Investigating the Effects of the Medulloblastoma Secretome on Neural Stem Cell Function

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Graduate Department of Molecular Genetics
University of Toronto

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Abstract

Medulloblastoma (MB) is the most common malignant pediatric brain tumour. MB survivors are often left with cognitive deficits that persist throughout life, which are attributed to the side-effects of treatment and other factors such as hydrocephalus. However, recent studies suggest that nearly half of the patients spared of radiation still develop cognitive impairments. Neural stem cells (NSCs) in the ventricular-subventricular zone (V-SVZ) contribute to oligodendrogenesis and therefore to cognitive development. This V-SVZ niche is supported by local and systemic circulating growth factors. Here, I have examined whether the MB secretome directly affects NSC function in the postnatal V-SVZ. The MB secretome results in a decrease in neural precursor cell proliferation one week after a single intraventricular administration. As well, olfactory bulb neurogenesis was perturbed in mice with MB xenograft tumours. These findings demonstrate that the MB secretome can act on V-SVZ NSCs and perturb their function, potentially disturbing forebrain development.
Acknowledgments

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Publications and Author Contributions

While not discussed directly in my thesis, I was a co-author on the published article “Interleukin-6 Regulates Adult Neural Stem Cell Numbers during Normal and Abnormal Post-natal Development,” in *Stem Cell Reports* 2018, doi:10.1016/j.stemcr.2018.03.008. I helped carry out the experiments, alongside Dr. Mekayla Storer who led the project. I contributed to Figure 2 where I worked on the neurosphere experiments. I also performed immunostaining and imaging of V-SVZ sections for Figures 2, 5 and 6.
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List of Abbreviations

BBB – blood-brain barrier

BMP-2 – bone morphogenetic protein-2

BrdU - bromodeoxyuridine

CC3 – cleaved caspase 3

CM – conditioned medium/media

CMS – cerebellar mutism syndrome

CNS – central nervous system

CSF – cerebrospinal fluid

CT – computed tomography

DCX – doublecortin

DG – dentate gyrus

DIV – days in vitro

EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

EMX1 – empty spiracles homeobox 1

FGF – fibroblast growth factor

GFAP – glial fibrillary acidic protein

GC – granule cells

ICV – intracerebroventricular

IGF – insulin-like growth factor
IL-6 – interleukin 6
IL-8 – interleukin 8
LV – lateral ventricle
MRI – magnetic resonance imaging
MASH1/ASCL1 – mammalian achaete-scute homolog 1
MB – medulloblastoma
MBP – myelin basic protein
NEUN – neuronal nuclei
NF1 – neurofibromatosis 1
NPC – neural precursor cell
NSC – neural stem cell
NT-3 – neurotrophin-3
OLIG2 – oligodendrocyte transcription factor 2
OPC – oligodendrocyte precursor cell
Ptc - patched
PDGFRα – platelet derived growth factor receptor alpha
PGC – periglomerular cells
RP – radial precursor
SDF1 – stromal cell-derived factor 1
SHH – sonic hedgehog
SGZ – subgranular zone

SOX2 – SRY (sex determining region Y)-box 2

TA cell – transit amplifying cell

TAM – tumour-associated macrophage

VCAM1 – vascular cell adhesion protein 1

VEGF – vascular endothelial growth factor

V-SVZ – ventricular-subventricular zone

WISC – Wechsler Intelligence Scale for Children
Chapter 1
Introduction

1 Chapter 1 – Introduction

1.1 Medulloblastoma

1.1.1 Medulloblastoma (overview)

Medulloblastoma (MB) is the most common malignant pediatric brain tumour and the most common type of embryonal tumour of the central nervous system (Ostrom et al., 2014, Story et al., 2017). Although the age of prognosis for MB ranges from infancy throughout adulthood, most cases occur during the first decade of life, with a slight male bias in pediatric cases (Ostrom et al., 2014, Smoll and Drummond, 2012). MB tumours originate in the posterior fossa, located in the back of the brain (Chang et al., 1969). In some cases, the tumour will metastasize to the leptomeningeal surface of the brain and spinal cord, through either the cerebrospinal fluid (CSF) or by hematogenous dissemination (Garzia et al., 2018). Tumours often occur in the cerebellum, an area in the posterior fossa important for coordination and balance, therefore, many patients experience dizziness and imbalance. Other symptoms include those associated with increased intracranial pressure, such as nausea, vomiting and headaches. Patients also present with vision problems, behavioural changes and lethargy, with the number of symptoms increasing as the tumour develops (Wilne et al., 2012). When patients present symptoms typical of MB, the first-line of diagnostic tests are brain imaging by head CT or brain MRI. An MRI of the whole spinal axis and a lumbar puncture may also be performed to assess for leptomeningeal metastasis (De Braganca and Packer, 2013).

Medulloblastoma is a heterogenous cancer. One characteristic of this is the distinct histological phenotypes. In addition to classic MB, others include desmoplastic-nodular, large-cell-anaplastic, and medulloblastoma with extensive nodularity (Louis et al., 2016). The classic subtype is the most common variant (Park et al., 1983).

