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Exploring atypical locations of mammalian neural stem cells: the human filum terminale

M. VARGHESE[1,2], H. OLSTORN[1,2], W. MURRELL[1], I.A. LANGMOEN[1,2]

[1] Vilhelm Magnus Laboratory, Institute for Surgical Research, University of Oslo, Norway;
[2] Department of Neurosurgery, Oslo University Hospital, Ullevål, Norway

ABSTRACT

Neurogenesis is a multifactorial event determined by local environmental cues, inherent cellular program as well as cellular milieu and may not necessarily be restricted to the SVZ and SGZ. NSCs have been isolated from or neurogenesis has been demonstrated in traditionally non neurogenic regions. This more permissive view of neurogenesis, however, is not widely accepted due to concerns regarding the methodologies used. Furthermore, it is compounded by the fact that the basal levels of increased neurogenesis in such regions has not been completely confirmed and thus precludes a paradigm shift. Were this non limited view of neurogenesis to be generally accepted after thorough investigation, it would open new avenues for regenerative medicine and stem cell therapy.

Key words

Neural stem cells • Human • Neurogenesis • Filum terminale

Introduction

Neurogenesis is defined as the de novo generation of functionally integrated neurons from neural progenitor cells (Emsley et al., 2005). The genesis of new neurons was considered to be prevalent in invertebrates and in non mammalian vertebrates such as fish, reptiles and birds (Gage, 2000). In contrast, neurogenesis in the adult mammalian brain was thought to be nonexistent (Colucci-D'Amato et al., 2006) and the prevailing view was "no new neurons after birth" (Ramon y Cajal, 1913). This dogmatic view in neuroscience existed for almost a century (Rakic, 2002).

Novel developments in cell labeling techniques using radioactive thymidine, which incorporates into replicating DNA, identified for the first time dividing neurons in three day old mouse brains (Smart, 1961). This pioneering study showed for the first time ongoing postnatal neurogenesis in an adult central nervous system (CNS). Later, similar dividing neurons were identified in the adult rat CNS (Altman, 1966); in the lining of ventricles of songbirds (Goldman and Nottebohm, 1983) and primates (Rakic, 2002). However, others did not find any such evidence for neurogenesis in the adult brain (Rakic, 2002).

The introduction of bromodeoxyuridine (BrdU), a synthetic thymidine analogue allowed for combined immunocytochemical and phenotypic identification of de novo neurons (Gratzner, 1982). Using the aforementioned technique, Eriksson et al. (1998) demonstrated that new neurons (positive for BrdU and neuronal marker NeuN) were generated from dividing progenitor cells in the sub granular zone (SGZ) of the dentate gyrus of the post mortem adult human hippocampus. This pioneering study as well as other similar studies provided unequivocal evidence for the first time of postnatal neurogenesis in the adult human CNS (Alvarez-Buylla and Lim, 2004; Sanai et al., 2004; Curtis et al., 2007). Recently, neurogenesis has also been

Corresponding Author: Mercy Varghese MD, PhD, Vilhelm Magnus Laboratory for Neurosurgical Research, Institute for Surgical Research, Sognsvannsveien 20, Rikshospitalet, 0027 Oslo, Norway - Email: mercy@me.oslo.no

documented in the live human brain using magnetic resonance brain imaging techniques (Manganas et al., 2007).

Neural Stem Cells

It has been shown that a small subset of cells residing in the sub ventricular zone (SVZ) and SGZ of the adult CNS are responsible for the de novo generation of postnatal neurons (Gage, 2000). These cells are known as neural stem cells (NSCs) and exhibit cardinal stem cell properties of self renewal, proliferation and trilineage differentiation into astrocytes, oligodendrocytes (collectively known as glia) and neurons (Alvarez-Buylla and Lim, 2004). Neurons are the functional components of the CNS, responsible for information processing and transmission whereas glia are primarily regarded as supporting cells essential for proper functioning of the CNS (Ming and Song, 2005).

