliferation in vivo, I next set up infusion experiments. This work was done in collaboration with D McNay, and was largely carried out by him in the Division of Endocrinology, Diabetes, & Metabolism, Harvard Medical School, Boston. Mice were given a continuous infusion of PBS (control) or FGF-2 directly into one of the lateral ventricles, together with BrdU to label proliferating cells. After a week, the mice were sacrificed and the distribution of newborn cells was examined.

The results of this first experiment are summarised in Figure 6.7. Control mice (n=2) displayed some background proliferation, demonstrating that new cells are generated even in the absence of exogenous stimulating factors. The majority of $BrdU^+$ cells were found scattered throughout the parenchyma of the hypothalamus (Figure 6.7 A, B), and a small minority were detected in the ependymal layer. It is interesting to note that these scattered newborn cells were only observed in the hypothalamic region (plus other areas known to be neurogenic such as the DG and the SVZ), and not throughout the entire CNS, indicating that this is not a global phenomenon (data not shown). Within the hypothalamus, there was no obvious A/P or D/V bias.

In contrast, the FGF-treated mouse displayed a clear upregulation in proliferation at or near the ependymal layer (Figure 6.7 C, D), specifically at the level of the ME. Proliferative cells appeared to be clustered, perhaps relating to different cell types found at different points on the D/V axis. The largest of the clusters could be described as a ventral cluster, and was located in the lateral recess of the third ventricle at the approximate level of vα2 tanycytes. This was clearly visible at both mid and posterior levels of the ME. The other clusters were a little more diffuse, and located further dorsally, in regions thought to be occupied by α1 tanycytes and ependymocytes. Conversely to the ependymal

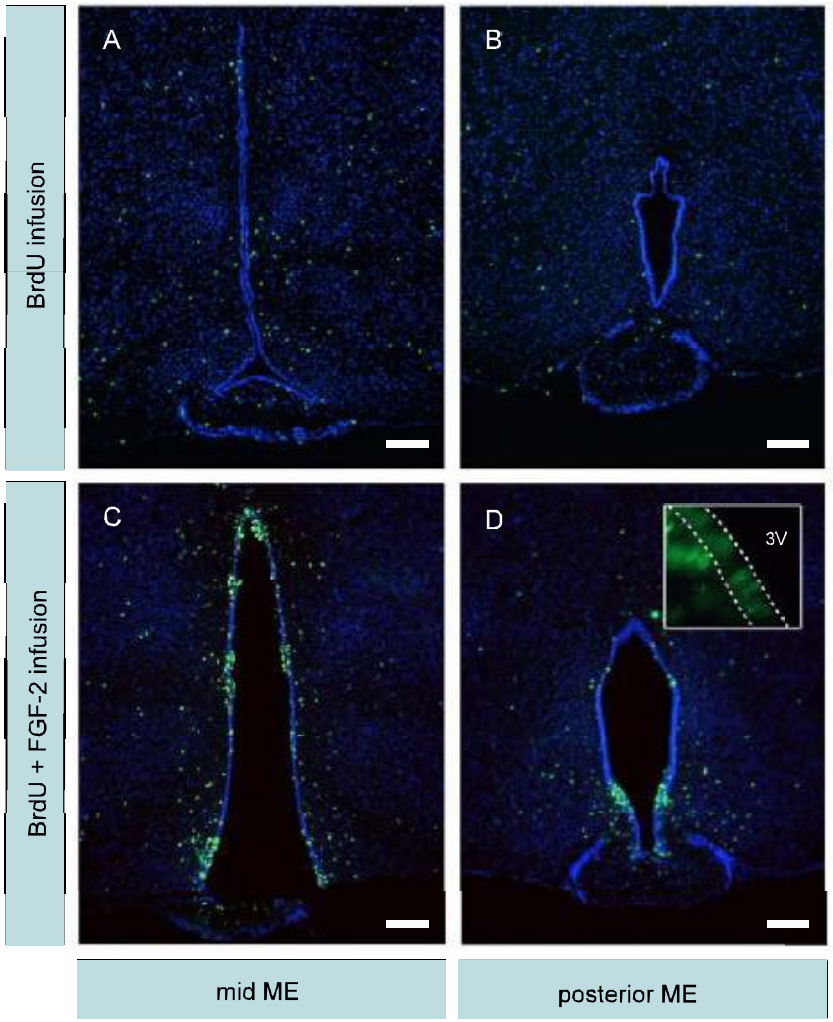
Figure 6.7

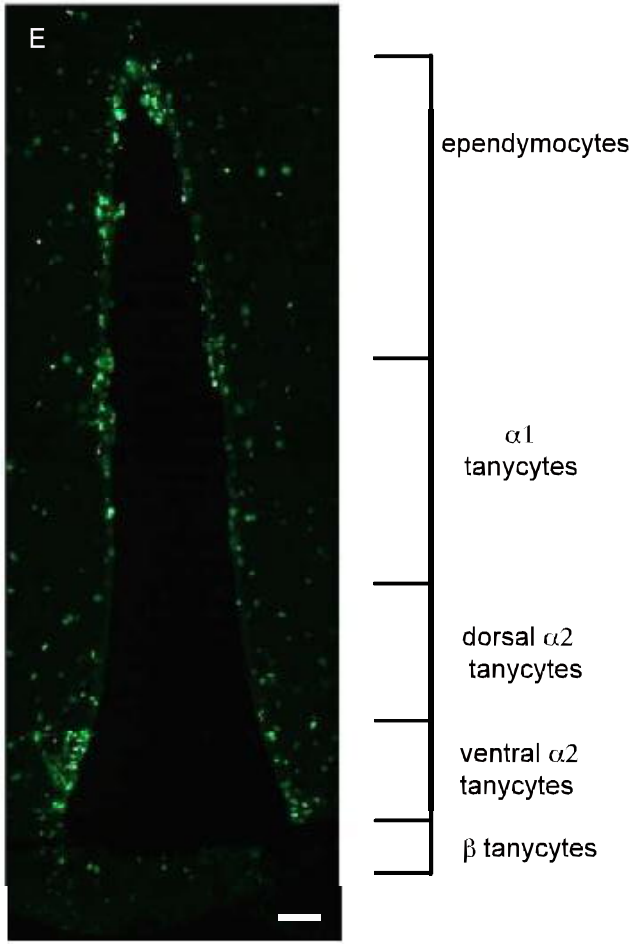
Effect of FGF-2 infusion on proliferation in vivo

A, B: BrdU labeled sections of the mid (A) and posterior (B) ME region of mice infused with BrdU only for 1 week. Scale bars = 100μm.

C, D: BrdU labeled sections of the mid (C) and posterior (D) ME region of mice infused with BrdU plus FGF-2 for 1 week. D inset is an enlargement showing that $BrdU^+$ cells can be found both within and adjacent to the ependymal layer (dotted white lines). Scale bars = 100μm.

E: High power magnification of the section shown in C. The approximate locations of tanycyte subtypes are shown alongside. Scale bar = 50μm.


A
B
C
D
3V
BrdU infusion
BrdU + FGF-2 infusion
mid ME
posterior ME


E
ependymocytes
α1
tanycytes
dorsal α2
tanycytes
ventral α2
tanycytes
β tanycytes

proliferation, parenchymal proliferation was not upregulated in the FGF-2 treated mouse, in fact it appeared slightly reduced.

This experiment was later repeated, using at least five further mice each for control and FGF-treated conditions (work performed by D. McNay and M. Kokoeva). Two more conditions were also examined, in which mice were treated with CNTF, and FGF-2 + CNTF. The results upheld the patterns described above, and strengthened many of the observations (Figure 6.8). It was clear that BrdU uptake in response to infused FGF-2 was greatest in the ventral cluster of cells (Figure 6.8 F-J). Mid/dorsal level ependymal proliferation was also consistently seen, although it generally involved a much smaller number of cells. The ventral cluster was confirmed as being present in the region harbouring vα2 tanycytes; when sections were double labelled with GFAP antibody, the BrdU was found in the $GFAP^-$ region just ventral to the strongly $GFAP^+$ dα2 tanycytes (Figure 6.8 U). It was also clear that the parenchymal proliferation detected in control mice (Figure 6.8 A-E) was indeed reduced in FGF-2 treated mice (Figure 6.8 F-J).

The pattern of proliferation induced by CNTF matched that described in previous publications [62, 64], and was clearly very different from FGF-induced proliferation. Instead of being focused at the ependymal layer, newborn cells were scattered throughout the parenchyma (Figure 6.8 K-O). The pattern seemed much more comparable to that of the constitutive proliferation seen without growth factors (Figure 6.8 A-E), but with higher numbers of cells involved. Interestingly, this parenchymal proliferation was not seen in mice infused with both FGF-2 and CNTF (Figure 6.8 P-T). The pattern in these mice mirrored that of the FGF-2 only mice, with increased ependymal proliferation, particularly in ventral regions. It is not possible to be sure without greater numbers of mice, but there appears to be a trend for a greater number of newborn cells in these two regions in the CNTF + FGF-2 mice, relative to mice given only FGF-2.

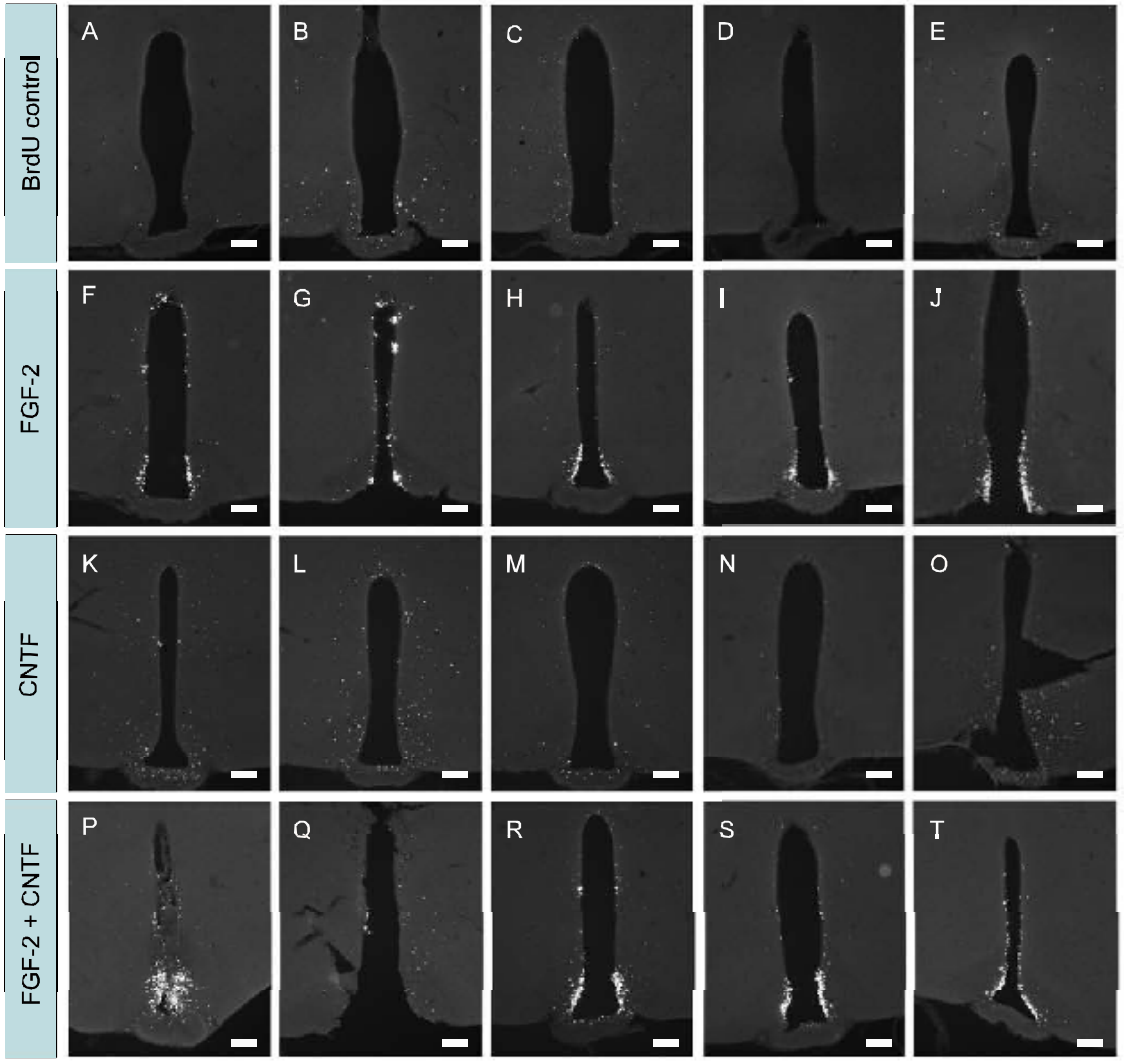
Figure 6.8

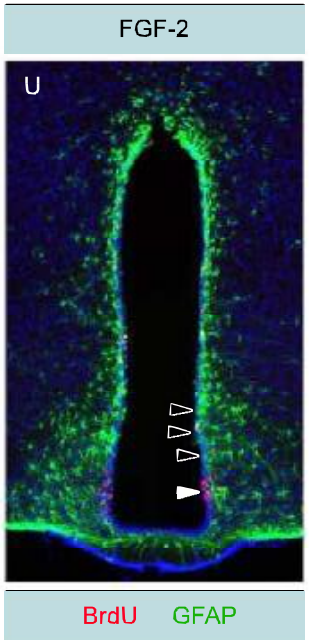
Proliferation in response to FGF-2 and CNTF in vivo

A-T: BrdU labelling in the ME-level hypothalamus of mice infused with growth factors and BrdU. Repeat experiments using five different mice are shown for each condition. A-E: BrdU only (controls). F-J: FGF-2 infusion. K-O: CNTF infusion. P-T: FGF-2 and CNTF joint infusion. Scale bars = 100μm.

U: BrdU/GFAP double labelling in an FGF-2 infused mouse. The ventral cluster of $BrdU^+$ cells (solid arrowhead) is located below the region of high GFAP that marks dα2 tanycytes (outlined arrowheads), and is instead found in the region containing $GFAP^-$ vα2 tanycytes.

These images were produced by D. McNay and M. Kokoeva.


BrdU control
FGF-2
CNTF
FGF-2 + CNTF
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T


FGF-2
U
BrdU GFAP

6.2.5 FGF signalling in neurospheres

The in vivo experiments detailed above clearly demonstrate that the addition of exogenous FGF-2 is sufficient to induce proliferation in cells which I have previously shown to exhibit NS/PC-like properties. In order to determine whether FGF signalling is also necessary for proliferation, I returned to the neurosphere culture. Neurospheres are typically cultured in medium containing both FGF-2 and EGF. This makes the assay capable of selecting FGF or EGF responsive cells, but does not guarantee that either growth factor is required.

I first determined whether active FGF signalling is likely to be occurring in neurospheres, by labelling them with an antibody to pErk1/2. This was found to be strongly expressed in both primary and passaged neurospheres (Figure 6.9 F, G).

In order to investigate the requirement for FGF and EGF signalling further, I studied the effects of omitting one or both growth factors from the neurosphere medium. I set up primary neurosphere cultures in medium containing both growth factors, FGF-2 or EGF alone, or neither. All four of these conditions produced spheres, but with clear differences in both their number and size (Figure 6.9 A-E). The highest number of spheres were produced in medium containing both growth factors (127.9±3.8 spheres per well), followed by FGF-2 (107.1±3.6) and EGF (76.5±6.1). The largest spheres were also produced using both factors (Figure 6.9 B), with spheres from either factor alone appearing to be smaller (Figure 6.9 C, D), although this effect was not quantified. Although a few spheres were technically produced in wells with no added growth factors, they were extremely small (Figure 6.9 E), and were insufficient for passaging.