1.1.2 Subgroup

Over the years, genetic and molecular characterization of MB has been on the rise in an effort to improve treatment and survivability. This has led to the classification of the four major
subgroups, each presenting different molecular and genetic profiles and clinical outcomes (Taylor et al., 2012, summarized in Table 1). The WNT MBs are characterized by the activation of the WNT pathway, and patients in this subgroup have the best prognosis with an approximate 95% patient survival rate. The SHH subgroup is characterized by increased Sonic Hedgehog signaling and these patients have an intermediate prognosis. The Group 3 tumours are associated with an increase in MYC amplification and have the highest rate of metastatic dissemination compared to the other subgroups, thus patients in this group have the worst survivability. Group 4 is the most prevalent subgroup and has an intermediate overall survival of 75% (Taylor et al., 2012). Tumours from these individual subgroups have been shown to arise from distinct developmental origins (Gibson et al., 2010). Recently, these subgroups have been further subdivided into 12 subtypes, identified by combined gene expression and DNA methylation data (Cavalli et al., 2017, Table 1). This new classification of tumours may have future clinical significance in how patients are treated for MB.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>WNT</th>
<th>SHH</th>
<th>Group 3</th>
<th>Group 4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WNT α</td>
<td>WNT β</td>
<td>SHH α</td>
<td>SHH β</td>
</tr>
<tr>
<td>Age</td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
<td><img src="image" alt="Image" /></td>
</tr>
<tr>
<td>Survival (at 5 years)</td>
<td>97%</td>
<td>100%</td>
<td>69.8%</td>
<td>67.3%</td>
</tr>
<tr>
<td>Metastatic rate</td>
<td>8.8%</td>
<td>21.4%</td>
<td>20%</td>
<td>33%</td>
</tr>
<tr>
<td>Expression</td>
<td>WNT signalling</td>
<td>SHH signalling</td>
<td>MYC/Retinal signature</td>
<td>Neuronal signature</td>
</tr>
<tr>
<td>Histology</td>
<td>Classic</td>
<td>LCA Desmoplastic</td>
<td>Desmo-plastic</td>
<td>MBEN Desmo-plastic</td>
</tr>
</tbody>
</table>

Age (years): ![Image](image) 0-3 | ![Image](image) >3-10 | ![Image](image) >10-17 | ![Image](image) >17

**Table 1: Summary of the 12 medulloblastoma subtypes.** Schematic representation of key features of each MB subtype within the previously identified four medulloblastoma subgroups (Adapted from Taylor et al., 2012, Cavalli et al., 2017). LCA = large-cell-anaplastic; MBEN = medulloblastoma with extensive nodularity.
1.1.3 Treatment

Over the past number of years, there has been an increase in survival rate with the improvement of treatment methods. Maximal surgical resection is the first-line of treatment to remove as much of the tumour as possible while avoiding brain damage (De Braganca and Packer, 2013). Patients with minimal residual tumour following surgery often have a better prognosis (Albright et al., 1996). For children 3 years of age and older, postoperative craniospinal radiation is common, with an additional boost radiation dose at the tumour bed to reduce the risk of recurrence (Gajjar et al., 2006). Chemotherapy is often given along with radiation to increase radiosensitivity and is administered again after the completion of radiation (Gajjar et al., 2006, Packer et al., 2006). Various chemotherapy treatments are used, all of which must be able to cross the blood-brain barrier (BBB). Common chemotherapeutic agents include vincristine, cisplatin, cyclophosphamide, lomustine, etoposide, methotrexate, temozolomide and carboplatin (Taylor et al., 2005, De Braganca and Packer, 2013). In recent years, treatment has been tailored to the severity of the cancer, the age of the individual, the presence of metastasis and different MB subtypes. For instance, a multicenter study found that gross total resection was beneficial compared to partial resection for only Group 4 tumours (Thompson et al., 2016). They also observed no improvement in progression-free or overall survival for greater extent of resection of WNT, SHH or Group 3 tumours. Therapies that accommodate for the heterogeneity of this disease will help to reduce tumour burden for these patients.

1.2 Late effects of cancer

1.2.1 Treatment strategies contribute to the adverse late effects of cancer

Patients that are cured of MB, as well as survivors of other types of cancer, experience side-effects that persist throughout life. Some of these include fertility problems, cardiovascular problems, hypothyroidism, endocrinologic deficiencies, subsequent neoplasms, hearing impairments, vision problems, feelings of social isolation and neurocognitive deficits (Gurney et al., 2003, van Casteren et al., 2009, Ribi et al., 2005, Neglia et al., 2006, Armstrong et al., 2009, Gurney et al. 2006, Packer et al., 2003).

Treatment has been well-documented as a key source of these late effects. Patients who have undergone chemotherapy treatment have reported experiencing cognitive dysfunction,
fatigue, forgetfulness and problems with concentration – symptoms which are collectively described as ‘chemobrain’ or ‘chemofog,’ (Pierson et al., 2016, Schagen et al., 1999, Brezden et al., 2000). These side-effects are experienced in cancer patients, irrespective of whether the tumour is in the central nervous system (CNS) or not. Standard-dose systemic chemotherapy results in poorer cognitive function, especially in verbal memory and psychomotor function, compared to local therapy (surgery with local radiation) in long-term survivors of breast cancer and lymphoma (Ahles et al., 2002). This suggests that dosage and intensity of treatment may also play a role in these late-effects seen in cancer survivors.

Surgical intervention has also been identified as a source of long-term effects in cancer survivors. Surgical resection of pediatric cerebellar tumours, the majority of which include MB tumours, lead to cognitive deficits in many cases (Levisohn et al., 2000). One complication that largely contributes to these effects is the development of cerebellar mutism syndrome (CMS), a postoperative syndrome where individuals have reduced speech that progresses to mutism, emotional lability, ataxia and hypotonia (Robertson et al., 2006). CMS occurs in approximately one-fourth of MB patients who undergo surgical resection and can lead to long-term consequences such as nonmotor speech and language deficits, neurocognitive problems and ataxia (Huber et al., 2006, Robertson et al., 2006).