The identity of NSCs in the CNS is elusive even today. Johansson et al. (1999) provided evidence for ependymal cells (cells lining the ventricle) as putative NSCs, while Doetsch et al. (1999) identified glial fibrillary acidic protein (GFAP) positive astrocyte like cells, which reside beneath the ependymal layer in the SVZ as the more likely NSC candidate. The current prevalent view posits that GFAP positive astrocytes are NSCs (Ming and Song, 2005). However, the debate is still ongoing, with a recent study providing evidence in support of an ependymal NSC paradigm (Coskun et al., 2008).

NSCs were first isolated from mouse striatum (Reynolds and Weiss, 1992). Later, similar NSCs were isolated from the ventricular wall (Morshead et al., 1994) and hippocampus of adult humans (Kukekov et al., 1999). When grown in a defined culture medium consisting of mitogens such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF), NSCs exhibited self renewal and proliferation to generate free floating multicellular spheres, called neurospheres (Gottlieb, 2002). This neurosphere assay is currently the *in vitro* standard for determining the presence of NSCs (Reynolds and Weiss, 1992).

Withdrawal of mitogens or exposing NSCs to factors including retinoic acid (Jang et al., 2004; Kim et al., 2009), induces NSCs to differentiate into neurons (electrically active bipolar or multipolar cells) and glia (electrically passive flat pancake like or protoplasmic cells) (Gage, 2000). This has been shown both *in vitro* (Moe et al., 2005) and *in vivo* (Olstorn et al., 2007). We and others have highlighted the discrepancy that exists between the cellular morphology of differentiated cells and their functional profile (Ming and Song, 2005; Moe et al., 2005). In fact, studies have shown that morphology and immunocytochemistry are not necessarily predictive of the physiological properties of a cell (Ming and Song, 2005). This makes functional analysis using electrophysiology an important tool in investigating NSCs (Song et al., 2002) given that future clinical applications depend on the differentiation of NSCs into functional cells (Gage, 2000). We have used whole cell patch clamp studies and demonstrated that NSCs isolated from adult human CNS can indeed differentiate into functional neurons that exhibit mature regenerative action potentials and communicate via synaptic transmission (Moe et al., 2005). This is a characteristic hallmark of neurons (Reh, 2002) and is absent in glial cells (Moe et al., 2005). Taken together, we have shown that NSCs isolated from the adult human ventricular wall develop into morphologically and functionally mature neurons (Moe et al., 2005).

Neurogenic regions and the neurogenic niche

A neurogenic region promotes neuronal differentiation and integration of both transplanted as well as intrinsic cells (Kempermann, 2006). In the adult CNS there are two regions where neurogenesis occurs constitutively *in vivo*; the SVZ of the lateral ventricle wall and the SGZ of the hippocampal dentate gyrus (Gage, 2000). The neurons generated in the SVZ migrate anteriorly through the rostral migratory stream into the olfactory bulb and become interneurons (Alvarez-Buylla and Lim, 2004). The neurons in the dentate gyrus of the hippocampus develop into new granule neurons (Ming and Song, 2005) and may play an important role in learning and memory (Leuner et al., 2006; Wu et al., 2008).

Neurogenic regions harbor NSCs within a unique neurogenic niche (Morrison and Spradling, 2008). The neurogenic niche is defined as a microenviron-

ment consisting of specific factors that intimately support and tightly regulate stem cell property and behavior (Alvarez-Buylla and Lim, 2004). Moreover, the niche provides a permissive environment for differentiation and integration of new neurons (Zhao et al., 2008) and also safeguards the stem cells from excessive cell production which may lead to cancer (Moore and Lemischka, 2006). Though detailed anatomical and functional components of the neurogenic niche in both SGZ and SVZ remain elusive, studies suggest that the cytoarchitecture of the niche includes astrocytes, extracellular matrix and blood vessels (Morrison and Spradling, 2008).

NSC behavior in the niche is regulated by signaling molecules, including growth factors, neurotransmitters and hormones (Doetsch et al., 1999). Briefly, signaling pathways including Notch, Eph/ephrins, Sonic hedgehog, Wnt and Bone morphogenetic proteins regulate important aspects of NSC proliferation and differentiation (Lim et al., 2007). Sonic hedgehog has been shown to regulate proliferation and maintenance of NSCs as well as neurogenesis of SVZ and SGZ NSCs (Alvarez-Buylla and Lim, 2004). Similarly, studies have shown that infusion of EGF and FGF onto SVZ cells, *in vitro* and *in vivo*, dramatically expands the NSC population (Gage, 2000). Moreover, bone morphogenetic proteins and their antagonists, acting alone or in combination with leukemia inhibitory factor, maintain SVZ NSCs (Reynaud-Deonauth et al., 2002) suggesting that investigating the factors that regulate NSC fate determination 1) is important for *in vitro* NSC expansion to sufficient numbers for transplantation paradigms; 2) allows us to mobilize endogenous NSCs for neural repair using niche factors (Lim et al., 2007).