On passaging, the effect of omitting one or other of the growth factors was even more pronounced (Figure 6.9 A). Neurospheres grown in the presence of FGF-2 +EGF were kept for five passages, by which time they had reached a steady rate of approximately 30 spheres per well on each passage. Spheres kept in FGF-2 alone produced only a very low number of secondary spheres (8.4±0.9), which failed to regenerate into tertiary spheres when passaged again. Those grown in

Figure 6.9

Requirement for FGFs in hypothalamic neurosphere cultures

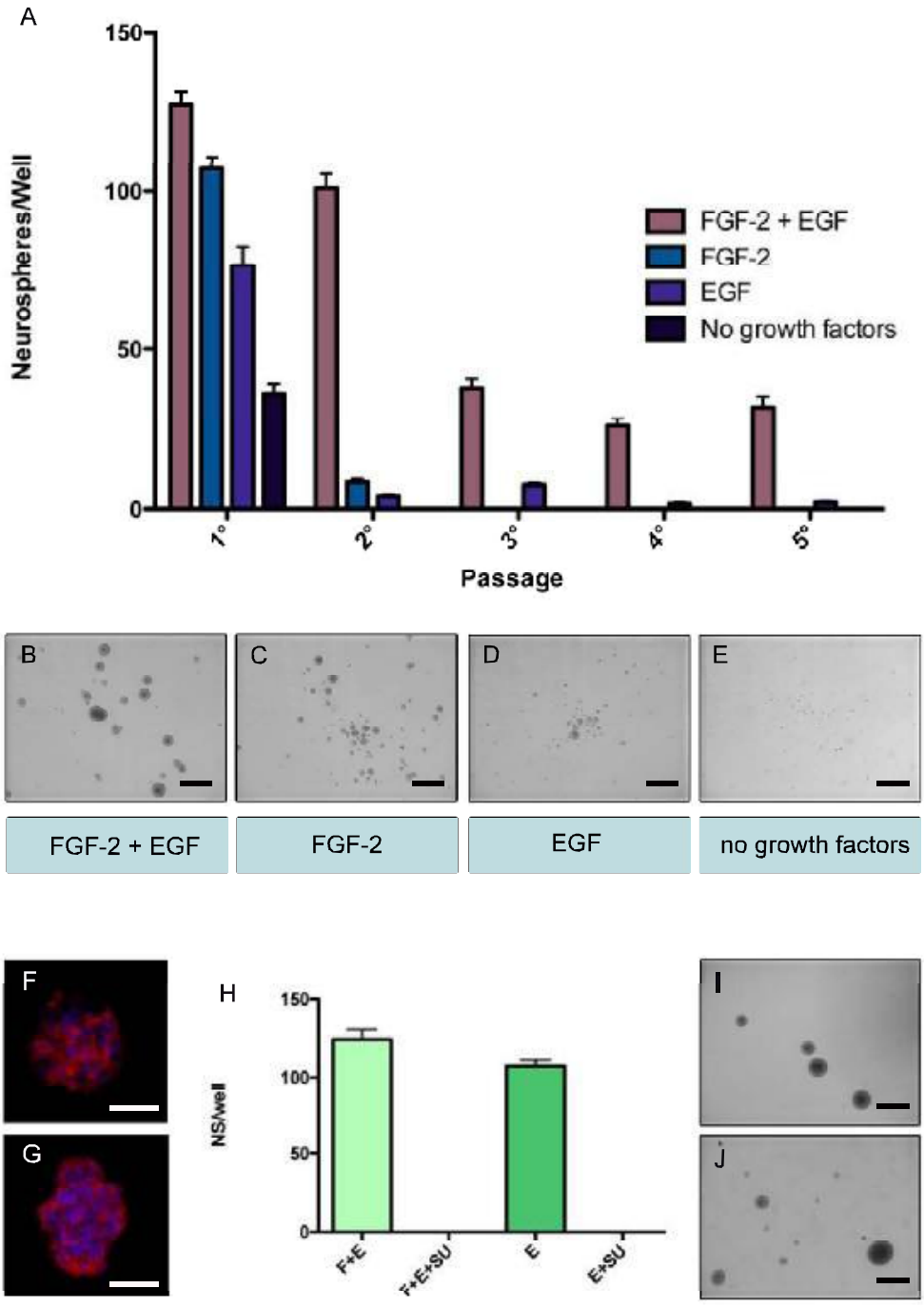
A: Primary neurospheres were grown in the presence and absence of FGF-2 and EGF, and the number of neurospheres generated per well was counted. The graph shows a single representative experiment (n=3).

B-E: Pictures comparing the size and density of primary neurospheres grown in FGF-2+EGF (B), FGF-2 only (C), EGF only (D) or without additional growth factors (E). Scale bars = 500μm.

F, G: Primary neurospheres grown in FGF-2+EGF expressed pErk1/2, a marker of active growth factor signaling (F). pErk1/2 is maintained in passaged (tertiary) spheres (G). Scale bars = 50μm.

H: SU5402 was added to primary neurospheres grown in either FGF-2+EGF or EGF alone. In both cases the FGF inhibitor prevented neurosphere formation.

I, J: The hypothalamic cells were spun out of the FGF+EGF+SU culture after 10 days, and replated in fresh FGF+EGF medium without FGF inhibitor. After 10 days, new neurospheres were observed (I), of a comparable size to secondary neurospheres never exposed to FGF inhibition (J). Scale bars = 500μm.


A
150
100
50
0
Neurospheres/Well
FGF-2 + EGF
FGF-2
EGF
No growth factors
1°
2°
3°
4°
5°
Passage
B
C
D
E
FGF-2 + EGF
FGF-2
EGF
no growth factors
F
G
H
150
100
50
0
NS/well
F+E
F+E+SU
E
E+SU
I
J

EGF alone dramatically reduced in number when passaged (4.0±0.5 secondary spheres per well), although they could be maintained at these low numbers for the full five passages.

It is clear from these results that FGF signalling plays an important role in neurosphere formation. However the observation that neurospheres can be passaged over an extended period of time, albeit in very low numbers, in EGF alone indicates that sphere formation is not entirely reliant on the provision of exogenous FGFs. There are a number of possible explanations for this phenomenon:

- Hypothalamic neurosphere-forming cells may be a mixed population, the majority FGF-dependent and the minority FGF-independent.
- There may be some redundancy between the actions of FGF and EGF in neurosphere-forming cells, due to overlap of their downstream pathways. This would result in them having a cumulative effect with each other.
- FGF may play a non-essential role. Cells may be able to proliferate at a low level without the influence of FGF signalling; however it may still act to increase the frequency or speed of cell divisions when present.
- FGF may play some role in neurosphere-forming cells other than that of a mitogen, for example acting to increase the chance that cells will survive.
- FGF may be necessary for survival or proliferation, but be produced at low levels by the neurospheres themselves.

A simple way to rule out some of these options is to investigate the effect of abolishing all FGF signalling in the spheres, using a chemical inhibitor. SU5042, an inhibitor that acts at the level of FGF receptors to block all downstream signalling cascades, is ideal for this. It is not thought to interfere with EGF signalling, or any other growth factor pathways [110].

Neurospheres in either FGF-2+EGF or EGF alone were derived with or without the addition of SU5402. Whether or not exogenous FGF-2 was present, the inhibitor completely prevented all neurosphere formation (Figure 9.6 H). This unequivocally demonstrates that FGF signalling is required for neurosphere formation. In order to rule out a non-specific toxic effect of the SU5402, the unproliferated cells were spun out of the FGF-2+EGF+SU5402 medium after ten days, and replated without the inhibitor. After a further ten days, a number of neurospheres formed (Figure 6.9 I), of an equivalent size to secondary spheres never exposed to FGF inhibitor (Figure 6.9 J).

6.3 Discussion

It is clear from the FGF inhibitor experiments detailed above that FGF signalling is required for neurosphere formation (Figure 6.9 H). This rules out the theories that FGF signalling may be redundant with signalling from another growth factor, that it may play a non-essential role, or that a small population of FGF-independent proliferative cells is present. Instead, the difference between the neurosphere forming capacity of cells grown simply without the provision of exogenous FGF-2 and those in which FGF signalling was inhibited gives credence to the idea that FGF may be produced by the neurospheres themselves. It seems likely that the FGF signalling in this scenario would be cell-autonomous, as a non-autonomous mechanism would be expected to produce a long lag before neurospheres started to appear, relative to cultures already containing FGF-2. This would be due to the low starting density of cells, which would require a long time to secrete enough FGF into the medium to produce the first cell divisions. The difference between neurospheres cultured with and without exogenous FGF (but in the presence of EGF) suggests that the neurospheres can secrete only limited amounts of FGF, as they cannot fully compensate for its absence.

Although the experiments above do not directly address the question of what function FGF plays in neurosphere-forming cells, there are some clues. If the absence of signalling from a particular factor results in a total loss of neurosphere formation, as in this case, it is likely that that factor was either required for cell survival, or for proliferation. The absence of a cell survival factor would result in the death of neurosphere-forming cells before they could begin proliferation, whilst the loss of a proliferation factor would result in quiescence, where the original cells would remain in single cell suspension without expanding. The fact that primary cells could be incubated for ten days in the presence of SU5402 before going on to form spheres in fresh medium strongly suggests that FGF signalling is not necessary for their survival (Figure 6.9 I). Thus it is highly likely that FGFs influence neurosphere formation by either permitting or inducing proliferation.

The in vivo data clearly demonstrates that FGF-2 is sufficient to induce proliferation in the adult mouse hypothalamus (Figure 6.7 and 6.8). Proliferation occurs in a defined pattern, within a number of discernable zones (discussed further in chapter 8). In the absence of exogenous stimulation, newborn cells are scattered throughout the hypothalamic parenchyma, and only rarely seen at the ependymal lining. Previous studies, confirmed again here, have shown that when CNTF is added to stimulate proliferation, the resulting pattern of newborn cells is not altered, just increased in number ([64] and Figure 6.8). However, our results using FGF-2 show an increase specifically in the vicinity of the ependymal lining of the third ventricle, coupled with a decrease in new cells in the parenchyma. It is clear from this that the FGF is not stimulating the same population of cells that respond to CNTF, or the same cells that provide the majority of constitutive proliferation. One theory that could explain this is the idea that there are separate populations of stem/progenitor cells in the hypothalamus: a parenchymal CNTF-responsive population and an ependymal FGF-responsive one. This would fit with the observation of scattered pErk1/2^{+} cells in both of these locations. One attractive possibility is that these two populations represent different stages along a linear progression. Given the

relatively low levels of ependymal proliferation seen in the absence of mitogenic stimulation, the FGF-responsive cells would be a good candidate for the true stem cell, which is likely to have a relatively quiescent character. These could then give rise to CNTF-responsive progenitor cells, which would migrate out into the parenchyma, and divide more rapidly. This idea is supported by the observation that there is a decrease in parenchymal proliferation in the presence of FGF, even when CNTF is also applied. This could be caused by an FGF-induced increase in symmetrical self-renewing divisions amongst the stem cell population, leading to a subsequent depletion of the progenitor cell pool. Of course, this is not the only possible explanation, but it serves to highlight the kind of theories that would be interesting to test in the future.

The combination of data demonstrating that FGF-2 is sufficient to induce proliferation in vivo (Figures 6.7 and 6.8), together with that showing that active FGF signalling is necessary for the proliferation of hypothalamic cells in vitro (Figure 6.9), strongly suggests that FGF signalling is a crucial component of the hypothalamic stem/progenitor cell niche. This raises the question of which FGFs might act as the endogenous ligand under normal conditions. There are at least 22 FGFs and four FGFRs, plus subtypes produced by alternative splicing. The high level of cross-reactivity of most FGFs for the various different FGFRs means there can be significant amounts of functional redundancy between FGFs. Although FGF-2 was used throughout this study due to its association with many other stem cell systems, this is no guarantee that it also functions as the endogenous ligand. Although this question remains far from answered, a good starting point is to find out which FGFs and receptors are normally present.

Data from the Allen Brain Atlas can give clues as to which FGF(s) may act to regulate constitutive proliferation (Figure 6.2). However, it must be acknowledged that there are a number of limitations with this system. The variations seen between different picture series of the same gene, as noted for FGF1, indicate that the system used to generate these in situ's may not always be optimised for a particular probe. In particular, it would be dangerous to

automatically rule out all the genes that appear to be absent, as it is likely that the system may produce many false negative results. With this in mind, this sort of database search should be used only as a starting point to identify genes with particular potential. The presence or absence of each factor should then be confirmed, either through trying to replicate the result, or by searching the literature for convincing evidence. Another constraint of this approach is that it takes no account of splice variants. The FGFRs previously reported in the hypothalamus were specifically noted to be of the IIIc isoforms, with IIIb receptors not detected [20]. As the isoform is a significant determinant of affinity for the different FGFs, this is very useful information.

Using the database search as a starting point, I identified two FGFs – FGF-10 and FGF-18 - which are expressed in the adult mouse hypothalamus (Figures 6.4 and 6.5). FGF-10 is known to play a role in the development and maintenance of several adult stem/progenitor cell systems, including in the lungs, mammary glands and continuously-growing incisors of mice [111],[112],[113]. In all these locations it is thought to signal via its principle receptor, FGFR2IIIb [114]. In the hypothalamus, FGF-10 has been reported to be present in the ventral ependymal lining at the level of the ME [20]. The authors hypothesized that FGF-10^{+} cells in this region are progenitors, which subsequently downregulate FGF-10 and migrate into the arcuate nucleus. My findings here are consistent with the reported distribution in this paper.

FGF-18 was first identified in 1998. It is part of the FGF8 subfamily of FGFs, although unlike the other members of this subfamily (FGF8 and FGF17) it does not exist as functionally distinct splice variants [115]. Although it has not yet been characterized as thoroughly as some other FGFs, it is known to play a significant role in skeletal development, by influencing chondrogenesis and osteogenesis [116]. FGF-18 has been implicated in several developmental systems such as the specification of left-right asymmetry, as well as being present in early somites [117],[118]. In the developing brain, FGF-18 is expressed in the midbrain, where, in co-ordination with FGF-8 and FGF-17, it

controls early patterning events [119]. In adult mice, FGF18 is thought to play a role in the growth and maintenance of skin hair follicles [120]. There are no known reports of FGF-18 being expressed in the adult CNS of any species. Analysis of the binding patterns of FGF-18 demonstrate that it has a high affinity for the IIIc isoforms of FGFR-1, -2 and -3, and can also bind FGFR4 [115]. Its effects in bone are generally linked to signalling via FGFR3, considered to be its main receptor [121]. It does not bind to any of the IIIb receptor isoforms.

This is the first time that FGF-18 expression has been reported in the adult hypothalamus. Of particular interest is the fact that its expression is limited to the ventral $\alpha 2$ tanycyte population, the exact region that shows the greatest proliferative response to exogenous FGF-2. The reason for the specific co-localisation of FGF-2 responsive cells with FGF-18^{+}, but not FGF-10^{+}, regions may lie in the receptor specificity of these two growth factors. When FGF-10 was characterised in the adult hypothalamus, the authors also examined the distribution of receptor subtypes [20]. The ME/posterior region of the hypothalamus was reported to contain largely IIIc isoforms of FGFR1 and FGFR2, but not the IIIb isoform of either subtype. This puzzled the authors, as FGF-10 is known to signal mainly through IIIb isoforms, leading them to conclude that FGF-10 signalling in this region was not cell autonomous. However, both FGF-2 and FGF-18 express a preference for binding to the IIIc, rather than IIIb, isoforms of FGFR1 and FGFR2 [122],[115]. The presence of both FGF-18 and at least one of its receptors suggests that under normal conditions this pathway is likely to regulate proliferation of ventral $\alpha 2$ tanycytes. As FGF-2 binds to the same receptor isoforms, it is likely that the FGF-2 infusion experiments hijacked this pathway. The higher levels of proliferation seen in the presence of exogenous FGF-2 suggest that the pathway is not usually saturated under normal conditions. Previous studies reported the presence or absence of FGFR isoforms based on RT-PCR data, and so the precise location of these receptors is not clear. It is likely that the FGFR1IIIc receptors are the same as those identified in the β tanycyte region by non-specific FGFR1 probe, whereas it is not possible to speculate on the location of FGFR2IIIc. In order to confirm

the soundness of the theory suggested above, it would be necessary to determine the precise distribution of FGFR1IIIC and FGFR2IIIc in this region. As the relative expression patterns of different isoforms is not necessarily fixed, but may be up- or down-regulated in response to both FGF and non-FGF growth factor signalling [123], it would be interesting to examine whether FGF-2 infusion actually alters the pattern .

In addition to the presence of FGF-10 and FGF-18 as reviewed above, there may be other FGFs present in the hypothalamus that have not yet been analysed. While I failed to find any evidence for the expression of FGF-2, reports in the literature contradict this. Other studies have observed FGF-2 mRNA to be present in the lateral walls of the third ventricle, a region approximately equivalent to α tanycytes [124]. The same study also observed FGFR1 mRNA to have a much wider distribution than the one I described, being expressed in the entire ependymal lining at the ME/posterior level, plus many cells of the ME, ARC and VMH. Whether this could be due to my probe having unintentional specificity for some unknown subset of FGFR1, or their probe having some non-specific binding, is not clear. Therefore it is wise to be cautious in interpreting the results based on the presence or absence of particular growth factors, when it is likely that there is much to still be confirmed or discovered.