Furthermore, the long-term adverse effects of radiotherapy have been well-documented in a variety of cancers. Patients who receive radiation treatment have an elevated risk for many of the side-effects mentioned earlier, specifically hearing and vision problems, endocrinologic defects and development of subsequent neoplasms, compared to those who only received surgical treatment (Packer et al., 2003, Gurney et al., 2003, Gurney et al. 2006, Armstrong et al., 2009, Neglia et al., 2006). As well, radiotherapy is well known for its long-term effects, especially in the pediatric brain, and these effects may continue into adulthood. Specifically, radiotherapy is associated with neurocognitive damage, poor overall health and poor social functioning in adults who were previously treated for pediatric CNS tumours (Armstrong et al., 2010). These neuropsychological outcomes were shown to have different long-term outcomes depending on the region exposed to radiotherapy. As a result, children below the age of three typically do not receive radiotherapy.
Several studies have looked at reducing radiotherapy dosage or limiting radiation to the tumour bearing region by using proton beams to reduce the side-effects of MB treatment (Yuh et al., 2004, Merchant et al., 2008). Recent strategies also include reduced-dose radiotherapy administered in combination with adjuvant chemotherapy. While reduced-dose radiotherapy with chemotherapy does lead to better intellectual outcomes compared to with conventional radiotherapy doses, these patients still presented with poor intellectual outcomes (Ris et al., 2001).

1.2.2 Pediatric brain tumours and neurocognitive deficits
Survivors of pediatric CNS tumours are often left with neurocognitive deficits that persist throughout life (Liu et al., 2015, Moxon-Emre et al., 2016). To assess these cognitive impairments in these patients, a battery of tests such as the Wechsler Intelligence Scale for Children (WISC) is used. The WISC assessment includes the following indices: The Full Scale Intelligence Quotient (IQ) measures overall cognitive function, the Verbal Comprehension Index measures verbal reasoning and the Perceptual Reasoning Index assesses interpretation of visual/nonverbal information. Working memory measures concentration abilities and the Processing Speed Index evaluates graphomotor and mental processing (Wechsler, 2003, Moxon-Emre et al., 2016).

Pediatric brain tumour survivors show a decline in cognitive functions such as impaired memory, attention, information processing speed, executive function and multitasking (Armstrong et al., 2009, Palmer et al., 2013). This neurocognitive decline is still evident into adulthood. Approximately 18 years after diagnosis, MB survivors were shown to have lower full scale IQ scores and, in some cases, executive function deficits (Brinkman et al., 2012). These poor intellectual outcomes make daily tasks a challenge for these survivors and because of this, many of these children have difficulty graduating from high school, are less likely to be employed and have an overall reduced quality of life (Armstrong et al., 2009).

While treatment, such as craniospinal irradiation has been reported as a major contributor of these long-term effects, recent work has shown that a proportion of pediatric low-grade glioma patients who did not undergo radiation treatment also experienced cognitive deficits as compared to healthy controls (Liu et al., 2015). This suggests that tumour-specific mechanisms
or other patient-specific factors, described further below, may be contributing to the structural and neurocognitive changes observed in pediatric brain tumour survivors.

MB patients were shown to have subgroup-specific intellectual outcomes (Moxon-Emre et al., 2016). Patients with SHH tumours appeared to have the most distinct cognitive outcomes, where processing speed declined less in these patients than in Group 3 patients. However, the SHH group also had a lower incidence of cerebellar mutism and motor deficits. Furthermore, whereas WNT and Group 4 patients benefited from reduced treatment, individuals with SHH and Group 3 tumours did not see an improvement in intellectual outcomes with a reduced dose. This suggests that there are tumour-specific effects that contribute to these long-term intellectual outcomes.

There are several therapies to help manage these cognitive impairments. Cognitive rehabilitation and occupational therapy are used in some cases to manage the long-lasting cognitive impairments and help improve quality of life for survivors. Cognitive behavioural therapy that emphasizes the acquisition of new behaviours and cognition to compensate for memory deficits, has been shown to be effective in breast cancer survivors who experience chemotherapy-related cognitive impairments (Ferguson et al., 2012). Additionally, exercise training has recently been explored as a strategy to improve neurocognitive function, such as short-term memory and processing speed, in long-term pediatric CNS tumour patients treated with radiotherapy (Szulc-Lerch et al., 2018). These rehabilitative strategies, in combination with more targeted or reduced-dose therapies, may aid in reducing the long-term cognitive side-effects these cancer survivors are left with.

1.2.3 Late effects of pediatric brain cancer on brain structure

Pediatric brain cancer survivors experience a multitude of long-term effects. To better understand the neurocognitive and behavioural outcomes observed, research has been done to investigate whether perturbations in brain structure occur.

One region that has been shown to be affected in pediatric brain tumour patients is the white matter consisting of myelin-wrapped axons that enables rapid and efficient communication between neurons (Partanen et al., 2018). However, these affects were largely attributed to the
effects of irradiation. What was even more interesting is that a proportion of pediatric low-grade glioma patients that did not receive radiotherapy were presented with damage to the white matter (Liu et al., 2015). This white matter compromise correlated to poor cognitive outcomes, demonstrating that the tumour itself may be contributing to the neurocognitive and structural changes seen in these patients.

This work has expanded to look at other brain structures, including the hippocampus, the region important for learning and memory (Dupret et al., 2008). MB survivors have been shown to have reduced white matter volume and smaller hippocampal subfield volumes compared to healthy control, which were associated with memory impairment (Riggs et al., 2014, Decker et al., 2017). Damage was also observed in the uncinate fasciculus, a white matter tract that connects part of the limbic system such as the hippocampus and amygdala with the frontal cortex, approximately 5 years after diagnosis (Riggs et al., 2014).

1.2.4 Other sources of cognitive impairments

While treatment is a major contributor to the late effects in cancer survivors, there are many other factors that can account for these poor outcomes. Interestingly, some of these late effects have been observed even before treatment. In one study, about one-third of breast cancer patients exhibited cognitive impairment before receiving chemotherapy treatment (Wefel et al., 2004). Neurocognitive deficits were also seen in some pediatric CNS tumour patients at diagnosis (Margelisch et al., 2015). This suggests that there may be other factors, aside from therapeutic intervention, that are contributing to the late effects of cancer.

Demographic characteristics may predispose individuals to cancer-related cognitive impairment. For instance, factors such as younger age at diagnosis, low socioeconomic status and individuals whose parents had a low education level are associated with poor neurocognitive outcomes in pediatric CNS tumour patients (Liu et al., 2015, Moxon-Emre et al., 2016). While these characteristics have been well-documented, the neurobiological basis behind these deficits is largely unknown.