Atypical neurogenesis

As mentioned earlier, there are two constitutive neurogenic sites in the adult CNS, the SVZ and SGZ (Gage, 2000). The remainder of the CNS is considered to be non neurogenic (Kempermann, 2006). An emerging view, however, suggests that progenitor cells from regions previously considered to be non neurogenic may in fact be capable of proliferation (Kempermann, 2006). In fact, Gould et al. (1999) has shown parenchymal neurogenesis in the primate frontal cortex in adult macaques. Similar neurogenesis has also been identified in the substantia nigra (Shan et al., 2006; Zhao et al., 2008) and brain stem (Bauer et al., 2005) of rodents. Moreover, progenitors with proliferative capacity have recently also been isolated from the cortex (Arsenijevic et al., 2001; Palmer et al., 1999), white matter (Nunes et al., 2003), spinal cord (Yamamoto et al., 2001; Shihabuddin, 2008) and substantia nigra (Lie et al., 2002) of rodents. These studies support the concept of atypical neurogenesis.

Several factors are known to promote neurogenesis including exercise, electroconvulsive therapy, antidepressants and antipsychotic drugs (Madsen et al., 2000; Malberg et al., 2000; Wang et al., 2004). Recent evidence points to niche astrocytes and factors they release, including sonic hedgehog (Jiao and Chen, 2008) and interleukins (Barkho et al., 2006), as being key regulators. Importantly, enhanced neurogenesis has also been reported in human patients with Alzheimer's disease (Jin et al., 2004). The aforementioned findings suggest that a neurogenic program may be elicited in any CNS region provided with the appropriate stimulus.

General acceptance of a widespread neurogenic environment in the adult CNS has not gained mainstream acceptance for a number of reasons including reports of neurogenesis that have been difficult to reproduce as well as studies publishing contradictory findings (Zhao et al., 2003; Frielingsdorf et al., 2004). Furthermore, the results may be subject to misinterpretations with several concerns regarding the methodologies used (Frielingsdorf et al., 2004). These caveats notwithstanding, one should be cautious in dismissing the paradigm of a more widespread neurogenic environment in the CNS based on our knowledge of NSCs.

The spinal cord and filum terminale

A non neurogenic region that has recently been shown to harbor NSCs is the spinal cord (Shihabuddin, 2008). In fact, NSCs have been isolated from all levels of the adult spinal cord (Liu, 2006), first from rodent spinal cord and recently also in primates (Vessal et al., 2007). As with the SVZ and SGZ, the identity as well as the location of NSCs in the spinal

cord remains elusive. Kraus-Ruppert et al. (1975) reported the presence of mitotic activity in 8.8% of ependymal cells in the central canal of the spinal cord of young mice and Frisen et al. (1995) demonstrated an increase in mitotic activity resulting from nestin positive cells in a region close to the central canal. In keeping with these studies, the putative location of NSCs within the spinal cord may be the region around the central canal. The central canal extends into the terminal part of the spinal cord, the filum terminale (Fig. 2). However, almost no data is present regarding the neurogenic potential of filum terminale (Varghese et al., 2009).

In adults, the filum terminale is a 15 cm long, elastofibrous structure extending from the caudal end of the spinal cord to the first coccygeal vertebrae (Fig. 1) (Larsen, 1993; Salbacak et al., 2000). Traditionally, the filum terminale has been regarded as a fibrovascular tag of clinical significance only if it resulted in the overstretching or compression of the spinal cord which gave rise to the tethered cord syndrome (Yamada et al., 2004). This syndrome is characterized by various symptoms, including back pain radiating to both legs, neurologic deficits and bowel and bladder dysfunction (Iskandar et al., 1998). Treatment is often surgical untethering, wherein the filum terminale is divided to untether the spinal cord. Studies have shown that patients may benefit from the surgical intervention (Al-Holou et al., 2009; Garces-Ambrossi et al., 2009).