Although the precise role of the specific FGFs and FGFRs remains to be elucidated, it is clear that there are a number of components of the FGF pathway present in the adult mouse hypothalamus. The data from the infusion of FGF-2 confirms that these components can respond to FGF stimulation by producing a proliferative response. In addition, the presence of pErk1/2 in the hypothalamus under normal conditions suggests that this pathway is constitutively active in some areas.

With regard to my original hypotheses, I have demonstrated:

1. That various components of the FGF pathway, and evidence of active growth factor signalling, are present in the adult hypothalamus.
2. That exogenous FGFs are capable of inducing proliferation in vivo.
3. That neurospheres are influenced by (and require) active FGF signalling.

In light of this data, it seems fair to conclude that FGFs act to regulate the proliferation of hypothalamic stem/progenitor cells. However many aspects of this regulation remain to be discovered, such as how the production and secretion of FGF is itself regulated, by which cells and in response to which biological conditions.

Chapter 7

Chapter 7 – Hypothalamic Slice Cultures

7.1 Introduction

Whilst the neurosphere assay can give a wealth of useful data on how stem cells behave, it is limited by its highly artificial, in vitro selection nature. For example, it is ideal for answering questions such as how many cell types can be produced from NSCs isolated from the brain given ideal conditions, but cannot produce information for examining how many of those cell types are actually produced in a normal in vivo situation. The ideal situation is to work in vivo, but animal licensing constraints mean this is not always an option. In addition, the adult brain, and particularly the hypothalamus, is extremely inaccessible, and mice lack the genetic tracing techniques that might allow a systematic in vivo conditional genetic approach. Many techniques (for example electroporation of DNA constructs) are much more practical to perform in vitro. With this in mind, I aim to establish a protocol for ex vivo culture of adult hypothalamic tissue.

Ex vivo cultures are commonly used for giving easy access to brain tissue without the need for live animals. Their most common application is probably in electrophysiology, as neurons can retain their normal firing patterns for days or even weeks in culture. The two main forms are explants – tiny pieces of tissue usually maintained in collagen, and slice cultures, which are much larger slices of brain tissue. Given the importance of both A/P and D/V positioning described in chapters 5 and 6, I decided to use slice cultures because they are capable of providing much clearer spatial information.

7.2 Results

7.2.1 Basic methodology

Although slice cultures are a commonly used procedure, there is very little in the literature relating specifically to hypothalamic slices. One example is documented by Ikeda and Allen, but refers to neonatal tissue [125]. There is relatively little variation between slice culture methods from different labs, as most protocols share their core features. As it is unlikely that hypothalamic tissue as a whole requires different conditions from other regions, a cerebellar slice protocol was adapted [72].

After dissection of the brain, the tissue needs to be sectioned into a number of slices. The two main options are using a tissue chopper or a vibratome, and both of these were found to be suitable for hypothalamic tissue. The tissue chopper had the advantage of speed and was easier to sterilize, however care had to be taken to embed the tissue in precisely the correct density of agarose to avoid it sticking to the blade. The vibratome was much more gentle, and although it was hard to keep sterile no obvious problems with infection were noted in the cultures. Although some of the early experiments presented here used slices from a tissue chopper, the vibratome may ultimately prove to be best at minimising tissue damage.

All slices were cultured on micropore inserts, a standard technique. The inserts sit level with the top of the culture medium, and the medium diffuses through to form a thin layer surrounding the slice. This provides more structural support for the tissue than simply leaving it submerged in media.

7.2.2 Culture medium

A major question was which culture medium would best support the slices. Most slice protocols are not aimed at stem cell studies, and so the media used would not necessarily be suitable for maintaining NSCs. The important role of the niche in stem cell control is indicative of a delicately balanced system

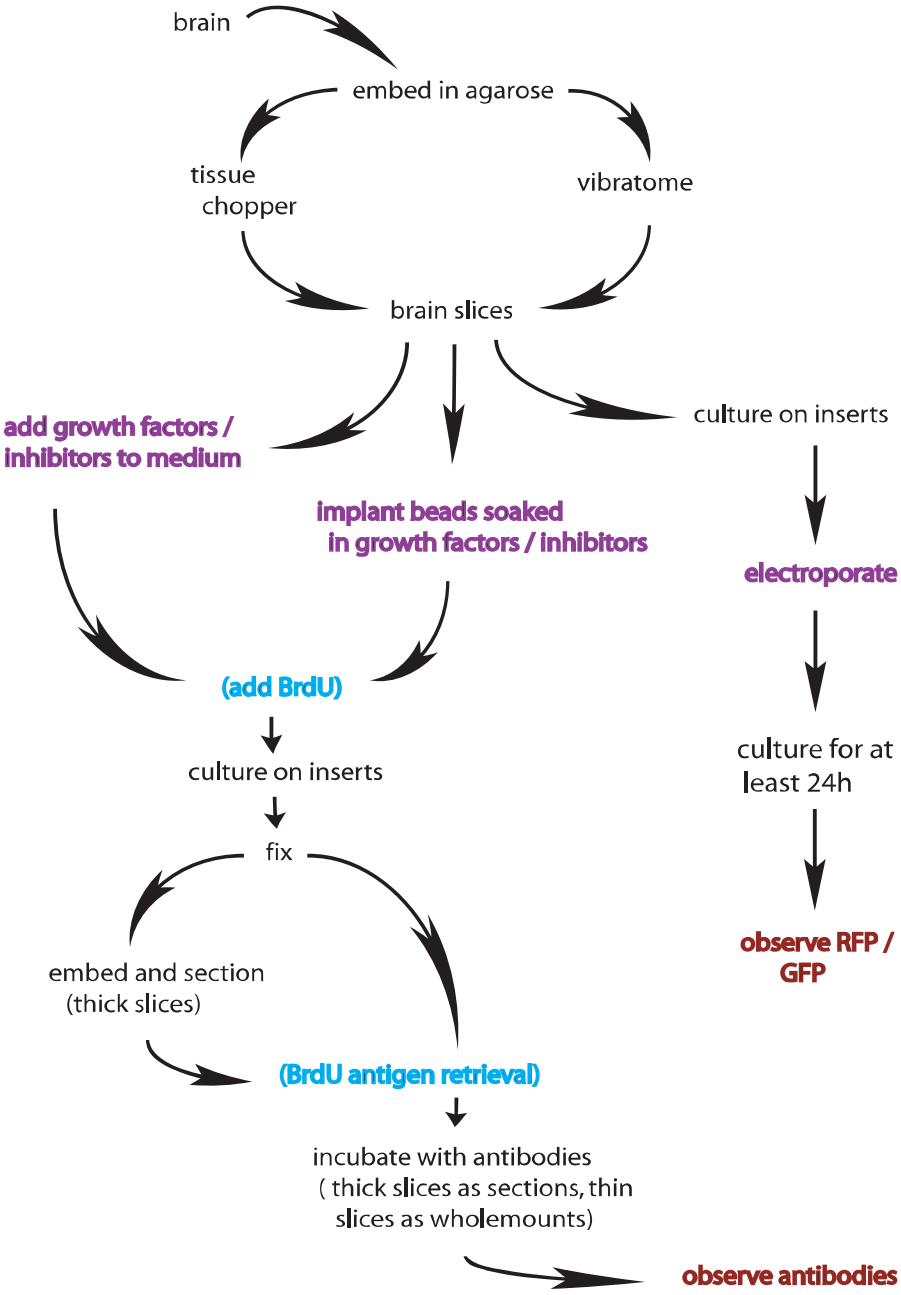


Figure 7.1

Methods for hypothalamic slice cultures

Flow diagram of the methods used to derive and culture hypothalamic slices. Purple highlights indicate ways in which the cultures can be manipulated, blue indicates how BrdU labeling can be included in the protocol, and red shows the end information.

allowing both self-renewal and differentiation. Ideally any ex-vivo culture would try not to upset this. Many slice cultures contain a high volume of serum, which contains many undefined factors. As one of my aims for these cultures is to use them in conjunction with specific growth factors or their inhibitors to investigate how NS/PCs are regulated, I wanted to avoid its use if possible. As an initial starting point I tried six different media variations, and simply observed whether the slices appeared healthy and unchanged to the naked eye 24 hours later. Three of the media used were DMEM:F12 +N2 +B27 (the standard self-renewal medium used for neurospheres), either alone, +FGF-2 +EGF, or + hypothalamus conditioned media. Two OptiMEM based solutions were also tested, with either 2.5 or 25% foetal bovine serum (FBS), plus BME + 25% HBSS. No differences were observed between slices in any of these conditions. In addition, when they were subsequently fixed and stained with DAPI healthy nuclei were seen in every case (data not shown). Subsequent experiments were performed using DMEM:F12 +N2 +B27 (referred to as SR medium in future) as a base media, as it contains supplements designed to support both immature and mature neural cells.

In order to establish whether serum was a necessary addition to slice culture medium, I looked at how a variety of markers were maintained in both serum and serum free media. I first investigated the effect of serum on Sox2 maintenance in three serum protocols: serum free, 25% foetal bovine serum (FBS) and reducing serum in which FBS was initially added at 25%, reduced to 15% the following day, then 5% and finally 0% by day 3. Slices were cut from a Sox2-eGFP mouse, so that Sox2 levels could be tracked at several timepoints in the same slice. At least one slice was taken at the level of the ME, and one more anteriorly, for each condition. Slices were examined for Sox2 expression at the time of plating (d0) and on the 4 subsequent days. It should be noted that the eGFP produced in these mice is relatively stable, and so there may be a lag of around 24 hours between loss of Sox2 protein and loss of eGFP signal. The results are shown in Figure 7.2. In both anterior (Figure 7.2 B,C) and ME (Figure 7.2 Q,R) level slices, it seems that Sox2 is lost 2-3 days after plating without

Figure 7.2

The effect of serum on Sox2 maintenance in slices cultures

Slices were cut from either the anterior (P-Y) or ME (A-O) hypothalamic areas of Sox2-eGFP transgenic mice. They were cultured in one of three serum regimes: without serum ('no FBS' – A-E, P-T), with 25% FBS (K-O, U-Y), or with an initial supplement of 25% FBS that was sequentially reduced on subsequent days ('reducing serum – F-J). GFP expression was recorded at the start of culture (D0) and then at 24-hour intervals for the next 4 days.

A-E: Sox2 expression is lost at the level of the ME in unsupplemented media.

F-J: Sox2 is maintained at the level of the ME in reducing levels of FBS.

K-O: Sox2 is difficult to distinguish, but may be lost after 3 days in culture with 25% FBS.

P-T: Sox2 is lost in the anterior hypothalamus in unsupplemented media.

U-Y: Sox2 is maintained in the anterior hypothalamus in media supplemented with 25% FBS.

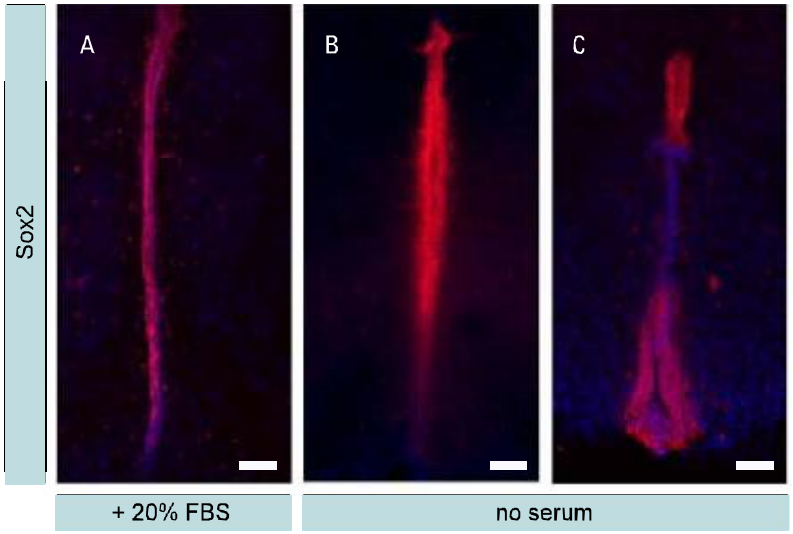
All scale bars = 100μm.

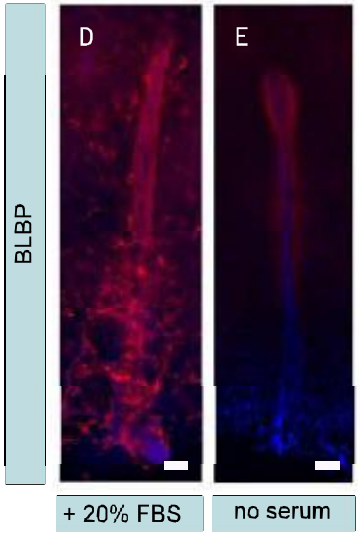
Median Eminence
Anterior
SR (no FBS)
SR + reducing FBS
SR + 25% FBS
SR (no FBS)
SR + 25% FBS
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
U
V
W
X
Y
D0
D1
D2
D3
D4

serum. In contrast, it is maintained in reducing serum conditions in the ME (Figure 7.2 F-J) and sustained serum in the anterior hypothalamus (Figure 7.2 U-Y). It is not clear whether it is maintained in the ME slice in sustained serum – although it appears to get weaker the angle of the ependymal layer in this slice makes it difficult to discern the eGFP, even at the start of the experiment. These results suggest that serum is required for the maintenance of Sox2 in slice cultures, although it is not continuously required at a level as high as 25%.

This result appears very unambiguous, however it had been noted in preliminary experiments that Sox2 was detected by antibodies in slices cultured for several days in serum-free media. In order to resolve this matter, more experiments were conducted using slices from non-transgenic mice, with antibodies to detect Sox2 expression after five days in culture. The antibody results directly contradicted the results from the Sox2-eGFP mice, as it seemed clear that Sox2 was still expressed in the ependymal layer of slices from both ME (Figure 7.3 B) and posterior (Figure 7.3 C) levels.

In order not to place too much weight on contradictory results, other antibodies were compared in serum free and 20% FBS cultures. Labelling with GFAP, nestin and DARPP-32 revealed no major differences (data not shown), however BLBP revealed a major disregulation of cell organization in serum-treated slices (Figure 7.3 D) that was not seen in serum free conditions (Figure 7.3 E). In order to establish whether this disruption extended to the proliferative cells of the hypothalamus, cultures were treated with BrdU for 24 hours, then labelled with antibodies to both BrdU and Ki67, a cell cycle marker. The serum free slice showed a low but reasonable level of proliferation, with both $BrdU^{+}/Ki67^{-}$ single labelled and $BrdU^{+}/Ki67^{+}$ double labelled cells observed (Figure 7.3 H, I, green and orange arrows respectively). However no cells expressing either marker were seen in the 20% FBS condition (Figure 7.3 F,G), strongly suggesting that serum inhibits hypothalamic proliferation. As my intention is to study proliferation within the slice cultures, it was clear that serum-free conditions should be used for all future experiments.





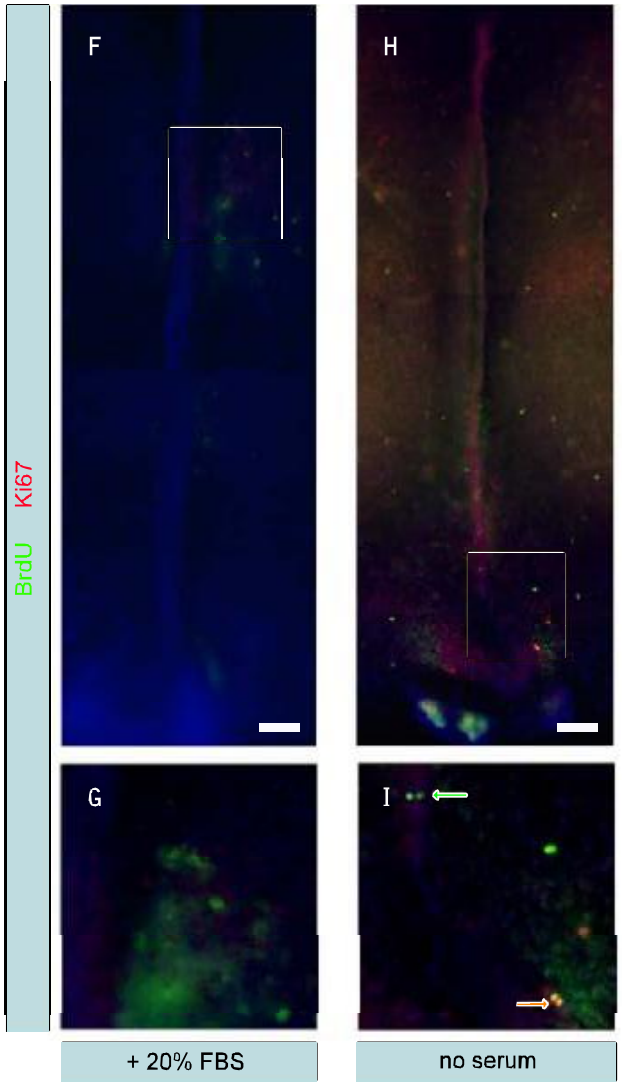


Figure 7.3: Comparison of serum vs. non-serum cultures

A-C. Sox2 expression in a slice cutlured with 20% FBS. (A), and anterior (B) and posterior (C) slices cultured in serum-free conditions..