Other contributors that are more specific to pediatric CNS tumours are hydrocephalus, which occurs when the tumour mass blocks the flow of cerebrospinal fluid, causing a buildup of
intracranial pressure. Hydrocephalus, as well as cerebellar mutism, have been shown to affect many aspects of intellectual functioning, including verbal comprehension, verbal reasoning, perceptual reasoning, working memory and processing speed. (Moxon-Emre et al., 2016). In addition, tumour location can also influence long-term outcomes. Neurocognitive impairment was found to be worse in children with cortical tumours, compared to those with supratentorial midline or posterior cranial fossa tumours (Iuvone et al., 2011).

Lastly, genetics also contributes to the long-term outcomes in pediatric CNS tumour survivors. For example, the presence of Neurofibromatosis 1 (NF1), one of the most common single-gene disorders that affects cognitive performance, is associated with worse intellectual outcomes in pediatric low-grade glioma patients (Shilyansky et al., 2010, Liu et al., 2015).

1.2.5 Tumour-specific mechanisms as a source for cognitive impairments
While many of the phenotypes seen in cancer survivors are largely attributed to the treatments these patients receive, there has been research supporting that tumour growth and development may also be a contributing factor. As mentioned earlier, multiple studies have observed cognitive impairments in cancer patients prior to treatment (Wefel et al., 2004, Margelisch et al., 2015). To better characterize these outcomes, a recent study investigated the effects that cancer treatment and tumour growth independently have on neurobiological mechanisms in a breast cancer orthotopic model (Winocur et al., 2018). Specifically, they showed that prior to treatment, transgenic tumour bearing mice made more errors in spatial memory and working memory tasks compared to non-tumour bearing controls. However, one limitation in this study is when they examined changes in frontal lobe and hippocampal volume, the effects of tumour and chemotherapy were in opposite directions and made their results difficult to interpret. Tumour bearing mice that did not receive chemotherapy had reduced hippocampal and frontal lobe volumes, while control mice that had received chemotherapy showed an increase in frontal lobe volume. Of note, they did observe a decrease in hippocampal neurogenesis in chemotherapy-treated mice. Why chemotherapy should increase frontal lobe volumes, especially in the non-tumour bearing mice, while at the same time decreasing hippocampal neurogenesis was not discussed.
**Tumour immune system**

The tumour immune system is one contributing factor to cancer prognosis. A recent study showed that tumour-associated macrophage (TAM) behaviour predicts poor prognosis in SHH MB tumours (Margol et al., 2015). Although they did not investigate the tumour immune system and its effects on cognition, this study provides insight into how tumour immune function may result in different prognoses and may also result in varying long-term intellectual outcomes in MB survivors (Moxon-Emre et al., 2016). A contributing factor may be cytokines overproduced by tumour cells and their associated immune cells, some that are known to perturb postnatal neural stem cell function (Storer et al., 2018).

**Cancer Secretome**

One source of tumour-specific mechanisms that contributes to long-term cognitive effects is the cancer secretome, a collection of cytokines and other proteins secreted by tumour cells. The cancer secretome plays a significant role in malignant disease progression and metastasis. Prior to treatment, cancer patients were shown to have a dysregulation of cytokine levels (Jebreel et al., 2007, Tsimberidou et al., 2008). Furthermore, the expression and secretion of its components correlate with cognitive impairments. Pro-inflammatory cytokines such as interleukin-6 (IL-6) are associated with cognitive impairment, fatigue and depression (Rohleder et al., 2012, Heyser et al., 1997).

One study investigated the relationship between increased circulating levels of cytokines and cognitive impairment in patients with acute myeloid leukemia or myelodysplastic syndrome prior to treatment. They showed that patients who had higher circulating levels of IL-6 at diagnosis were found to have poorer executive function, whereas higher levels of IL-8 were associated with better memory (Meyers et al., 2005). In addition to cytokines, other factors released by MB cells include chemokines, such as MCP-1, and Bone Morphogenetic Proteins (BMPs) which promote tumour malignancy and affect the surrounding niche (Garzia et al., 2018, Salsman et al., 2011, Bakhshinyan et al., 2018). Therefore, the cancer secretome, in combination with treatment strategies, results in an increase in cytokines and other secreted factors, which contribute to the long-term cognitive impairments. It is important to keep in mind, however, that immune cells that express these cytokines may also contribute to cognitive impairment.
To gain a better understanding of the tumour- and treatment-dependent effects on cognitive outcome, Winocur et al. looked at cytokine levels in the breast cancer mouse model. Tumour bearing mice exhibited a dysregulation in cytokine activity, with an increase production of pro-inflammatory and decrease in anti-inflammatory cytokines. They observed a direct relationship between increased pro-inflammatory cytokine levels and impaired spatial memory in the tumour group that received chemotherapy. They also reported changes in cytokine levels in the tumour group that did not receive treatment, but there was no correlation with cognitive performance (Winocur et al., 2018). This work suggests that the tumour itself is secreting cytokines into the surrounding environment, but whether it is enough to affect behaviour without treatment-contributing effects, requires further investigation. Additionally, the effects of the cancer secretome on cognition that I have described here are in peripheral tumours in the adult brain. Little is known about the tumour-specific effects of pediatric CNS tumours where these factors do not have to traverse the BBB. Perhaps pediatric CNS tumours would have more of an impact on the developing brain and result in worse cognitive outcomes.