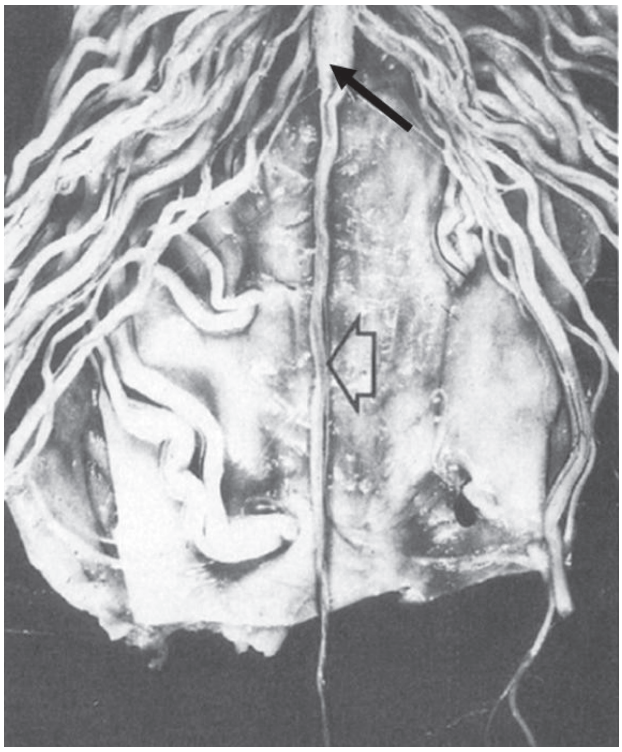

Fig. 1. - Gross photograph of the caudal end of the spinal cord (arrow) and the filum terminale (empty arrow) of an 18-year-old woman (reproduced after permission from Choi, 1992).

Recent histological studies have shown that the filum terminale, which is less than 2 mm wide (Smith and Schoenwolf, 1997), contains an ependyma-lined canal continuing from the spinal cord as well as glial cells and neuronal cell bodies (Choi et al., 1992). Moreover, the filum terminale shows strong reactivity for a stem cell marker, neural cell adhesion molecule (Alonso, 1999; George et al., 2003) and given the presence of neurons, glia and even an ependymal lined central canal where NSCs may reside (Gonzalez-Robles and Glusman, 1979; Choi et al., 1992; Storer et al., 1998; Rethelyi et al., 2004) suggests that the filum terminale may be a potential as of yet unknown source of NSCs with putative biological and clinical implications.

At our neurosurgical department filum terminale biopsies were obtained during surgical procedures. The filum terminale was identified (1) visually, based on its greyish hue, (2) based on texture, which was softer than the neighboring nerve roots, and (3) localization. The biopsies were transported in Leibowitz-15 (L15) medium at +4°C. The filum terminale samples were gently minced with a scalpel and chemically digested using trypsin-EDTA by incubating at 37°C for 6 min. The digested material was then centrifuged at 300 g, rinsed with L15, counted using a hemocytometer and trypan blue to exclude dead cells and cultured as described previously (Moe et al., 2005). Briefly, the cells were seeded at clonal density (20,000 cells/mL) in DMEM/F12 supplemented twice a week with B27-supplement, leukemia inhibitory factor, basic fibroblast growth factor and epidermal growth factor at 37°C in 6% CO_2 and 20% O_2. This resulted in the formation of floating aggregates (neurospheres) which were subsequently dissociated, as described above, into single cells before the centers became necrotic.