D,E. Expression of BLBP after 5 days in 20% FBS (D) or serum-free media (E).

F-I: Cultures exposed to 24hrs of BrdU, in the presence (F) or absence (H) of serum, and double labelled for Ki67. An enlargement of F shows no positive cells (G), while an elargement of H has both $BrdU^+$ cells (I, green arrow) and $BrdU^+$/ $Ki67^+$ cells (I, orange arrow).
All scale bars = 100μM

7.2.3 Validation of slices

In order for hypothalamic slice cultures to be a useful experimental tool, it is important to confirm that the tissue functions as it would in an intact animal. It is already known that the electrophysiological properties of neurons are generally maintained in slice cultures, however as we are aiming to study proliferative cells rather than mature neurons we decided to focus on cell markers. If a cell's function or behaviour were disrupted then it would be reasonable to expect a change in the protein markers it expresses, and so if these can be shown to be expressed normally it is likely the cell's function is also normal.

In addition to the validation, I hoped to establish the optimum conditions for slice cultures. One of the variables is the thickness of each slice. In this respect, two different options were investigated. Thin slices were cut to a maximum of 250μm thick, and were processed for antibody labelling as wholemounts. This is a fairly typical thickness to use for slice cultures. However, wholemount tissue often does not produce very clear images after antibody labelling, and so I also experimented with using thicker slices. These were cut 1mm thick, which meant that after culture and fixation they could be embedded and cryosectioned, giving clearer slide-based pictures.

Initial validation experiments were performed on thick slices. I first looked at whether regional neuropeptide markers were well-maintained in culture. Figure 7.4 shows a comparison of NPY and AVP labelling in vitro and in vivo. It should be noted that the NPY-expressing domain appears much more extensive than when it was used to verify subdissections (chapter 5) because the antibody labels projections as well as the cell body. Anteriorly both markers are present in the correct areas, although there may be a slight reduction in the level of NPY projections to the SCN (Figure 7.4 A,E). A normal pattern of expression is also seen at the level of the median eminence, with AVP projections seen in the ME (Figure 7.4 G) and NPY in the ARC and DMH (Figure 7.4 C). The apparent

Figure 7.4

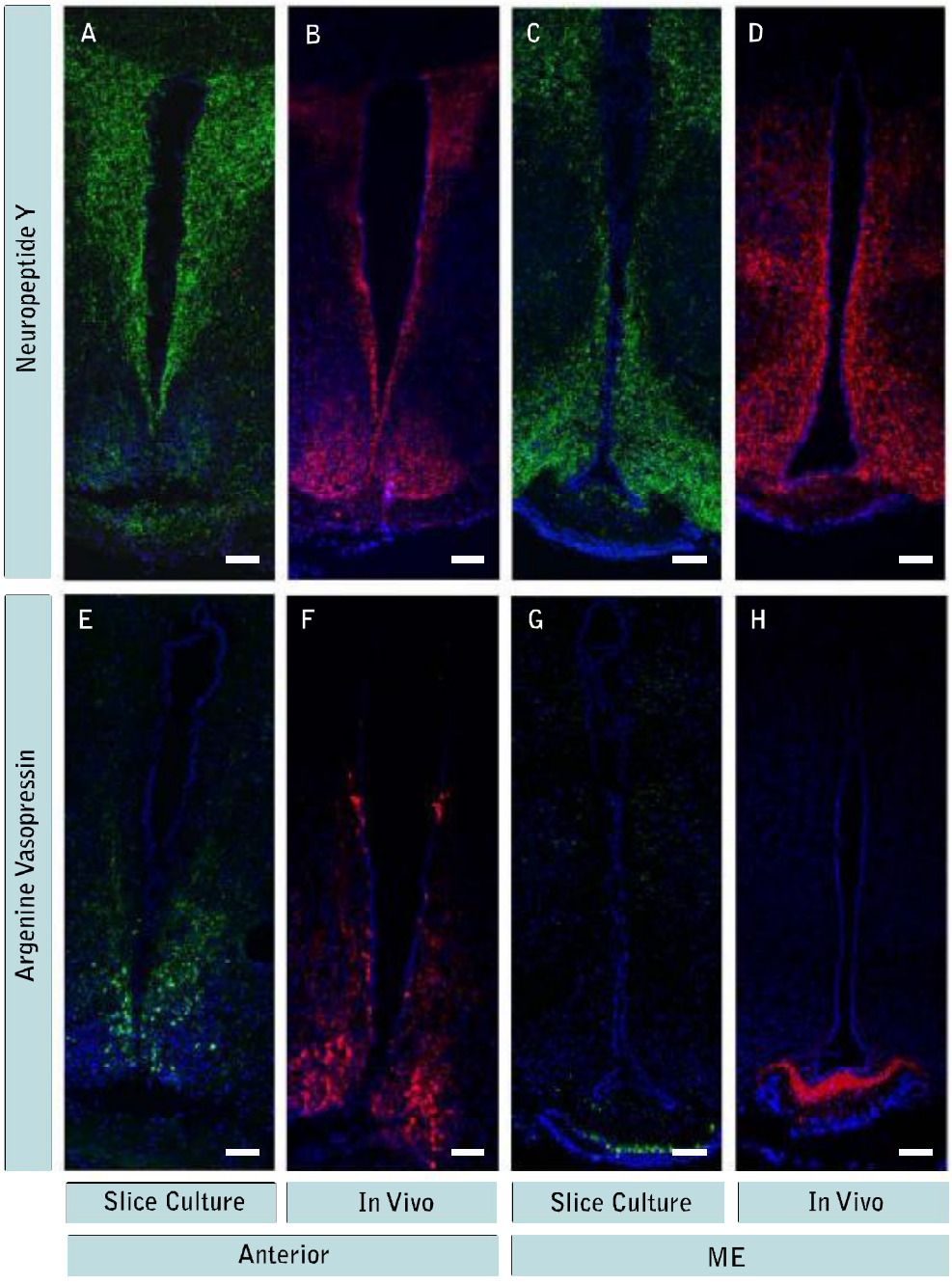
Maintenance of neuropeptide markers in thick hypothalamic slices

A, B: NPY expression in anterior sections of thick slices. In vivo NPY-expressing axons are seen in the SCN and PVH (B). This expression is maintained after culture in thick slices, although it may be weakened in the SCN (A). Scale bars = 100μm.

C, D: NPY expression in ME level sections of thick slices. In vivo NPY-expressing axons are seen in the ARC and DMH (D). This pattern is also seen in cultured slices (C). Scale bars = 100μm.

E, F: AVP expression in anterior sections of thick slices. AVP is limited to the SCN both in vivo (F) and after culture (E). Scale bars = 100μm.

G, H: AVP expression in ME level sections of thick slices. Some AVP expression is seen in the ME both in vivo (H) and in vitro (G). Scale bars = 100μm.


Neuropeptide Y
Argenine Vasopressin
A
B
C
D
E
F
G
H
Slice Culture
In Vivo
Slice Culture
In Vivo
Anterior
ME

difference around the level of the VMH in these pictures is assumed to be the result of a slightly different sectioning levels, rather than a change in expression.

The next step was to investigate whether stem cell and tanycyte markers were similarly well preserved in these cultures. Initial experiments looked at Sox2 and GFAP expression, again using thick slices. As is evident from Figure 7.5, these markers were massively disrupted. Sox2 expression appeared to be downregulated in the ependymal layer at both anterior and ME levels, although it was still weakly expressed in some cells (Figure 7.5 A, C). By contrast GFAP was upregulated throughout the slices, presumably indicating that high levels of gliosis were induced during culture (Figure 7.5 E, G). This response was in contrast to earlier results from thin slices (Figure 7.6 B). It was concluded that the hypothalamus is not suited to culture as thick slices, and all subsequent experiments were conducted on thin slices of no more than 250μm depth.

Stem cell and tanycyte markers were repeated, this time on thin slices. In contrast to the thicker slices, both Sox2 and GFAP were maintained (Figure 7.6 A, B), and remained restricted to the ventricular region in accordance with their in vivo distribution. This region also maintained expression of Six3 (Figure 7.6 C) and DARPP-32, which was seen only in the ventrally-located β tanycytes as expected (Figure 7.6 D). BLBP, which in vivo was observed in a separate cell population outside of the ependymal lining, could also still be detected (Figure 7.6 E). Vimentin antibody was also tested, to examine the morphology of tanycytes after culturing. At the level of the ME, vimentin$^+$ processes with the characteristic location and shape of ventral α2 tanycytes were observed (Figure 7 .6 F, arrow). Failure to observe more dorsal tanycyte subtypes is thought to be due to difficulties with penetration of the antibody into the tissue, and will require a repeat experiment for confirmation. At more posterior levels, tanycytic processes were seen protruding from the ventral two-thirds of the ventricular lining (Figure 7.6 G) as would be expected, indicating a normal morphology is present in this region.

Figure 7.5

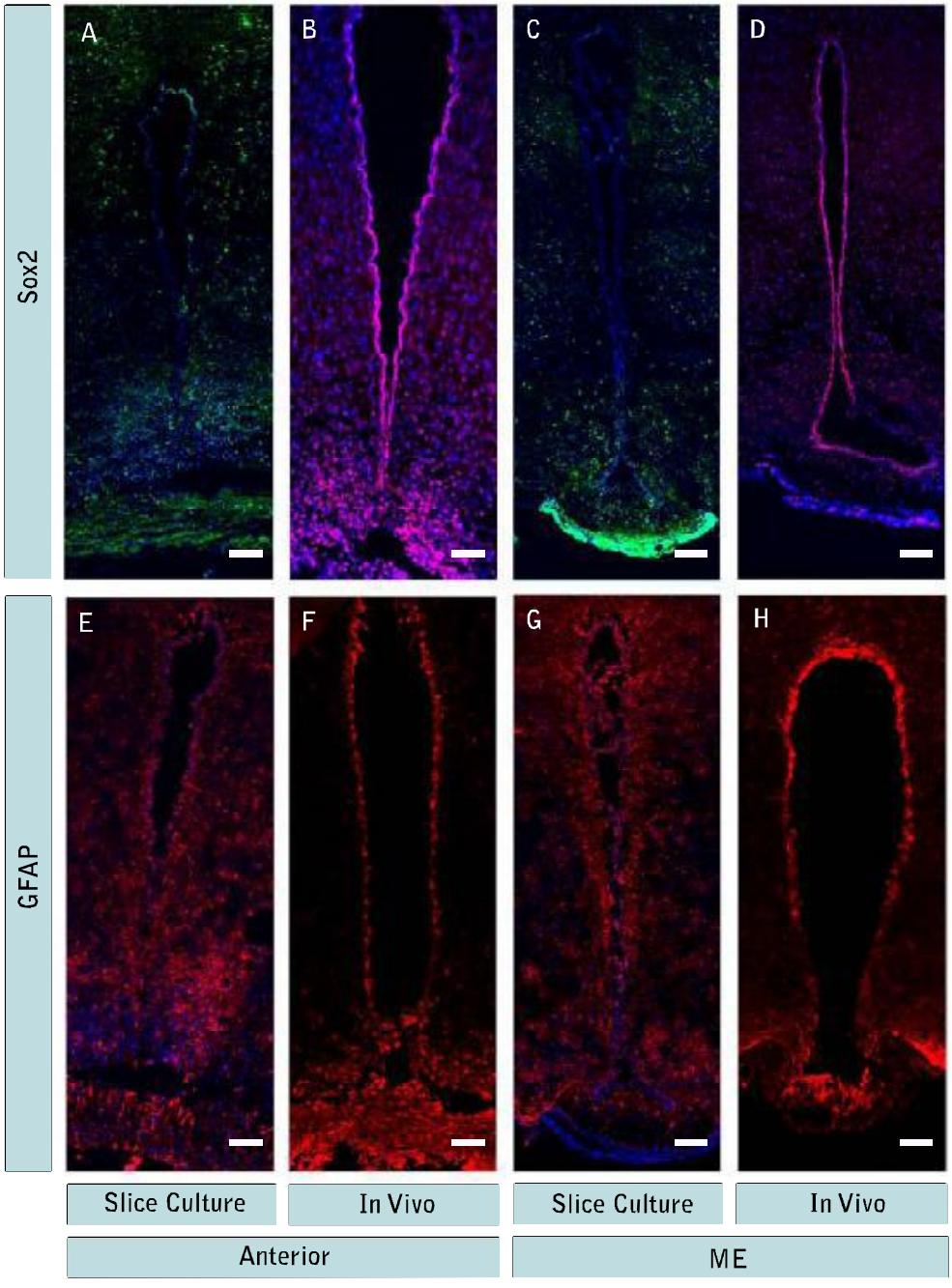
Stem cell markers in thick hypothalamic slices

A, B: Sox2 expression in anterior sections of thick slices. In vivo Sox2 is seen in the SCN and ventral ependymal lining (B). This expression is largely lost after culture in thick slices (A). Scale bars = 100μm.

C, D: Sox2 expression in ME level sections of thick slices. In vivo Sox2 is seen in the ventral ependymal lining, and scattered other cells (D). Ependymal expression is largely lost after culture in thick slices, although some other scattered cells remain $Sox2^+$ (C). Scale bars = 100μm.

E, F: GFAP expression in anterior sections of thick slices. In vivo GFAP is limited to the region around the 3rd ventricle and the SCN (F). After culture in thick slices GFAP appears to be massively upregulated in normally $GFAP^-$ regions, with expression extending across the slice (E). Scale bars = 100μm.

G, H: GFAP expression in ME level sections of thick slices. In vivo GFAP is limited to the region around the dorsal two-thirds of the 3rd ventricle and the ME (H). After culture GFAP appears to be massively upregulated throughout the slice (G). Scale bars = 100μm.