1.3 Neurogenesis

1.3.1 Postnatal brain development

The murine brain is an excellent model system for studying the development of the embryonic and postnatal mammalian brain. The cortex develops from a thin layer of neuroepithelium consisting of mitotically-active neural stem cells (NSCs). Before neurogenesis occurs, around embryonic day 9 (E9), the neuroepithelial stem cells and radial precursor (RP) cells divide symmetrically to amplify the precursor pool (Haubensak et al., 2004). Neurogenesis occurs between E11 and E17, when precursors start to divide asymmetrically, to generate one precursor cell to maintain their precursor pool and differentiate into a neuron directly or indirectly via intermediate progenitor cells (Englund et al., 2005; Noctor et al., 2004). A subset of slowly dividing RPs express a distinct transcriptional identity, which is maintained throughout their transition to a non-proliferative state during the time of neurogenesis and will eventually give rise to adult NSCs (Furutachi et al., 2015, Yuzwa et al., 2017). Finally, there is a transition to gliogenesis postnatally, first generating astrocytes followed by oligodendrocytes (Barnabe-Heider et al., 2005, Burns et al., 2009).
1.3.2 Neurogenesis in the postnatal mammalian brain

Once thought to be a process discontinued after development, the generation of new neurons, or neurogenesis, persists throughout life in the postnatal rodent brain, although whether humans maintain neurogenesis into adulthood remains controversial (Boldrini et al 2018, Sorrells et al 2018). Neurons are generated from neural stem cells (NSCs) that reside in two regions in the brain – the ventricular-subventricular zone (V-SVZ) in the subependymal region of the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus (Bond et al., Fig. 1).

**Figure 1: Location of the neurogenic niches in the adult murine brain.** The ventricular-subventricular zone (V-SVZ) resides in the forebrain along the lateral ventricle (LV). V-SVZ NSCs differentiate into neuroblasts and migrate to the olfactory bulb (OB) via the rostral migratory stream (RMS). The subgranular zone (SGZ) is caudal to the V-SVZ in the dentate gyrus of the hippocampus (adapted from Bond et al., 2015).

**Subgranular zone (SGZ)**

The SGZ resides in the dentate gyrus of the hippocampus. Sex determining region Y)-box 2 (SOX2)-expressing radial glia-like cells are the NSCs of the SGZ which differentiate into intermediate progenitors, which then gives rise to neuroblasts (Berg et al., 2015). These neuroblasts differentiate into mature neurons that integrate into the dentate granule cell layer (Sun et al., 2015). SGZ neurogenesis is important for memory formation and cognition throughout life (Leutgeb et al., 2007, Gu et al., 2012). Impaired adult hippocampal neurogenesis
is thought to contribute to neuropsychiatric disorders such as schizophrenia and depression (Duan et al., 2007, Boldrini et al., 2013).

**Ventricular-subventricular zone (V-SVZ)**

The V-SVZ niche resides in the subependymal region of the lateral ventricles. The V-SVZ has been shown to play an important role in olfactory bulb maintenance and odour-reward memories in the rodent brain and perhaps the infant human brain (Alonso et al., 2019, Sanai et al., 2011). The V-SVZ consists of NSCs that express glial fibrillary acidic protein (GFAP) and SOX2 (Zhang et al., 2015, Fig. 2A). The activated and quiescent populations of these GFAP+ cells co-exist in the neurogenic niche and are distinguished by the specific markers they express; activated NSCs upregulate epidermal growth factor receptor (EGFR) and Nestin, while quiescent NSCs are EGFR- and Nestin-negative (Codega et al., 2014). NSCs are surrounded by ependymal cells, forming a pinwheel structure, and the NSCs extend apically to receive contact from the ventricle (Mirzadeh et al., 2008). NSCs give rise to transit amplifying (TA) cells expressing mammalian achaete-scute homolog 1 (MASH1/ASCL1), which differentiate into neuroblasts, marked by the microtubule associated protein doublecortin (DCX) (Kim et al., 2007, Brown et al., 2003). Neuroblasts migrate through the rostral migratory stream to the olfactory bulb by chain migration where they differentiate into functional interneurons (Lois and Alvarez-Buylla et al., 1994).

**1.3.3 Glial cells in the V-SVZ**

In addition to making neurons, V-SVZ NSCs also generate a substantial proportion of astrocytes and oligodendrocytes during the early postnatal period and continues throughout life (Levison and Goldman 1993, Menn et al., 2006).

**Oligodendrocytes**

Neural stem cells (NSCs) in the ventricular-subventricular zone (V-SVZ) contribute to oligodendrogenesis. In addition to the large numbers of new neurons they produce, NSCs also generate a small amount of non-myelinating oligodendrocyte precursor cells (OPCs) and mature myelinating oligodendrocytes (Menn et al., 2006). In the murine brain, oligodendrogenesis occurs in three waves, with the third wave occurring perinatally (Kessaris et al., 2006). Oligodendrogenesis has
also been shown to continue throughout adulthood, where OPCs, which express oligodendrocyte transcription factor 2 (OLIG2) and platelet derived growth factor receptor alpha (PDGFRα) migrate from the V-SVZ into the corpus callosum, striatum, and fimbria fornix to differentiate into oligodendrocytes (Menn et al., 2006). These oligodendrocytes myelinate axons within these regions and are critical for the relay of signals between neurons. White matter maturation is important in cognitive development and function, and when myelination is perturbed, poor intellectual outcomes can arise (Nagy et al., 2004, Liu et al., 2015).

**Astrocytes**

Astrocytes, which express glial fibrillary acidic protein (GFAP), are generated during late embryogenesis and the early postnatal period (Burns et al., 2009, Sofroniew and Vinters, 2010). Astrocytes were originally recognized for providing support to neurons and to the neurogenic microenvironment (Lim and Alvarez-Buylla, 1999). However, astrocytes play a critical role in adult olfactory bulb neurogenesis, where they aid in the migration of neuroblasts along the RMS and promote neuron survival in the olfactory bulb (Kaneko et al., 2010, Khodosevich et al., 2013).