Using a panel of assays established in our laboratory for SVZ NSCs, we investigated the stemness as well as the neurogenic potential of the isolated filum terminale cells (Moe et al., 2005). The NSCs were positive for neural stem cell markers Sox2 and nestin (Lendahl et al., 1990; Ellis et al., 2004)

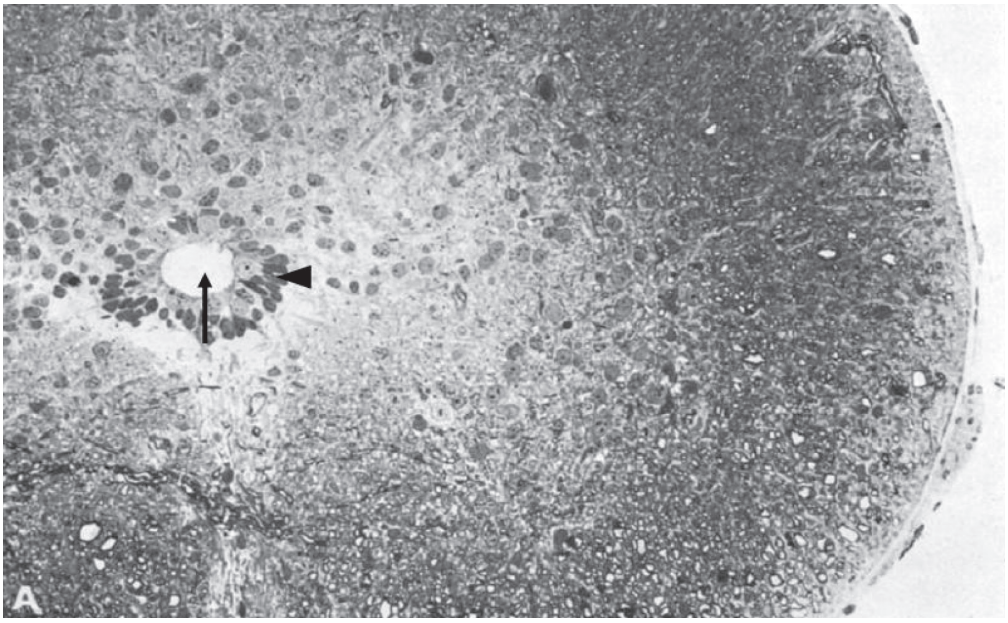

Fig. 2. - Light micrograph (x 200) of the frog filum terminale showing the central canal (arrow) and ependymal cells (arrowhead) lining the central canal (x 200) (reproduced after permission from Glusman, 1979).

and were capable of self renewal, proliferation and developed into neurospheres, even upon subculture (Varghese et al., 2009). The spheres were similar to SVZ spheres morphologically; however, detailed studies are needed before one can draw such a conclusion. When NSCs were cultured with serum and without growth factors, they exhibited multipotent differentiation into neurons, astrocytes and oligodendrocytes (Varghese et al., 2009). Interestingly, a glia:neuron ratio of 2:1 was observed in our filum terminale cultures, consistent with a report on filum terminale in situ (Chvatal et al., 2001). This contrasts the 2:7 glia:neuron ratio seen in NSC cultures obtained from the brain (Moe et al., 2005). As in the case of the spinal cord, this may reflect an internal programming specific for the cells isolated from filum terminale (Hitoshi et al., 2002). Using electrophysiology, we have shown that differentiated filum terminale NSCs exhibited two distinct populations; an electrically passive glial population and a β III tubulin positive neuronal population, where the neurons displayed characteristic mature action potentials (Varghese et al., 2009). This is the first report showing that stem cells isolated from non neurogenic adult human filum terminale develop into functional neurons.

For NSCs to be clinically viable they need to exhibit certain properties including survival and integration within the brain tissue after transplantation and differentiation into appropriate neural cells types (Lindvall and Kokaia, 2006). Moreover, the cells must not give rise to side effects and be genetically stable (Lindvall and Kokaia, 2006). To this end, filum terminale NSCs were transplanted into a global brain ischemia rodent model already established in our group (Olstorn et al., 2007). No tumor formation was seen in or adjacent to the graft area in our transplanted animals. A robust immune response to the transplanted cells was seen and this is in coherence with other reports of xenotransplantation (Duan et al., 1995). This of course can be circumvented by using auto or allografts (Duan et al., 1995) but lack of human material precluded this experimental paradigm. Despite the immune response, nestin positive NSCs were shown to survive and the survival of the transplanted cells estimated at 4-5% (Varghese et al., 2009). Also, the transplanted cells were shown to migrate towards the ischemic lesion, suggesting that NSCs respond to cues from the injured tissue (Varghese et al., 2009). A similar migratory trait has been shown by our laboratory for transplanted adult human ventricular wall NSCs (Olstorn et al., 2007) and by others for different stem cells (Windrem et al., 2002; Imitola et al., 2004).