Sox2
GFAP
A
B
C
D
E
F
G
H
Slice Culture
In Vivo
Slice Culture
In Vivo
Anterior
ME

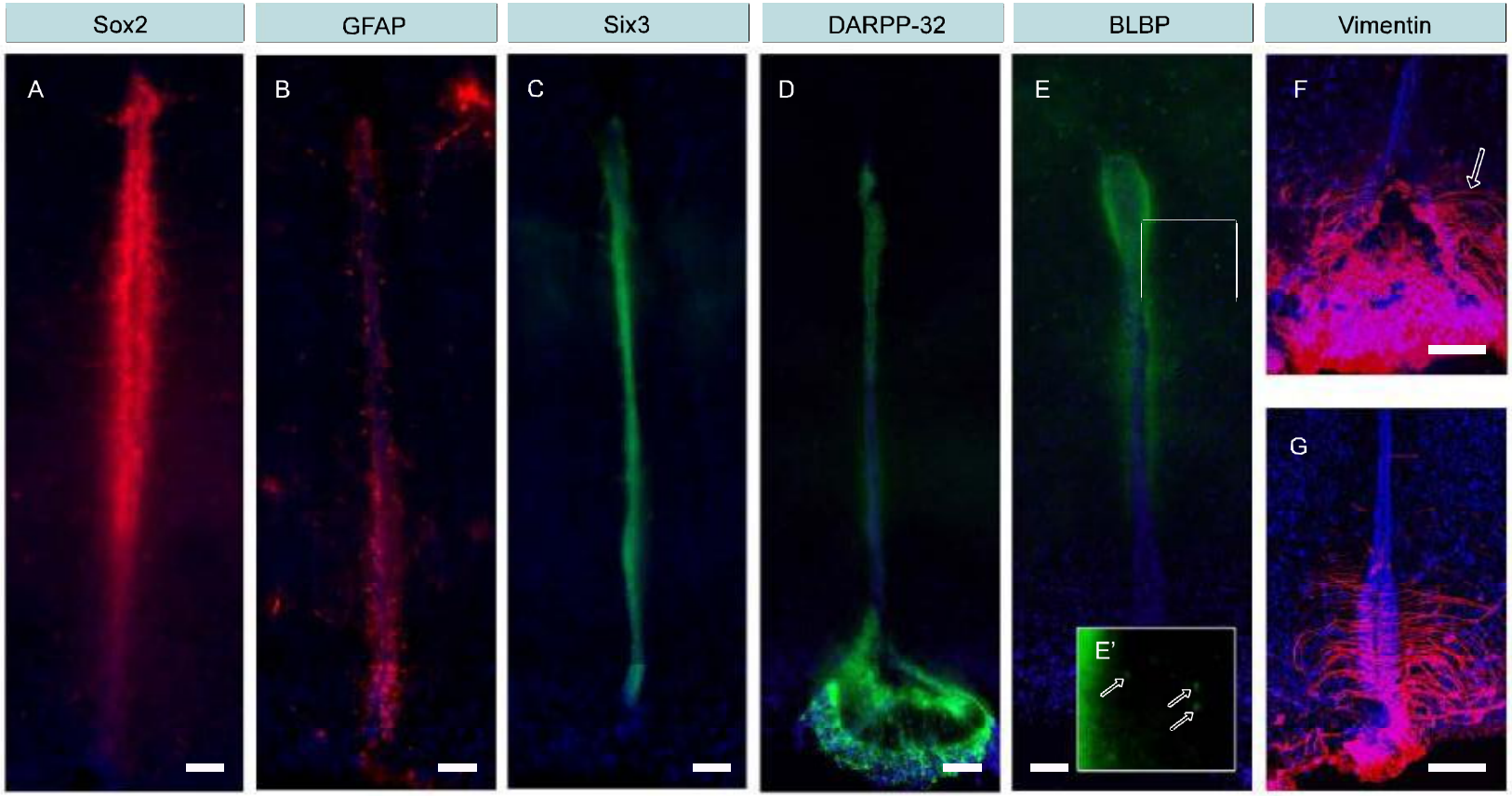


Figure 7.6 Maintenence of stem cell / tanycyte markers in thin hypothalamic slice cultures

A-F Antibody labelling on wholemount slice cultures. A: Sox2. B: GFAP. C: Six3. D: DARPP-32. E: BLBP. E': Enlargement of box in E, showing individual $BLBP^{+}$ cells (arrows) Scale bars = 100μM.

F,G Optical sectioning of vimentin-labelled slices shows maintenence of tanycyte morpholgy at ME (F, arrow indicates α2 tanycytes) and posterior (G) levels. Scale bars = 100μM.

7.2.4 BrdU labelling in slice cultures

It has been shown by several authors that in vivo BrdU labelling in the hypothalamus is ineffective when administered by the usual intraperitoneal route, and instead requires direct infusion into the brain ventricles [62],[60]. This makes the whole process considerably more complicated. Slice cultures may prove to be a viable alternative to intracerebral infusions, requiring less technical expertise as well as being possible without a specific mouse license. In order to test the viability of this I carried out several preliminary experiments.

Slices were incubated with BrdU for a minimum of period of 16 hours, to allow time for it to be incorporated into dividing cells. Two methods of antigen retrieval were tested – HCl treatment, or DNase treatment. Both methods were capable of detecting BrdU incorporation, although they differed in their compatibility with other antibodies (Figure 7.7 B-G). DNase gave slightly weaker BrdU staining, and although double-labelled antibodies usually came out well they also picked up a lot of background. HCl gave slightly weaker labelling of other antigens, but with very low background. The BrdU labelling itself was very clear, and on balance this seems the best method to use. An alternative for double labelling using both BrdU and another antibody would be to label the other antibody first, then begin the antigen retrieval and BrdU labelling.

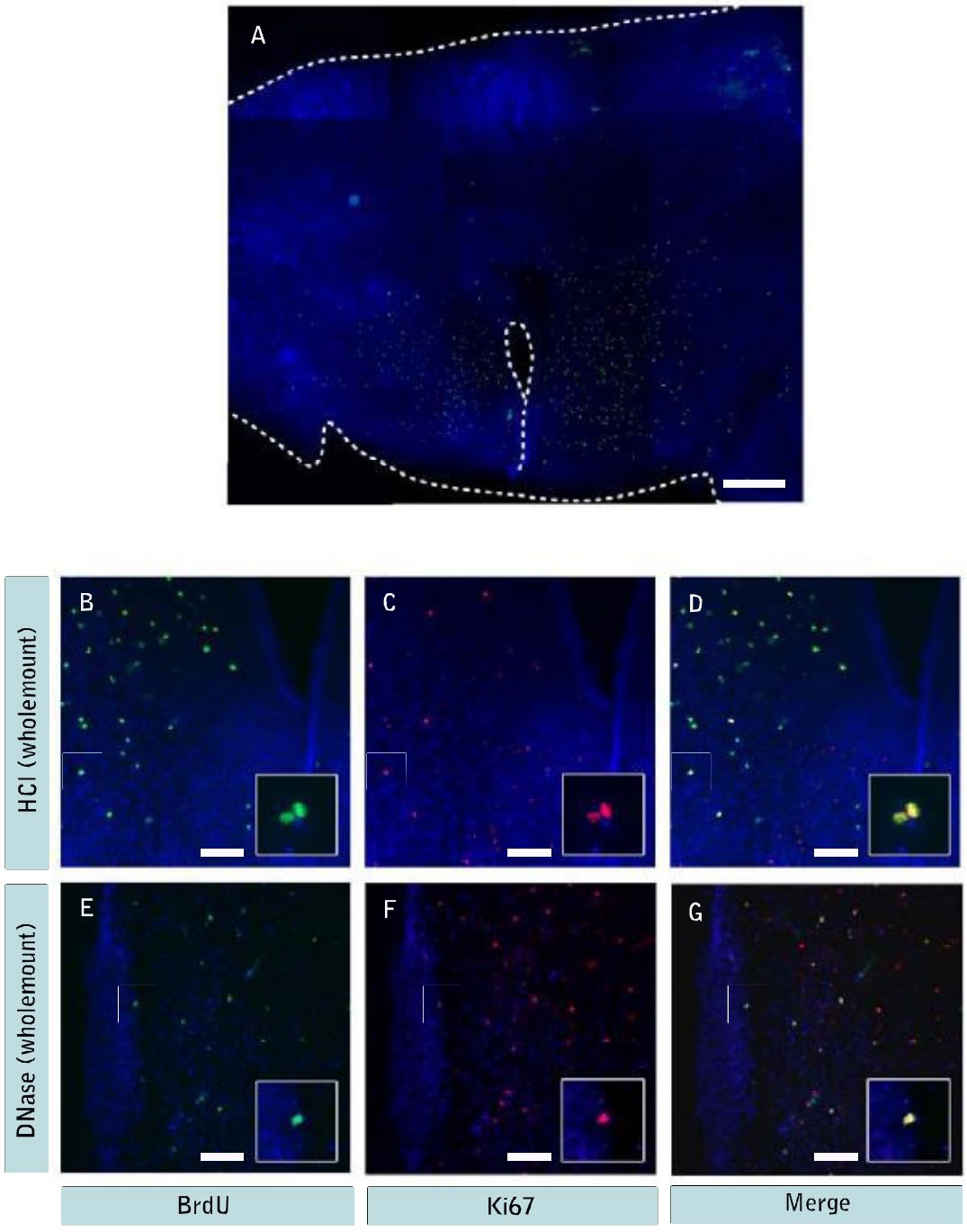
In order to confirm that BrdU was labelling dividing cells, slices were co-labelled with Ki67, a marker of proliferating cells. Given that the slices are exposed to BrdU for approximately 24 hours, it would not be unexpected to find some cells that label with BrdU but not Ki67. These represent cells that were proliferating only at the beginning of the BrdU incubation, and have completed the process before fixation. In contrast, there should not be any $Ki67^{+}$ cells that do not contain BrdU. This is because there is no washout period where cells continue to grow after BrdU removal, and the synthesis phase of the cycle is the stage at which both BrdU is incorporated into the DNA, and Ki67 is first detected (at least for cells not continuously proliferating, which may express

Figure 7.7

BrdU labelling in hypothalamic slice cultures

A: Low power magnification of BrdU labelling (green) counterstained with DAPI, on a whole brain slice. Only the hypothalamic region contains $BrdU^+$ cells.
Scale bar = 500μm.

B-G: BrdU / Ki67 double labelling, using two different BrdU antigen retrieval techniques. Using both HCl treatment (B-D) and DNase (E-G), BrdU (green) and Ki67 (red) can be detected. Inserts show magnifications of the double-positive cell nuclei in the regions denoted (white box). Using DNase gives higher background in the red channel; areas positive for Ki67 only are likely to be artefacts. Using HCl gives the predicted result, in which only a subset of $BrdU^+$ cells are also $Ki67^+$, and no $Ki67^+/BrdU^-$ cells are seen. Scale bars = 50μm.


A
HCl (wholemount)
B
C
D
DNase (wholemount)
E
F
G
BrdU
Ki67
Merge

Ki67 in some stages of G1 [93]). Thus any $Ki67^+$ cell progressing through the cell cycle at the time of fixation should also contain BrdU. When Ki67/BrdU double labelled slices were examined, these predictions proved to hold true. Although slices prepared using DNase antigen retrieval appeared at first glance to contain Ki67 labelling alone, on closer inspection this proved to be background interference, probably resulting from DNase-induced tissue damage (Figure 7.7 G). Slices using HCl antigen retrieval methods could be seen to contain a mix of BrdU single positive cells and BrdU/Ki67 double positive cells, but no Ki67 single positive cells (Figure 7.7 B-D). Thus it is convincing that BrdU can be used in combination with hypothalamic slice cultures to selectively identify proliferating cells.

Figure 7.7 A shows the overall pattern of BrdU incorporation for a whole coronal slice, taken at the level of the hypothalamus (note that at this level neither the dentate gyrus or the SVZ are present). It is clear that whilst $BrdU^+$ cells are numerous in the hypothalamic area, they are not seen elsewhere. This correlates well with the in vivo data (D McNay, M Kokoeva, personal communication). In addition, the absence of labelled cells elsewhere in the brain supports the idea that this is not simply astrocytic proliferation induced by cutting the slices, as that would be expected to be universal across all brain regions.

7.2.5 DNA electroporation in slice cultures

One of the advantages that slice culture should provide over in vivo work is ease of access for manipulations such as DNA electroporation. This powerful technique involves incorporating artificial DNA constructs into living tissue using an electric current. It can also be used to deliver small inhibitory RNAs (siRNAs) to examine the effects of gene knockdown. In order to test if this would work in practice in my slice cultures, I conducted preliminary experiments using DNA for RFP or GFP, enabling me to visualise the individual electroporated cells. The results are summarised in Figure 7.8.

The main practical issue was where to place the electrodes. It was clear that they could not be in contact with the tissue as it would burn, and so were placed outside the slice, parallel to the third ventricle (Figure 7.8 A). Although this required them to be much more widely spaced than usual, it did not seem to cause any problems. The cells I am most likely to want to target are the tanycytes, and their position in the ventricular wall makes things relatively simple. In this case, the DNA is injected into the third ventricle. Trials involving DNA injected into the parenchyma were much less successful, and only a single electroporated cell was ever detected, indicating that although this is possible it would be a more technically challenging method. The DNA itself was required at a relatively high concentration – good results were achieved using a concentration of 10μg/μL, approximately ten times that required for chick electroporations. Guidance on the levels of current required was taken from Murphy and Messer [73]. Nine 100mV pulses were administered, each 50ms long and approximately a second apart.

These methods gave reliable results, with most slices having at least a few electroporated cells. As expected, the DNA was usually incorporated into cells of the ependymal layer when initially placed in the ventricle. The RFP / GFP filled the entire cell, and so allowed a detailed study of their morphology. A bright cell body in the ventricular wall with a long laterally-extending process was often observed, which is consistent with the electroporated cells being tanycytes (Figure 7.8 B-E). The D/V position of the electroporated cells varied, but with experience it is likely that this could be controlled to better target the electroporation. In summary, it seems hypothalamic slice cultures are highly suited to manipulation by electroporated DNA constructs. This should prove to be a very useful tool in the future, for answering many of the questions relating to hypothalamic stem cells.

7.2.6 Other manipulations

In addition to the possibility of electroporating the slices, the culture medium also provides a very easy way of manipulating cell signalling. Incubating slices

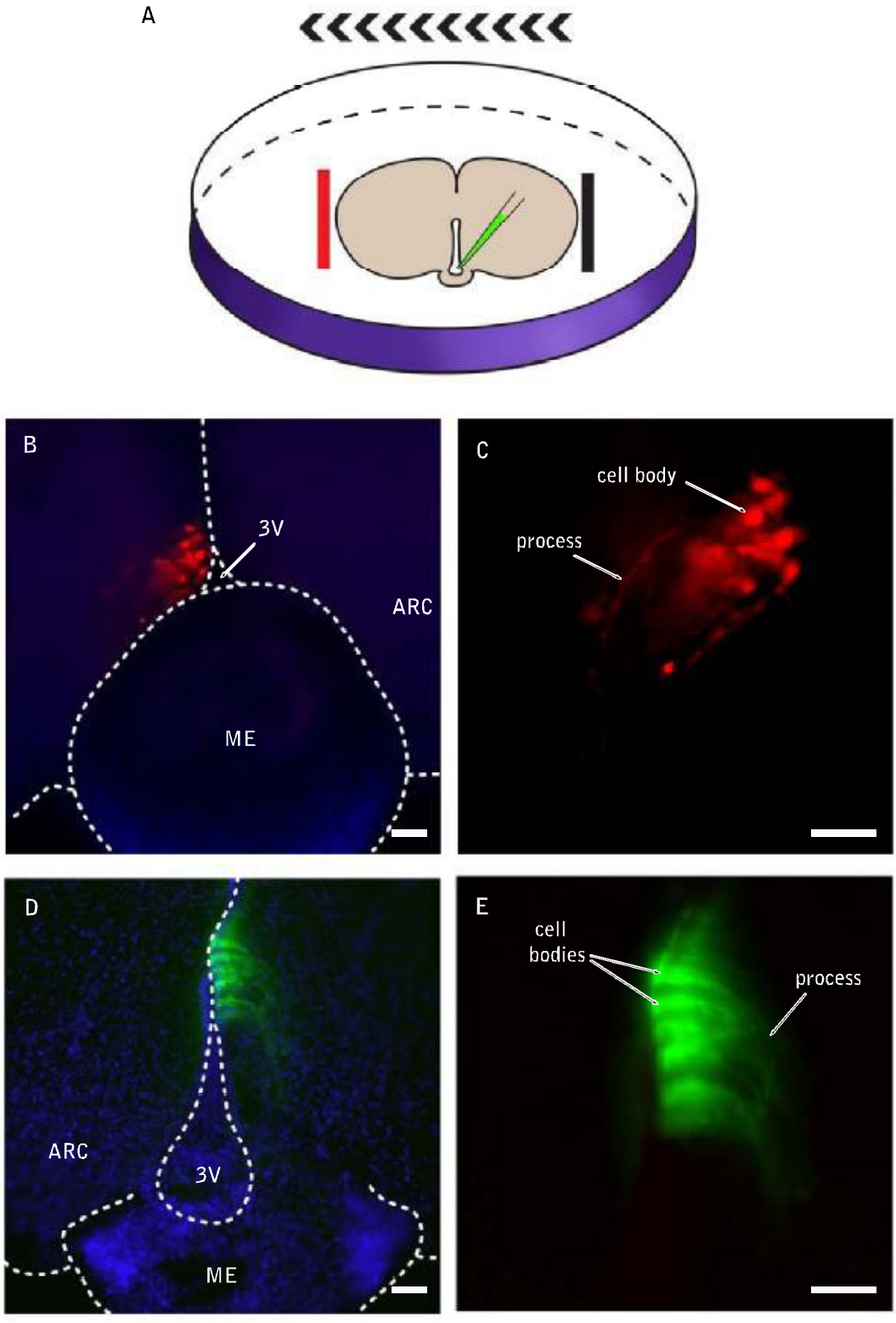
Figure 7.8

Electroporation of tanycytes in slice culture

A: Cartoon illustrating the electroporation of hypothalamic cells in slice culture. The insert is removed from the 6-well plate, and electrodes (black / red rectangles) are placed on either side of the slice. A small drop of medium is added, and DNA solution (green) is injected into the third ventricle. When short bursts of current are applied, the DNA moves in the direction indicated by the arrows, becoming incorporated into cells near the ventricle.