1.3.4 Region-specific differences in the V-SVZ

The postnatal V-SVZ has been shown to be regionally specified early in development, which accounts for the cellular heterogeneity in the adult neurogenic niche. Specifically, empty spiracles homeobox 1 (EMX1) progenitor cells that originate from the embryonic cerebral cortex reside in the adult dorsal V-SVZ, while the striatal V-SVZ arises from GSH2-expressing cells from the lateral and medial ganglionic eminence (Fuentealba et al., 2015, Young et al., 2007). Furthermore, different V-SVZ NSCs generate regionally-specified progeny. The dorsal V-SVZ is particularly dense with oligodendrogial progenitors and gives rise to oligodendrocytes in the corpus callosum and the white matter tracts of the striatum and the fimbria fornix (Ortega et al., 2013). This cellular heterogeneity also influences the types of neurons produced in the olfactory bulb. Specifically, the dorsal region gives rise to tyrosine-hydroxylase-positive periglomerular cells (PGCs) and superficial granule cells (GCs) in the OB, while the striatal NSCs give rise to calbindin-positive PGC and deeper GCs (Fuentealba et al., 2015).
1.3.5 V-SVZ microenvironment

The microenvironment is a critical component in the maintenance and proper function of V-SVZ NSCs and their progeny. The V-SVZ niche resides along the lateral ventricles, thus cells are in close contact with circulating factors in the cerebrospinal fluid (Lehtinen et al., 2011). NSCs receive signals from the choroid plexus, vasculature, and the ependymal cells lining the ventricular surface (Tavazoie et al., 2008, Delgado et al., 2014, Mirzadeh et al., 2008, Fig. 2B). Neighbouring cells are also important for the regulation of the V-SVZ niche, including the NSCs, NPCs, neuroblasts, astrocytes and oligodendrocytes mentioned above. Additionally, microglia, the resident CNS immune cells, have been shown to be important for neurogenesis and oligodendrogeneration in the early postnatal mouse brain by the release of cytokines (Shigemoto-Mogami et al., 2014).

**Growth factors and their influence on the V-SVZ**

Growth factors are another major component in regulating the V-SVZ niche. For instance, some factors in the V-SVZ microenvironment promote the quiescence of NSCs. BMP-2, locally expressed within the V-SVZ, promotes NSC quiescence by suppressing epidermal growth factor (EGF)-induced NSC activation (Joppe et al., 2015). Other quiescence factors include endothelial-derived neurotrophin-3 (NT-3) in the vasculature and CSF, and Wnt signaling via Rho-GTPase Cdc42 (Delgado et al., 2014, Chavali et al., 2018). Recent work has shown that heterogeneous expression of stromal cell-derived factor 1 (SDF1) influences NSC activity. Proliferating NSCs were preferentially associated with SDF1-positive vasculature, while quiescent NSCs were more associated with SDF1-negative vessels (Zhu et al., 2019).

Additionally, there are factors in the V-SVZ that activate the cells in the niche and promote the generation of differentiated cell types. Specifically, FGF2 is important for proliferation of V-SVZ progenitors, and the deletion of FGF2 results in a reduced precursor pool and smaller olfactory bulbs (Zheng et al., 2004). IGF-1 mediates neurogenesis in the V-SVZ and SGZ (Yuan et al., 2015). VEGF also promotes V-SVZ neurogenesis as seen when VEGF is injected into the ventricles of the rat brain (Jin et al., 2002). As well, PDGFRα is essential for oligodendrogeneration in a subpopulation of V-SVZ NSCs (Jackson et al., 2006).
However, when qualitative or quantitative alterations in these environmental cues arise, this can result in perturbed NSC function (Lehtinen et al., 2011). Recent work from our lab has shown that acute perturbations in levels of circulating IL-6 as observed in human pathological conditions such as infection, can have long-lasting effects on the adult NSC pools (Storer et al., 2018). In addition to these external sources, other sources of IL-6 that could influence V-SVZ function include microglia and precursors themselves (Lee et al., 2002, Gallagher et al., 2013).

Figure 2: The V-SVZ neurogenic niche and its microenvironment. A. The V-SVZ and major cell types that reside in the niche. B. The V-SVZ microenvironment receives signals from the choroid plexus, the cerebrospinal fluid (CSF), the vasculature and nearby cells (adapted from Bond et al., 2015, Chaker et al., 2016).

1.4 Hypothesis and Rationale
Alterations in environmental cues can have long-lasting consequences on the V-SVZ niche and NSC function. This led us to investigate whether alterations caused by cancer, specifically, factors released by medulloblastoma tumours, interact with the V-SVZ neurogenic niche, perturbing normal V-SVZ NSC function and maintenance. While various research groups have examined the effects of treatment – such as radiation – on neurogenesis, whether tumour-specific mechanisms directly affect V-SVZ neurogenesis is largely unknown (Beera et al., 2018). This thesis will aim to investigate whether the MB secretome directly affects endogenous forebrain NSCs and their progeny with two general aims: 1) to examine the local effects of MB cell-conditioned media on the postnatal V-SVZ NSCs and their progeny and 2) assess whether the MB secretome influences V-SVZ cell differentiation.
Chapter 2
Materials and Methods

2 Materials and Methods

2.1 Animals and injections

This study was approved by the Hospital for Sick Children Animal Care Committee, in accordance with Canadian Council on Animal Care guidelines. Mice were maintained on a 12-hr light/dark cycle, and food and water were provided ad libitum. For embryonic cortical culture experiments and intracerebroventricular injections, timed-pregnant wildtype CD1 mice were obtained from Charles River Laboratories.

For MB xenograft experiments, male NOD/SCID mice (4–5 weeks) were bred in house at the Max Bell Research Centre. Mice were injected with 2x10^6 ONS76 tumour cells at a 1:1 ratio with growth factor-reduced (GFR) Matrigel and basal DMEM. Control mice were injected with GFR-Matrigel and basal DMEM. Tumour volumes were monitored bi-weekly and measured using handheld calipers once they were palpable. For olfactory bulb neurogenesis experiments, mice were injected with 100 mg/kg bromodeoxyuridine (BrdU) every 2 hours for a total of 8 hours (five injections) and brains were harvested 21 days following injection.