In the transplanted environment, filum terminale NSCs were shown to differentiate into exclusively

GFAP positive astrocytes (Varghese et al., 2009). This contrasts our *in vitro* data as well as the multilineage differentiation seen when ventricular wall NSCs were transplanted in a similar milieu (Olstorn et al., 2007). Our results are in coherence with other studies showing that NSCs from non neurogenic adult spinal cord are ineffective in promoting neurogenesis (Shihabuddin, 2008). Factors precluding neuronal differentiation include, lack of trophic support, insufficient nutrition and oxidative stress (Le Belle et al., 2004). A less often considered factor may be the immune system (Galea et al., 2007). Traditionally, the CNS has been viewed as an immunologically privileged site (Galea et al., 2007). However, this concept has recently been challenged (Martino and Pluchino, 2007). In fact, it has been shown that the immune system may play a role in the outcome of differentiated progeny of xenografts (Carpentier and Palmer, 2009). Such factors notwithstanding, it has been demonstrated that when similar progenitors were transplanted into the hippocampus they developed into neurons (Shihabuddin, 2008) suggesting that it is premature to rule out the possibility of neuronal differentiation.

Taken together, one can conclude that neurogenesis is a multifactorial event dependent on 1) the interplay between the microenvironment and intrinsic properties of the grafted cells (Kelly et al., 2004); 2) instructions relayed via environmental cues (Lie et al., 2002; Shihabuddin, 2008), growth factors (Nakatomi et al., 2002) and even pathological processes (Thored et al., 2006), with all these events exerting a cumulative effect on the outcome of NSCs.

Promises and challenges

Sixty-three years after Ramon y Cajal stated that in a mature CNS neural tissues could not regenerate after damage (Ramon y Cajal, 1913), the dogma was proven erroneous when unequivocal evidence was provided for the existence of NSCs and postnatal neuro genesis in the SVZ and SGZ of the adult CNS (Kriegstein and Alvarez-Buylla, 2009). By virtue of the hallmarks of self renewal, proliferation and multipotent differentiation it is thought that NSCs may replace and repair neural cells damaged in disease systems of the CNS (Lindvall and Kokaia, 2006).

It has already been shown that endogenous neurogenesis increases in response to brain pathologies, such as stroke (Brown et al., 2003), Alzheimer's disease (Cameron and McKay, 1999) and Parkinson's disease (Zhao et al., 2003). This occurs in response to cues released from the injured tissue and niche, which enable NSC migration and differentiation to replace cells that are dead or damaged (Lindvall and Kokaia, 2006). However, the capacity of self repair is not sufficient to enable a complete functional recovery (Lindvall and Kokaia, 2006). One way of circumventing this may be by pharmacological manipulation of endogenous NSCs residing in the SVZ and SGZ (Kokaia and Lindvall, 2003). This may be achieved using growth factors or niche factors, as they have been shown to ameliorate cellular death and promote targeted differentiation (Jin and Galvan, 2007). Another contributing factor may be identifying new sources of NSCs such as the filum terminale (Jiao and Chen, 2008).

The arbitrary division of the CNS into neurogenic and non neurogenic regions may need revising given that we and others have either isolated NSCs from or demonstrated neurogenesis in non neurogenic regions (Yamamoto et al., 2001; Wang et al., 2004; Shihabuddin, 2008; Varghese et al, 2009). Concern regarding methodologies used to demonstrate atypical neurogenesis notwithstanding, the prospect of having a more permissive CNS environment is very promising. The intrinsic and extrinsic cues responsible for this are being currently investigated and in order to avoid misinterpretation of results, one needs to approach the topic with new tools and technologies that were not available earlier. In keeping with this evolving concept, it is worthwhile to note that stem cells and functional neurons have recently been induced from post mitotic cells including skin cells (Yamanaka, 2008; Nishimura et al., 2009; Vierbuchen et al., 2010). These induced pluripotent stem cells present a significant milestone in the field of stem cell biology and may have consequences not only for NSC biology but also for our understanding of the relation of NSCs to disease systems including tumors.

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