B, C: Ventral cells at the level of the ME can be electroporated (B). The presence of cell bodies at the ventricular surface and processes extending laterally suggest that these are ventral tanycytes (C) Red = RFP, blue = DAPI. Scale bars = 50μm.

D, E: more dorsal ependymal cells can also be electroporated (D). These cells also display the location and morphology of tanycytes (E). Green = GFP, blue = DAPI. Scale bars = 50μm.


A
B
3V
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bodies
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with either growth factors, signalling molecules or pharmacological inhibitors should prove a very useful tool for investigating hypothalamic stem cells. Alternatively, a much more spatially-restricted manipulation of signalling molecules could be achieved by implanting beads soaked in the relevant factors. These techniques have yet to be used in this culture system, but should provide a robust method useful for many different hypotheses.

7.3 Discussion

Although there is still more work to be done, this slice culture method has the potential to be a very useful tool to study hypothalamic stem cells in the future. The evidence suggests that slices can be cultured for up to five days, with no significant changes occurring in the character or morphology of the constituent cells. The many possibilities for artificial manipulation of the cell's internal or external environment is what makes this method so exciting. The demonstration that tanycytes can be easily electroporated with DNA constructs means that the effect of a single protein on their behaviour can be studied. In addition, the possibility for BrdU labelling provides an easy readout of the proliferative activity of individual cells. The next step for this work would to be to test a much wider range of markers for normal expression patterns, to verify that they are maintained appropriately. In order to confirm a normal pattern of proliferative activity, it would be useful to compare the results between slice cultures and in vivo experimentation, both in the presence and absence of stimulating factors such as FGF and CNTF.

Chapter 8

Chapter 8 – Discussion

8.1 Is there a stem cell population in the adult mouse hypothalamus?

It was assumed for many years that stem cells simply did not exist in the adult mammalian brain. Although this has now been conclusively proved incorrect, there is still the assumption by many researchers that only two adult niches are present: the SVZ of the lateral ventricles and the DG of the hippocampus. This idea persists, despite mounting evidence for proliferation and neurogenesis in a diverse range of other brain regions. One of the reasons for this is the relatively lower levels of BrdU incorporation seen in other regions, leading to the assumption that any neurogenesis is at levels insufficient to produce physiological effects. Another reason is the relatively scarcity of experiments definitively showing that new neurons are formed in vivo, as opposed to glial cell types.

8.1.1 Proliferation in the adult hypothalamus

The question of whether stem cells exist in other regions of the brain has recently begun to be addressed in the hypothalamus. A paper by Kokoeva et al demonstrated that new neurons can be produced in adult mice, and strongly suggested that this neurogenesis directly produced a physiological effect on body weight [64]. A follow-up paper also addressed the unresolved matter of the paucity of BrdU uptake in the hypothalamus, by asserting that intraventricular BrdU administration was significantly more efficient that interperitoneal injections, implying that previous studies were likely to have underestimated the levels of proliferation [62]. However, there is relatively little published material on hypothalamic neurogenesis in total. As robustness and reproducibility are two of the essential requirements for a scientific theory to become broadly accepted as true, anything which adds to the body of data in this field can only add weight to the hypothesis that neurogenesis occurs in the adult mouse hypothalamus.

My own data suggests the presence of stem cells in the adult hypothalamus. The formation of passagable neurospheres from the hypothalamus has been previously demonstrated [58],[59], however doubts have been raised about the stringency of this assay as a test for stem cells. Most commonly the argument is that the assay may also pick up precursor cells. If this were the case then it would be expected that different regions of the hypothalamus would give rise to spheres in a fairly uniform manner, as newborn cells have been observed throughout the whole hypothalamic parenchyma. The fact that my subdissection experiments show that only specific areas of the hypothalamus produce spheres suggests that more restricted cell populations are being picked up (Figure 5.4).

Although it is dangerous to rely on proposed stem cell markers to demonstrate the presence of stem cells, in addition with other evidence it can add to the case for the presence of stem cells. I observed many markers, linked to stem cells in other niches, in the hypothalamus. These include Sox1, -2 and -3, nestin, vimentin and GFAP (Figures 3.2, 3.3, 3.5, 3.9 and 3.4 respectively). There is still much debate as to the specificity of these markers, and it may well be that many of them are more indicative of general proliferative cells than true stem cells. In particular, Sox2 is often quoted as a stem cell marker, however I observed it both in numerous parenchymal cells in vivo (Figure 3.2) and in $MAP2^+$ neurons in vitro (Figure 4.4), indicating it may be far more widely expressed than is generally acknowledged (see also [47]). GFAP is another contentious marker – many people have hypothesized that it marks the original slow-cycling stem cell in the SVZ niche, and is lost in subsequent progenitors [41]. I observed that the FGF-responsive cells of the hypothalamus were located in both the $GFAP^+$ and $GFAP^-$ regions (Figure 6.8). Whatever doubts are raised regarding individual markers, it could be argued that their expression in combination in an individual cell is more compelling that the expression of a single marker. The expression of a number of prominent markers in both the in vivo hypothalamus

and in the hypothalamic neurospheres strengthens the evidence for the existence of hypothalamic stem cells.

8.1.2 Stem or progenitor cells?

In other proposed niches, much has been made of the debate over whether or not a true stem cell population of cells with an infinite capacity for self-renewal is present, as opposed to more restricted progenitors. It could be argued that this debate is a red herring, and that any population of cells with proliferative potential and a further differentiation capacity are worth studying. However, it is worth considering what type of proliferative cells are present in the hypothalamus. Stem cells and early progenitors are multipotent, capable of producing neurons, astrocytes and oligodendrocytes, whereas later progenitors may be restricted to just one or two lineages.

In vivo, newborn hypothalamic cells have been demonstrated to differentiate into neurons and oligodendrocytes, but not $GFAP^+$ astrocytes [62]. Rat, but not mouse, clonal hypothalamic neurospheres have been shown to produce all three lineages in vivo [59]. Although my neurospheres were unlikely to always be clonal at the plating density used, very few conjoined spheres were observed suggesting that the majority were likely to have been the product of a single original cell. Differentiated spheres were always observed to contain a proportion of cells from any lineage examined, irrespective of whether they were derived from a whole hypothalamic dissection or a subdissection. As no sphere actively missing a lineage was ever observed, it seems unlikely that the spheres were ever derived from only later lineage-restricted progenitors.

It is generally accepted that only neurospheres from a region containing stem cells can be passaged over the long term in vitro. While the cut off point is hard to define, it is clear that my spheres could be extensively passaged (Table 4.1). The maximum number of passages achieved was eight, however this experiment was terminated due to an incubator fault, and as the neurospheres had shown no reduction in number up until that point it seems likely that they

could have continued to passage well many more times. In addition, the passaging protocol was more rigorous that that used in some labs, where spheres may remain in substantial chunks rather than being reduced back to a single cell suspension on each passage.

8.2 Where is the niche located in the hypothalamus?

An important question concerns the location and character of hypothalamic stem cells. It has previously been suggested that tanycytes of the ventricular wall act as the original stem cell [59], although BrdU labelling marks cells scattered throughout the hypothalamus [62]. In order to resolve the question of which region(s) of the hypothalamus contain stem cells, I took a systematic approach to mapping the markers and neurosphere-forming potential of the different hypothalamic areas.

8.2.1 Stem cells and the anterior hypothalamus

One factor that has not been previously studied is the location of neurosphere-forming (ie proliferative) cells within the entire hypothalamus. Although previous studies made no mention of the A/P axis, it appeared from the pictures displayed that they focused on sections at posterior and ME levels, and did not examine the proliferation capacity of the anterior hypothalamus. I addressed this question using a combination of stem cell marker expression and hypothalamic subdissection experiments. By looking purely at markers, it appeared as though the anterior hypothalamus might be an interesting region (summarised in Figure 3.12). There were two main regions expressing an interesting combination of markers: the ependymal lining and the suprachiasmatic nucleus. The ependymal lining contained a few nestin$^+$ cells with a radial glial-like morphology, although tanycytes are documented not to exist in this region, and also expressed the three SoxB1 genes and GFAP. The SCN expressed SoxB1, GFAP, BLBP and Six3. There was an additional interesting region, only observed as having a high expression of pErk1/2, suggesting active growth factor

signalling (Figure 6.6). This was an ependymal region far anterior to the SCN, and cells here were also observed to have a radial glial like morphology. It is also known that newborn cells can be observed in the anterior hypothalamus using BrdU (D. McNay, personal communication). Despite the promising indications of marker expression, it was clear from the subdissection experiments that the anterior hypothalamus does not form neurospheres.

There are two possible explanations for these observations. Firstly, it may be that that no stem/progenitor cells with sufficient self-renewal capacity reside in the anterior hypothalamus. In this scenario, the stem cells markers in this region must be expressed either in late progenitors or mature cells, and the newborn cells observed by BrdU labelling would be the result of either glial proliferation or late stage progenitor cells that have migrated from elsewhere. Secondly, it is possible that a stem/progenitor cell population does exist in the anterior hypothalamus, but that it is not capable of forming neurospheres in the in vitro conditions used. This would make it unusual, as all the neural stem cells investigated so far can be induced to proliferate by either FGF and/or EGF. As well as a failure of proliferation, this effect could be the result of the lack of provision of a specific survival factor in the culture medium.

It would be interesting in the future to distinguish between these two hypotheses. The first theory could be tested by examining $BrdU^+$ cells in the anterior hypothalamus for activated glial markers, in addition to pulse-chase experiments to determine whether or not the newborn cells in this region have the fast-cycling characteristics of progenitor cells. Slice cultures may also be useful in examining this hypothesis, as separation of the anterior hypothalamus from the rest of the brain could be used to prevent additional progenitors migrating in, allowing only locally-derived cells to be studied. The second possibility could be tested by culturing the anterior hypothalamus in alternative culture media, containing extra growth or survival factors. However, it would not be practical to test every possible combination of factors. It would also be

interesting to see whether the infusion of factors other than FGF-2 and CNTF into the ventricle induced proliferation specifically in this region.

8.2.2 Stem cells and the ME/posterior hypothalamus

Most of the previous work on hypothalamic neurogenesis has focused on the region around the level of the ME, either because of the presence of tanycytes [59], or because it is the site containing the crucial nuclei of energy balance circuits [64]. My work complements and extends these observations, and provides strong evidence that stem cells are located at the level of the ME. Firstly, I was able to robustly derive neurospheres from this region (Figure 5.3 and 5.4). Secondly, a variety of stem cell markers were expressed, including SoxB1 genes, GFAP, vimentin, Six3, nodal and pErk1/2 (summarised in Figure 3.13, and 6.6). These markers were not only observed in vivo, but also in cultured neurospheres. The subdissection experiments separated out three A/P levels, with parts containing the ME separate from those located more posteriorly. The intention of this was to begin with an unbiased assay that roughly divided the hypothalamus into equal parts. However, from examining all the data it appears that this distinction has no biological relevance, and that there is no reason to think that posterior cells should be distinguished from ME-level ones. All the markers examined were continuous between the two regions. In addition, tanycytes are documented to be present as a continuous population, as confirmed by the morphology of cells labelled with vimentin antibody (Figure 3.9). For the rest of this discussion I will consider these two regions as one functionally discrete population. Where D/V issues are considered it is generally easier to consider data taken from the level of the ME, as the cell types are better documented at this level, and the ventricle is longer.

8.2.3 Ependymal vs. non-ependymal proliferation

The question of the precise location of stem cells is one that causes much debate regarding many niches. In the SVZ, it has long been thought that the original stem cell is located lateral to the ependymal layer, in a subependymal (or subventricular) layer [41]. While it seems relatively certain that the majority

of proliferating cells are located in this region, some researchers believe that the cell that gives rise to all other proliferating cells may in fact reside in the ependymal layer [126]. It has also been suggested that the DG stem cell niche is located in the ancestor of a ventricular zone, buried in the parenchyma due to folding of the tissue during embryogenesis [58]. These observations have led to the hypothesis that adult neural stem cell niches derive from the embryonic ventricular zone of proliferation, rather than the subventricular zone as originally thought. Therefore the question of whether or not hypothalamic stem cells are located in the ventricular wall may have implications for the neural stem cell field as a whole.

Although it does not definitively prove the theory, my data is certainly consistent with the idea that the stem cell is located in the ventricular zone. In subdissection experiments, cells dissected from lateral regions were never observed to form a single neurosphere (Figures 5.3 and 5.4). This suggests that both the stem cells and any early progenitors with sufficient self-renewal capacity to form neurospheres are never located distal from the ventricle. However due to the crudeness of subdissections, these experiments do not distinguish between cells of the ependymal lining and those in close proximity to it. This question has been partly addressed in rats, where it has been shown that cells taking up DiI from the ventricle, and therefore in contact with it, can form neurospheres [59]. However, it is possible that cells are located away from the ventricle, but access it via an apical process, an idea that has already been proposed in the SVZ [126]. Alternatively, there may be a number of different stem cell populations, which vary in their precise location. As with SVZ stem cells, this is a very difficult theory to prove either way.

8.2.4 Tanycytes as hypothalamic stem cells

If hypothalamic stem cells are indeed located in the ventricular wall, then tanycytes are a very good candidate for their identity. Theoretically they are attractive due to their similarity to the radial glia that proliferate to produce new neurons and glia during embryogenesis. As it is generally thought that they are

also actually derived from embryonic radial glia, it is not difficult to imagine that a developmental stem cell niche could have simply remained in a modified form in the adult organ.

In support of the idea of tanycytes functioning as stem cells is the observation that the neurosphere-forming regions of the hypothalamus exactly match the area containing tanycytes. Tanycytes are located in the wall of the third ventricle (Figure 3.9). They are first observed just anterior to the ME, and continue to the posterior limit of the ventricle. They are generally acknowledged to occupy the ventral two-thirds of the ventricle, whereas the dorsal third consists mainly of ependymocytes. I found neurospheres could be derived from all D/V levels of the ME-level hypothalamus (Figure 5.3). They were reliably produced from the MEV and MEM regions, and more variable from MED. This could be explained by the MED consisting of a mixture of tanycytes and ependymocytes, although we cannot exclude the possibility that ependymocytes may also be proliferative. Consistent with the co-localisation of neurosphere-forming regions in tanycytic areas, is the observation that FGF-2 induces proliferation in cells identified as tanycytes due to their position in the ventricular wall (Figures 6.7 and 6.8). Although this does not prove that they act as a stem cell, it demonstrates that they retain a proliferative capacity, and can enter the cell cycle under the influence of FGFs. As FGF ligands are present in the hypothalamus, it is likely that they can also undergo cell division under normal conditions (Figures 6.4 and 6.5). In support of this is the data on expression of pErk1/2 in the hypothalamus (Figure 6.6). In particular, it was observed that individual cells would occasionally have levels of pErk1/2 well above the baseline levels in surrounding cells. In one case, a cell was identified with extremely strong pErk1/2 labelling in the posterior hypothalamus. The cell body was located in the ventral ventricular wall, and it extended a long process into the parenchyma. This location and morphology leaves no doubt that the cell was a tanycyte. To follow up this observation, it would be helpful to combine pErk1/2 labelling with BrdU or Ki67, to confirm whether cells with such elevated pErk1/2 levels are proliferating, as seems likely.

8.2.5 Dorsoventral differences in hypothalamic stem cells

One of the more striking findings from the subdissection experiments was the difference in behaviour between neurospheres from different D/V levels. Whichever culture medium was used, it was found that ventral regions formed the highest number of spheres initially, but could be maintained for fewer subsequent passages. In contrast, mid/dorsal regions formed fewer primary spheres, but could be extensively passaged (Figure 5.3 and 5.4). Ventral neurospheres were initially largest, but were overtaken by mid/dorsal spheres at later passages (Figure 5.5). In addition, D/V differences were observed in FGF-induced proliferation in vivo, with greater number of $BrdU^+$ cells observed ventrally (Figure 6.7 and 6.8). No published work has yet looked at the possibility of D/V differences in hypothalamic stem cells, so it is an intriguing question to study further.