2.2 Intracerebroventricular (ICV) injections

Cells were plated at 8x10^5 in T75 flasks. 3 days later, media was changed to a 1:1 ratio of DMEM (1g/l glucose): F12 with penicillin-streptomycin. 24 hr later, conditioned medium was collected, spun down and filtered using low protein binding syringe filters (0.45 μm). Media was concentrated 40x using Amicon Ultra-15 Centrifugal Filter columns with a 3000 nominal molecular weight limit. Final media was exchanged to basal DMEM (1 g/L glucose). Basal DMEM was used as control medium. Postnatal day 0 (P0) or P1 CD-1 pups were injected with 4 uL total (2 uL per hemisphere) conditioned medium or control medium, into both lateral ventricles. Trypan blue was used at 0.05% as a tracer. 24 hr later or one week later, the brains were dissected and fixed in 4% paraformaldehyde overnight, or the subependyma of the lateral ventricles was dissected 24 hr following injection to perform neurosphere cultures.
2.3 Neurosphere cultures

For neurosphere cultures, the subependyma of the lateral ventricles was dissected from P2 brains and mechanically dissociated into small pieces using a scalpel. Tissue was then triturated into a single cell suspension using fire-polished glass pipettes. Cell density and viability were determined using trypan blue exclusion. Cells were cultured under clonal conditions at 10 cells/μl in 24 well ultra-low attachment dishes (Corning) in serum-free DMEM/F12 medium containing 2% B27 supplement (Gibco), 10 ng/ml FGF2 (Sigma-Aldrich), 20 ng/ml EGF (Sigma-Aldrich) and 2 ug/ml heparin (Sigma-Aldrich). Cells from each animal were cultured independently and four technical replicates were performed for each condition and each animal. Primary spheres ≥50 mm in diameter were quantified 7 days later. To assay self-renewal, neurospheres were mechanically dissociated into single cell suspensions using P1000 pipette tips and then cultured under the same conditions as the primary cultures, plating 2 cells/μl. Secondary spheres, ≥50 mm in diameter, were quantified 6 days after passaging.

2.4 Embryonic cortical cultures

Cortices were dissected from pooled E13.5 CD1 embryos from the same mother and cultured as described (Yuzwa et al., 2016). Briefly, meninges were removed, and the exposed neocortex was collected and mechanically triturated. Cortical cells were then plated onto microscope cover glass slips (Fisher) pre-coated with 2% laminin (BD Biosciences) and 1% poly-D-lysine (Sigma-Aldrich). Cells were cultured in Neurobasal medium (Gibco) containing 40 ng/ml FGF2 (Sigma-Aldrich), 2% B27 supplement (Gibco), 1% Penicillin and Streptomycin (Lonza), and 500 mM L-Glutamine (Gibco). The following day conditioned media collected from medulloblastoma cell lines (24 h conditioned in Neurobasal media + B27, FGF2, L-glutamine) was added 1:1 to the cultures. After 5 days in vitro (DIV), cells were fixed, permeabilized and prepared for immunostaining.

2.5 Immunostaining of embryonic cortical cultures

Cultured cells were washed with PBS for 10 min, fixed with 4% paraformaldehyde (PFA) for 15 min, permeabilized with 0.2% Triton-X-100 (Fisher) for 10 min, washed with PBS for 10 min, then blocked with 2% BSA and 6% normal donkey serum in PBS for one hour at RT. Cells were
then incubated with primary antibodies in blocking buffer for 2 hr at RT. Cells were washed with PBS for 15 min and incubated with fluorescently labelled secondary antibodies at RT for 1 hour. Cells were washed for another 15 min, then mounted with ProLong Gold Antifade Mountant with DAPI (Fisher). Cells were imaged using a Zeiss Axio Imager M2 system with an X-Cite 120LED light source and a C11440 Hamamatsu camera.

2.6 Neuroanatomy and immunostaining of sections

For immunocytochemistry, adult mice (NOD SCID) were transcardially perfused with PBS, followed by 4% PFA. Following dissection, both adult and perinatal brains were fixed in 4% PFA for 24 hr at 4°C and cryopreserved in 30% sucrose for 48 hr at 4°C. Brains were then snap-frozen in O.C.T. and sectioned coronally at 18 – 20 μm using a Leica CM 1850 cryostat (Leica Biosystems). Sections were washed with PBS for 10 min and blocked using 5% BSA (Jackson ImmunoResearch) and 0.4% Triton-X-100 (Fisher) in PBS for 1 hr at RT. Tissue sections were then incubated with appropriate primary antibodies diluted in 5% BSA in PBS, overnight at 4°C. For primary antibodies raised in mouse, a MOM (mouse-on-mouse) detection kit was used according to the manufacturer’s protocol (Vector Laboratories). Appropriate fluorescently labelled secondary antibodies (1:1000) were used for 1 hr at RT. For visualization of nuclei, sections were counterstained with Hoechst 33258 for 5 min and slides mounted using PermaFluor mounting media (Thermo Fisher). For BrdU staining, after incubation with fluorescently labelled secondary antibodies, sections were washed 3 times with PBS, post-fixed with 4% PFA for 10 min at RT and washed again for 15 min with PBS. Sections were then incubated with 1M hydrochloric acid (HCl) for 10 min at 4°C and 2M HCl for 10 min at RT and then 20 min at 37°C. After extensive washes with PBS, slides were blocked with 5% normal donkey serum, (Jackson ImmunoResearch), 0.4% Triton-X-100 and 1M glycine (BioBasics) for 1 hr at RT. Sections were then incubated with anti-BrdU (rat, ABD Serotec) antibodies diluted in PBS (1:300), overnight at 4°C and then with Cy3-conjugated anti-rat secondary antibody (Jackson ImmunoResearch) for 1 hr at room temperature. Sections were counterstained with Hoechst 33258 for 5 min and then mounted using PermaFluor mounting media (Thermo Fisher). z-stacked images were obtained using a Quorum Spinning Disk confocal microscope system or a Zeiss Axio Imager M2 system with an X-Cite 120LED light source and a C11440 Hamamatsu.
camera. Images were taken with an optical slice thickness of 0.5 μm and projected z-stacked images are shown.