It is reported in the literature, and confirmed by my own studies of stem cell markers, that there are D/V differences in tanycytes, that can lead to them being classified into 5 subtypes. If, as seems likely, the tanycytes are acting as stem cells in the hypothalamus this leads to two alternative modes of D/V variation, represented in Figure 8.1. In developmental systems a spectrum of behaviours is often the product of a graded signal, with different intensities of signal resulting in different outcomes (Figure 8.1 A). Alternatively, D/V differences may be linked to the various tanycyte subtypes, resulting in discrete areas of defined behaviour (Figure 8.1 B). In order to distinguish between these options, both the subdissection and in vivo data must be analysed together. Ideally, in vivo data would look at background levels of ependymal proliferation, rather than that in response to FGF infusion. However, constitutive levels of cell division are low, and it would require a much larger sample size than that available here to draw out concrete patterns. Therefore this section does not address the question of how FGF-independent behaviour varies in the D/V axis, and can only use the response to a large excess of exogenous FGF to speculate about the endogenous role of FGFs.

When trying to integrate the in vivo neurosphere data with that from in vivo FGF infusions, the first question is what the implications of the different observations are, and whether they agree with one another. The data can be split into two types – data that give information on the immediate proliferative behaviour of cells, and those that concern a longer-term ability to continue proliferating. Information on this longer-term capacity to proliferate can be gained by studying passaged neurospheres, while primary neurospheres and the response to FGF-2 infusion are more related to immediate responses. It was found that mid/dorsal regions contained modest numbers of newborn cells after FGF-infusion (Figures 6.7 and 6.8), but that when cultured as neurospheres could be maintained for the highest number of passages (Figures 5.3 and 5.4). In contrast, the ventral region contained a zone that readily proliferated in response to FGF (Figures 6.7 and 6.8), and formed many initial neurospheres with limited self-renewal (Figures 5.3 and 5.4). Looking more closely at the in vivo FGF data, it seems there are two ways of interpreting the greater number of $BrdU^+$ cells seen ventrally.

1. It could be that there are a larger number of $BrdU^+$ cells because the proliferative cells in this region have all produced a higher number of progeny, by cycling faster than more dorsal stem cells. This would correlate well with the observation that ventral regions produce larger primary neurospheres than mid/dorsal regions (Figure 5.5).

2. Another option is that more newborn cells are found ventrally because a higher proportion of ventrally-located cells have been induced to proliferate. This is supported by the fact the majority of ventricular cells in the ventral region are $BrdU^+$, whereas dorsally $BrdU^+$ cells are widely scattered amongst a majority of inactive cells (Figures 6.7 and 6.8). This would also fit well with the data showing that higher number of primary neurospheres are produced from ventral areas (Figure 5.4).

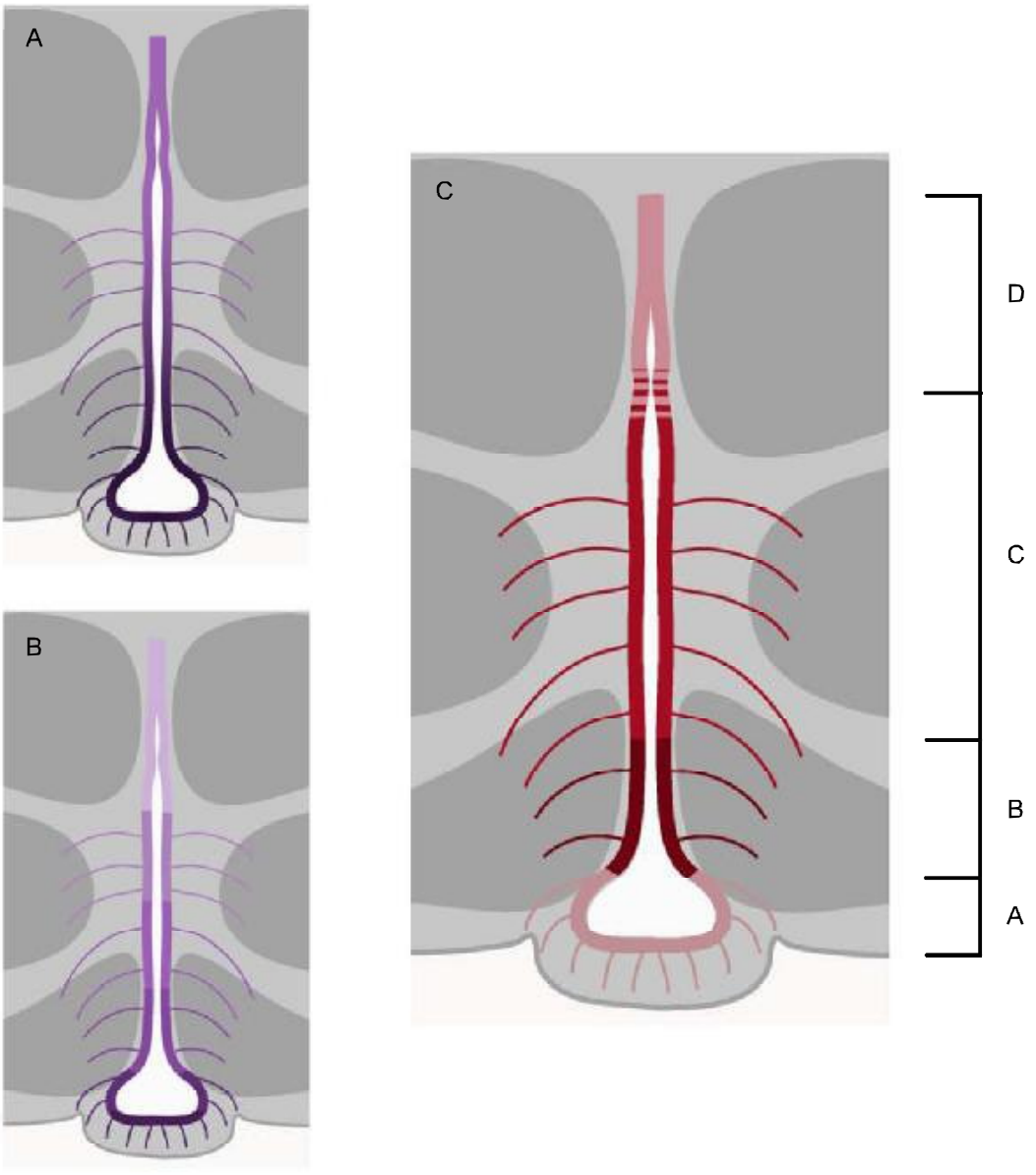

Figure 8.1

Models of dorsoventral differences in hypothalamic stem cells

These cartoon illustrate three alternatives for how stem cells might vary in their proliferation, with darker colours representing greater proliferation. A. A ventral to dorsal gradient. B. A ventral to dorsal gradient, with discreet zones based on tanycyte subtypes. C. Four distinct zones, with high levels of proliferation in zones B (vα2 tanycytes) and C (dα2 and α1 tanycytes).

These two options are not mutually exclusive, and either of them or both may turn out to be true. At first glance the neurosphere data seems to suggest that they both have an influence to some extent, however care needs to be taken with the neurosphere interpretation. It is not clear what the exact relationship is between number and size of neurospheres, but it unlikely that they are entirely independent of each other. For example, in wells with a high density of spheres they are presumably more likely to collide and merge together, producing larger spheres.

The implications of data on the long-term proliferation of cells is simpler to interpret. It seems clear that the ability to repeatedly form new neurospheres after passaging must be related to the ability of a cell to self-renew. The fact that mid/dorsal neurospheres consistently survived for more passages than ventral neurospheres suggests that they have a greater self-renewal capacity (Figure 5.4). Whether it is truly unlimited is not yet clear, but it seems fair to conclude that dorsally-located cells can undergo a greater number of self-renewing divisions than ventral cells. As this effect is seen under in vitro conditions, it further demonstrates that the effect is intrinsic, and not reliant on external niche factors from other cells.

Drawing all this together, the ME/posterior hypothalamus can be sensibly split into 4 distinct zones, as illustrated in figure 8.1 C. Zone B corresponds to the highly FGF-responsive ventral region described above, and also to a portion of the ventral neurosphere subdissections. In terms of tanycytes subtypes, the lack of GFAP in this region indicates that it is at the level of ventral $\alpha 2$ cells. The cells here appear to readily respond to FGF signalling with high levels of proliferation, however they have a limited capacity for self-renewal. This region expresses FGF-18 in vivo, which is likely to be the endogenous ligand controlling proliferation in this system.

Zone C is located dorsal to this, at the approximate level of GFAP-expressing dorsal $\alpha 2$ and $\alpha 1$ tanycytes. The cells here are still capable of proliferation in

response to FGF signalling, but respond less readily. It is not clear whether all the tanycytes in this region have this ability, or whether it is limited to a subset of them. Cells here appear to proliferate more slowly that those in zone B, however they have a much higher capacity for self-renewal.

Zone D is much less well defined, and indeed it is not clear whether there is a good case for classing it separately to C. It is based largely on the knowledge that the region contains mostly ependymocytes as opposed to tanycytes, rather than any observable difference in behaviour from cells in the C zone. Part of the difficultly comes from the issue of how to define the border with the tanycyte rich region below it. It is not clear whether there is a distinct boundary where tanycytes give way to ependymocytes, or a more gradual one with the two cell types intermingling in dorsal regions of the ependyma. An intermingled border would lead to confusing results, with dorsal tanycytes responsible for the formation of dorsal subdissection neurospheres. Alternatively, ependymocytes may also be induced to proliferate by FGF-2. Arguing against this possibility is the fact that no differences in marker expression were found between dorsal neurospheres and those from other regions, indicating that they are all likely to be derived from very similar cell types.

Finally, zone A represents the ventral-most region of the ventricle, where β tanycytes can be found. Data from growth factor infusion experiments indicates that this region does not proliferate in response to FGF-2. The standard subdissection is not useful for drawing conclusions on this area, as it is combined with zone B in ventral subdissections. I am currently working to overcome this by altering the subdissections to distinguish α tanycyte from β tanycyte regions (Figure 5.6). Some preliminary experiments do suggest that the β tanycyte / A zone does not readily form neurospheres. I will continue to persevere with this line of experimentation in the future, to answer conclusively whether this region of the hypothalamus is proliferative.

One possibility that merits further investigation is that the region is indeed proliferative, as proposed by other authors [20], but is not responsive to FGF-2. This is supported by the in situ data, showing that FGF-10 but not FGF-18 is expressed in this region. As FGF-10 signals through different receptor isoforms from those that bind to FGF-2, it may simply be that cells in this region are incapable of responding to FGF-2. In order to test this theory, the first step would be to carry out a thorough investigation of which FGFR isoforms are expressed in which locations. If the ventral zone A does indeed lack FGF-2 responsive IIIc receptors, then it would be interesting to repeat the infusion experiments using FGF-10 instead. It would also be possible to repeat the neurosphere subdissections using FGF-10 supplemented media.

The significance of the four zones outlined above is not yet clear. It may be that they represent four distinct populations of NS/PCs, each with unique properties and functions. Alternatively, it may be that they represent different stages in the progression of a single cell lineage. Future work into this question could use cell tracking techniques to determine whether individual cells ever migrate between the different compartments.

8.3 Regulation of hypothalamic stem/progenitor cells

Factors governing the regulation of proliferation, migration and differentiation have been extensively studied in other stem cell niches, but little has been done concerning hypothalamic cells. One study injected a range of growth factors into rats, and analysed the total number of newborn cells by BrdU incorporation [59]. They reported a modest trend towards an increase in $BrdU^+$ cells using N2, BDNF or EGF, and a significant increase using either FGF-2 or FGF-2 + EGF. A separate study demonstrated a large up-regulation of proliferation in response to CNTF in mice [64]. However, neither of these studies looked closely at whether these mechanisms were likely to be employed in normal animals, or whether they were necessary for proliferation.

8.3.1 An in vivo role for FGFs?

In this study, I have focused on the potential role of FGFs in regulating hypothalamic stem/progenitor cells. In other systems, FGF-2 is known to be capable of exerting a mitogenic effect, and it seems likely that it plays the same role in the hypothalamus. Neurospheres are grown in FGF-2 supplemented media, and it is possible that many of the observations noted above, for example the D/V differences in behaviour, are actually the product of a differential response to FGFs. It is clear that there are cells located in the ependymal lining of the third ventricle that are capable of responding to FGF-2 with a proliferative response (Figures 6.7 and 6.8). The majority of these cells are in the region containing tanycytes, however it cannot be ruled out that more dorsal ependymocytes may also respond. In contrast, parenchymal cells do not seem to respond to FGF-2. This correlates with the information gained from neurosphere experiments, which shows that only midline regions containing ependyma are capable of forming neurospheres (Figure 5.4). Furthermore, inhibitor experiments clearly demonstrate that these cells cannot proliferate in the absence of FGF signalling, and that it is likely that neurospheres can produce some FGFs themselves (Figure 6.9). In combination, this data strongly suggests that FGF signalling is likely to be an active method of stem cell regulation in the normal hypothalamus.

Two different FGFs were observed to be expressed by the hypothalamic ependyma: FGF-10 and FGF-18 (Figures 6.4 and 6.5). In addition, previous reports have looked for various FGFR isoforms in the hypothalamus. Little or no FGF-10-binding IIIb receptors were present, leaving the role of FGF-10 unclarified [20]. However, they do demonstrate the presence of two receptors capable of binding FGF-18: FGFR1IIIc and FGFR2IIIc. Although there is currently no information on the precise distribution of these receptors, their affinity for FGF-2 would suggest that they may be present in the portions of the ependyma that responded to FGF-2 infusion (Figures 6.7 and 6.8). The large 'ventral cluster' of proliferative cells coincides exactly with the observed region

of FGF-18 expressing cells. The presence of an FGF ligand in this region, together with the apparent cellular machinery to respond to it, strongly suggests that FGF-18 is the endogenous ligand that normally acts to regulate proliferation in this area.

8.3.2 The role of other growth factors

Although I have focused on the role of FGFs, the involvement of several other growth factors is implicated by my work. Most notable is the observation that exogenous EGF is required for neurosphere formation to be sustained over even just a single passage (Figure 6.9). A previous study noted that the infusion of EGF produced a modest increase in the number of $BrdU^+$ cells observed in vivo, and that in combination with FGF it contributed to a larger increase [59]. However, it did not produce an effect of the magnitude to that seen with FGF-2 alone. It seems unlikely that FGF-2 and EGF are simply required to activate the same mitogenic pathway, as the neurosphere results do not suggest a redundancy of function between them. Perhaps a more likely proposition is that EGF may act as a survival factor for either stem or progenitor cells, or may act to modify the effects of FGF. What exactly the role of EGF is in this system cannot be elucidated purely on the evidence available here, however it would make an exciting topic for a future study.

The differences in neurosphere growth and passagability between the two different media formulations also hints at a role for the insulin pathway (Table 4.1). Changes to insulin signalling caused by disease states or genetic modifications are reported to have complicated effects on hypothalamic proliferation (D McNay, personal communication). It is clear from the neurosphere data that switching the media formulation from one containing abnormally high insulin levels to one containing a more physiologically normal level of IGF dramatically increases the speed of neurosphere growth and the number of passages that can be maintained. Again, the data available here is insufficient to anticipate what precisely the role of insulin may be in this system, but it will be interesting to see what is deduced in the future.

8.3.3 The role of Six3

Another intriguing question in the regulation of hypothalamic proliferation is what factors might be involved in the differences observed between dorsal and ventral tanycytes (summarised above in section 8.2.5). The fact that differences could be observed in neurosphere passaging suggests a cell intrinsic effect, which does not rely on an external niche. Therefore, it is sensible to look for factors which are differentially expressed between dorsal and ventral tanycytes, and which are maintained in neurospheres. The investigation of potential stem cell markers revealed few D/V differences, with one of the exceptions being in the expression of Six3 (Figure 3.6). This appeared in an approximate ventral to dorsal gradient, possibly linked to tanycyte subtypes. It was strongly expressed in the most ventral regions, presumed to equate to β tanycytes. A reduced expression zone was seen adjacent to this, roughly equivalent to the α2 area. Further dorsally where α1 tanycytes and ependymocytes reside, it was not clear whether it was expressed weakly or not at all. Interestingly, expression also varied in neurospheres, with mRNA levels ranging from absent to strongly positive in different spheres (Figure 4.7). The next step would be to determine whether Six3 expression in neurospheres correlates with the region from which they are derived, with the expected result being high expression in ventral spheres and low in mid/dorsal ones. Preliminary results have been inconclusive, however it is a simple experiment to repeat. If a correlation were established, it would be interesting to consider whether Six3 might in fact have a causative role in the capacity for neurospheres to be repeatedly passaged. It has been reported previously that Six3 may act to shorten cell cycle length in developmental systems [81], and this would be an ideal mechanism to produce the kinds of effects seen in the neurospheres. For example, if ventral cells expressing high Six3 had a shorter cell cycle, it would explain how neurospheres from this region initially grow larger than more dorsally derived ones, as each cell could undergo more divisions in the same period of time. In addition if most cells (the progenitor cells) can only undergo a certain total number of divisions, it could explain why this limit is reached sooner in ventral

cells, resulting in neurospheres failing to passage. In order to test this theory, it would be interesting to alter the expression of Six3 in neurosphere forming cells. If electroporation techniques could be used with a DNA construct to express Six3 in mid/dorsal cells, and with siRNAs to inhibit it in ventral spheres, then its effect could be determined.

8.4 A model for hypothalamic stem and progenitor cells

When the data on the requirement for FGF signalling presented here is combined with information from the literature regarding the role of CNTF in hypothalamic neurogenesis, one potential basic model for hypothalamic neurogenesis stands out. I have demonstrated that FGF-responsive cells are located in the ependymal lining (Figures 6.7 and 6.8). By contrast CNTF-responsive cells are found throughout the hypothalamic parenchyma ([64] and Figure 6.8). Thus, it is clear that they are separate and distinct populations. However, several lines of evidence point to them actually being two different stages of a stem to progenitor cell progression. Firstly is the idea first suggested by Xu et al, that the daughter cells produced by ependymal divisions could migrate into the parenchyma along tanycytic processes [59]. Second is the observation that when CNTF and FGF are infused together, the same pattern is seen as when FGF alone is used, ie high ependymal and low parenchymal proliferation (Figure 6.8). This suggests that FGF can inhibit CNTF-responsive cells, either actively, or by reducing their numbers. Third is their apparent difference in character, as noted from in vivo BrdU studies without additional FGF-2 (Figure 6.7). Constitutive proliferation is more commonly seen in the parenchyma than in the ependymal lining, although both can occur. This suggests that the ependymal cells cycle much slower than those in the parenchyma do. It is well established that in other stem cell systems there is a relatively quiescent stem cell, which gives rise to more rapidly proliferating progenitors. If the hypothalamic niche follows this precedent, it points to the tanycytes acting as the stem cell, and the parenchymal cells being progenitors.

Finally, it has been shown that newborn cells in the hypothalamic parenchyma are unlikely to have migrated from other regions of the CNS [62], and so the alternative is that they must derive from somewhere within the hypothalamus. In the absence of any other proposed stem cell niches in this region, it is logical that the parenchymal progenitors derive from the tanycyte stem cells.

Putting all this data together, I propose a model whereby tanycytes in the ependyma act as neural stem cells (Figure 8.2). In accordance with other adult stem cell systems, it is likely that they are usually relatively quiescent, and capable of asymmetric division to give rise to a replacement tanycyte, plus a daughter progenitor cell. These progenitors do not retain the ability to proliferate in response to FGF, but instead can be induced to divide by exposure to CNTF. They migrate into the parenchyma, and rapidly divide a number of times before their progeny differentiate into neurons and oligodendrocytes.

Within this basic model, there are many possible permutations. Below I suggest four alternatives for how the FGF-2 + CNTF and neurosphere data could fit into or modify this basic model:

1. FGF influences symmetric versus asymmetric stem cell division:
Tanycytes may be capable of both symmetric self-renewing divisions that produce only more tanycytes, and asymmetric divisions that give rise to a tanycyte and a progenitor. In this model, high levels of FGF (such as those produced by experimental FGF infusion, rather than normal physiological levels) induce only self-renewing divisions, at the expense of producing progenitor cells. This would lead to a depletion of parenchymal progenitors, and thus the lack of proliferative response to CNTF. In this case, parenchymal proliferation would be reduced, but not completely removed due to the presence of progenitors from before the start of growth factor infusion. A close inspection of the data reveals that this is in fact the case: some parenchymal BrdU is present, although it is often much weaker than the in the ependymal cells. If all the parenchymal BrdU is located in rapidly-proliferating progenitors,

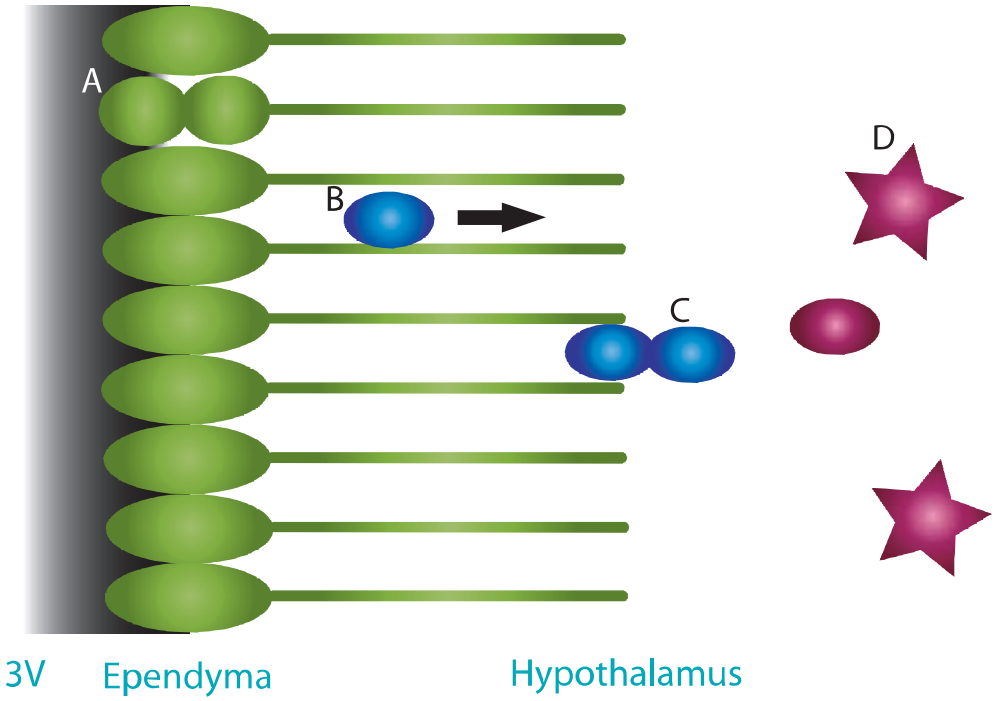

Figure 8.2

A model for hypothalamic stem and progenitor cells

Tanycytes (green) are capable of responding to FGF, but are generally quiescent. When they do divide, (A) the daughter cell becomes a CNTF-responsive progenitor (blue) and migrates laterally into the parenchyma (B). Here it can divide further (C), before giving rise to differentiated cell types (pink, D).

it is likely that cells will have continued to divide after the period of BrdU administration, diluting the level of BrdU. It is important to consider how the neurosphere data relates to this model. Generally, it is thought that neurospheres contain a small percentage of stem cells, while the bulk of tissue comprises of the various progenitor and precursor stages [127]. It is unlikely that a neurosphere could be composed entirely of stem cells, as their slower cycling time would be unlikely to produce sufficient numbers of cells, casting doubt on the practicality of this idea. This model could be tested if a method of measuring of asymmetric versus symmetric division could be found for tanycytes. It may be related to the plane of division as with other polarized systems, with symmetric divisions parallel to the plane of the ventricular surface, and asymmetric divisions perpendicular to it. If so, it should be possible to combine BrdU or Ki67 labelling for proliferating cells with a spindle marker to show the plane of division, in the presence or absence of FGF. This would be possible either in vivo, or in slice cultures. However, a drawback is that an extremely high sample number would be needed to provide sufficient cells caught in the act of division, especially in the absence of FGF.

2. FGF influences progenitor lineage:

It may be that there is more than one progenitor lineage, and that FGF influences this fate choice. In this scenario, FGF directs stem cell progeny away from becoming CNTF-responsive progenitors in favour of an alternative progenitor lineage. This idea is better supported by the neurosphere data, although it is not yet complete. In support of the idea that there could be multiple progenitor lineages is the fact that all three neural lineages can be produced from neurospheres (Figure 4.3), but only neurons and oligodendrocytes are seen in vivo [62]. However, this could just as easily be the result of differing environmental factors between neurospheres and in vivo cells. Assuming neurospheres do contain both stem and progenitor cells, then using only the basic model most cells in a hypothalamic neurosphere would be expected to be CNTF-responsive. However, if FGF directs precursors away from

a CNTF-responsive lineage, this would be reversed. Preliminary experiments failed to find any evidence for a response to CNTF in neurospheres, with neurospheres grown in CNTF-supplemented media growing at the same rate and frequency as those without (data not shown). It should be noted that this is not conclusive proof of a lack of CNTF responsiveness, as it is possible that neurospheres already produce sufficient CNTF to saturate the system, or that it can be driven by another factor already present in the media. EGF or IGF would be the most likely candidates for this. In order to resolve this question, it would be useful to ascertain whether neurospheres express CNTF and CNTFR. Unfortunately, it does not seem to be possible to selectively inhibit CNTFR signalling pharmacologically, and the downstream pathways share too many components of other growth factor pathways to be viable targets. However, it may be possible to produce a blocking antibody, and it would be interesting to test how that would influence neurosphere formation.

3. FGF inhibits proliferation of progenitors:

It is possible that progenitors are still capable of responding to FGF stimulation, but react in some way that actually reduces cell division. This could be either a direct inhibition of proliferation, or by promoting alternative choices, for example entering quiescence or differentiation. If this effect were dominant to that of the CNTF, then it would explain the lack of parenchymal proliferation when FGF and CNTF are applied together. However, this is not supported by the region's ability to form neurospheres. If this model were true, then it would be unlikely that neurospheres would ever form, as progenitors would always be prevented from undergoing a phase of rapid divisions by the FGF-2 present in the cell culture media. This stage is thought to be essential for producing enough new cells to give rise to a large neurosphere. One modification to this theory that may negate the issue of neurosphere formation, is if the effect of FGF on progenitors is indirect, requiring another cell type present in the hypothalamus but not in neurospheres to act as intermediary.

4. FGF inhibits progenitor migration:

Finally, the question may not be one solely of proliferation, migration may also be involved. In this model the excessively high levels of FGF used in these experiments inhibits the migration of newborn progenitors, leaving them clustered in the vicinity of the ependymal region. If they were to ectopically proliferate from this position in response to the CNTF, then it would be predicted that the total ependymal proliferation would be greater for both factors than for FGF alone. Although the data has not been quantified with this question in mind and a larger sample would probably be required, the pictures may indicate that there is in fact a trend for a greater number of $BrdU^+$ cells when CNTF is applied in conjunction with FGF-2, relative to FGF-2 alone (Figure 6.8). Future experiments to back up this observation would not be simple as migration is difficult to assess in vivo, but slice cultures may provide a viable alternative. Ideally this would be done using marked ependymal cells, or even time-lapse video if performed in slices, to see whether tanycyte progeny migrate into the parenchyma in the presence and absence of exogenous FGF. Although it may require a large sample size as the number of proliferating cells in the absence of FGF is very low, a similar experiment (not assessing the effect of FGF) has been successfully reported in the past [59].

8.5 Neurospheres and stem cells

An unresolved issue with the neurosphere assay is whether the cells it identifies as stem cells due to their proliferation capacity are the same as those identified in vivo by techniques such as BrdU labelling. Stem cells are generally accepted to be highly dependant on their surrounding niche, both in terms of regulatory factors secreted by neighbouring cells, and through cell surface contacts with other cells, the ECM, the CSF and the vasculature. In contrast, the neurosphere assay selects strongly for cells capable of both surviving and proliferating in the absence of all these supporting influences. Therefore it should be confirmed that the neurosphere forming cells selected in vitro are the same as the BrdU

incorporating cells identified in vivo before the two techniques can be convincingly combined to prove the existence of a new stem cell population.

This project makes extensive use of the neurosphere assay, a commonly used but still controversial cell culture technique. It is sometimes claimed that neurospheres are cited as proof of the existence of an in vivo stem cell population without due consideration for how the assay actually works and what biases need to be acknowledged. The major drawback of this assay is it's highly artificial nature, whereby cells are removed from their natural environment to be separated into single cells, and so are tested for proliferative capacity either in the presence of no other cells at all (first divisions), or in the presence of only their progeny (later neurospheres). Given the highly significant role attributed to the stem cell niche in most systems, this would be expected to have a substantial effect on the cells present. It may be that this effect underpins the usefulness of the neurosphere assay; in particular in very quiescent niches, the removal of surrounding cells with an inhibitory effect may be necessary to reveal the proliferative potential of a stem cell that is only rarely active in vivo. However, it may also be true that the artificial nature of this assay can reveal proliferation in cell populations that never undergo mitosis in their in vivo situation, and so should not be considered stem cells.

For the reasons outlined above, it is vital to combine neurosphere results with in vivo data, especially in the case of a novel niche. It is clear from both the work presented here and previous studies that proliferating cells are present in the hypothalamus under normal conditions, in addition to the extra proliferation that can be induced by FGF-2 or CNTF (Figures 6.7 and 6.8, and [62]). The circumstantial evidence strongly suggests that tanycytes are the source of both in vivo proliferation and in vitro neurosphere formation, however the argument would be strengthened considerably if it could be conclusively shown that tanycytes comprise the exclusive neurosphere forming cell in this system. Two complimentary approaches could be used here. Firstly, it should be possible to selectively label tanycytes with DiI from their basal processes using slice

cultures. This approach is superior to previous experiments using DiI incorporated from the ventricular side [59], as both ependymocytes and any subventricular astrocytes with a ventricular contact would be excluded. Labelled cells could then be sorted using a FACS machine, and grown into neurospheres proving that tanycytes are capable of initiating neurosphere formation. Taking the converse approach, it may be possible to selectively ablate tanycytes from the dissected cell suspension. Tanycytes have been reported to express the glucose transporter GLUT2, which is known to confer cells with sensitivity to the toxic effects of the antibiotic streptozotocin. If immunohistochemistry confirms that GLUT2 is found only on tanycytes within the hypothalamus, it would be predicted that brief treatment of the cell suspension with streptozotocin before culture would prevent neurosphere formation, demonstrating that tanycytes are absolutely required for neurosphere formation. If purified tanycyte populations could be cultured using the DiI approach outlined above, an additional corroborating experiment would be to use spheres known to be derived exclusively from tanycytes to repeat the results of Markakis et al, who demonstrated the generation of all uniquely hypothalamic neural cell types from whole hypothalamic spheres [58].

Additionally, there are a number of exclusively in vivo experiments that would considerably strengthen the argument for a hypothalamic stem cell niche. One robust way to identify a stem cell is by it's ability to repopulate a depleted niche, and so it would be interesting to repeat the successful SVZ experiments of Doetsch et al [128] in the hypothalamus, by depleting the fast cycling progenitors using an agent such as Ara-C. If tanycytes do act as the stem cell in this system, it would be predicted that they would be relatively unscathed by the initial Ara-C treatment, but would then proliferate to replace the lost parenchymal progenitor cells. Lineage tracing techniques would be expected to show they could ultimately contribute new mature neurons within the hypothalamus. Finally it may be useful to conduct label-retaining experiments in the hypothalamus, to confirm that tanycytes are slow cycling cells. However it should be noted that questions have been raised about the accuracy of this

method for identifying stem cells [129], and how it is affected by the discovery that many stem cells selectively segregate one strand of DNA to be retained in the self-renewed daughter cell [130].

In summary, the work presented here and elsewhere can currently only suggest the presence of a stem cell niche in the hypothalamus. However, experiments such as those suggested above may provide a way to increase the impact by more strongly linking the in vitro and in vivo data. Only once experiments such as these have been conducted is it likely that the hypothalamus will be accepted to contain a true stem cell niche.

8.6 Concluding remarks

The possibility that hypothalamic stem cells exist, and have an important physiological role to play, has been largely ignored until recently, and work into this phenomenon is still at a very early stage. However, all the evidence suggests that this emerging field has the potential to yield many exciting new discoveries. The work detailed here adds to evidence that there is a tightly regulated niche of stem and progenitor cells in the hypothalamus. I provide evidence that self-renewing cells are located only in defined areas of the hypothalamus, and suggest some markers that may be valuable in identifying and characterizing them. In addition, I introduce data to suggest that FGFs play an important role in the regulation of this niche. This information, together with the techniques I have developed to study hypothalamic proliferation, begins to answer some of the huge questions raised by the presence of a novel neurogenic niche. However, ultimately, this project raises more questions than it provides solutions to, and I hope that future researchers will be able to use some of these questions to expand the knowledge in this field even further.

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