2.7 Antibodies

The following primary antibodies were used:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Dilution</th>
<th>Source (identifier)</th>
</tr>
</thead>
<tbody>
<tr>
<td>goat anti-SOX2</td>
<td>1:250</td>
<td>R&amp;D Systems (AF2018)</td>
</tr>
<tr>
<td>rabbit anti-Ki67</td>
<td>1:250</td>
<td>abcam (ab15580)</td>
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<tr>
<td>mouse anti-OLIG2</td>
<td>1:250</td>
<td>Millipore (AB9610)</td>
</tr>
<tr>
<td>mouse anti-MASH1</td>
<td>1:1000</td>
<td>BD Pharmingen (556604)</td>
</tr>
<tr>
<td>rat anti-VCAM1</td>
<td>1:250</td>
<td>Millipore (CBL1300)</td>
</tr>
<tr>
<td>rat anti-GFAP</td>
<td>1:3000 (used in vivo)</td>
<td>Invitrogen (130300)</td>
</tr>
<tr>
<td>rabbit anti-GFAP</td>
<td>1:1000 (in vitro)</td>
<td>Dako (Z0334)</td>
</tr>
<tr>
<td>rat anti-MBP</td>
<td>1:300 (in vitro)</td>
<td>Millipore (MAB386)</td>
</tr>
<tr>
<td>mouse anti-NEUN</td>
<td>1:500</td>
<td>Millipore (MAB377)</td>
</tr>
<tr>
<td>rat anti-BrdU</td>
<td>1:300</td>
<td>Bio-Rad AbD Serotec (Clone BU1/75 (ICR1))</td>
</tr>
</tbody>
</table>

Secondary antibodies were AlexaFluor 488-conjugated donkey anti-rabbit and anti-rat IgG, AlexaFluor 555-conjugated donkey anti-mouse and anti-rabbit IgG and AlexaFluor 647-conjugated donkey anti-goat IgG (1:1000, Life Technologies). AffiniPure Cy3-conjugated donkey anti-rat IgG was also used (1:500 – diluted in glycerol, Jackson ImmunoResearch). If the MOM kit was used, AlexaFluor 488-conjugated streptavidin (Jackson ImmunoResearch) was used at 1:1000.
2.8 Quantification of sections

For analysis of the olfactory bulbs and V-SVZ, serial coronal 18 – 20 μm sections were collected spanning the rostral-caudal extent of the olfactory bulbs and V-SVZ. For quantification, every 6 – 10th section was selected for a total of 4 – 12 representative sections per olfactory bulb and SVZ, depending on the age of the mice (for P2 mice n = 4 sections; P8 mice n = 6 sections; >P21 mice n = 12 sections), which were matched in terms of neuroanatomical level. Every positive cell was counted on these sections in all cases except those located within the rostral migratory stream. To obtain the relative total number of SOX2-positive cells in the V-SVZ or the total number of NEUN-positive cells that were also positive for BrdU in the olfactory bulb, the total number of SOX2-positive cells or double positive cells was multiplied by the number of slides collected (P2 mice n = 5 slides; P8/P9 mice n = 6 slides; for P21 mice n = 8 slides; for >P21 mice n = 8 slides) to account for the sampling frequency. To quantify the proportion of SOX2-positive or VCAM1-positive cells that were also positive for MASH1 or Ki67 within the V-SVZ, 4 – 6 sections were sampled as above and the total number of cells positive for both or a combination of three markers were counted and expressed as a percentage of double-positive/single-positive cells.

2.9 Statistics

For embryonic cortical culture experiments, three random fields of view per sample were captured with a 20X objective and quantified. All statistical parameters are presented as scatterplots showing means ± SEM. In all cases, Prism (version 5.01) was used. In the figures, asterisks denote statistical significance marked by *p < 0.05, **p < 0.01, and ***p < 0.001. For embryonic cortical cultures, repeated measures ANOVA was used with Tukey's post-hoc test. For all other analyses, statistics were performed using a two-tailed unpaired Student’s t-test where a p-value < 0.05 was considered significant.
Chapter 3
Data chapter

3 Data Chapter – Investigating the effects of the medulloblastoma secretome on neural stem cell function

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Author Contributions:

JVS performed all experiments and analyzed the data, apart from Figure 1 which was performed by AG, and Figures 2 and 7, which were performed in collaboration with AG. AG, DRK, and FDM supervised the experiments.

3.1 Introduction

Medulloblastoma is the most common malignant pediatric brain tumour, which originates in the posterior fossa (Gibson et al., 2010). Over the past number of years there has been an increase in survival rate, with current treatment strategies including surgery, craniospinal irradiation and chemotherapy (Ramaswamy et al., 2016). Despite these improvements, MB survivors and survivors of other pediatric brain tumours are often left with cognitive deficits that persist throughout life. This was originally thought to be solely a consequence of irradiation and chemotherapy treatment (Ris et al., 2001, Mabbott et al., 2005). However, recent work has shown that a proportion of pediatric low-grade glioma patients who did not undergo radiation treatment also experienced cognitive deficits as compared to healthy controls (Liu et al., 2015). The neurocognitive changes in these patients may be due to tumour-related mechanisms, hydrocephalus, age of the individual or immune activation, or a combination of these factors (Mabbott et al., 2005, Hanzlik et al., 2015, Moxon-Emre et al., 2014).

Poor intellectual outcomes of medulloblastoma patients were associated with changes in brain structure, specifically, damage in the white matter (Liu et al., 2015). However, what is not
known is whether and how MB or other pediatric brain tumours may directly affect neural stem cells (NSCs) responsible for aspects of brain development. Neural stem cells reside in two regions in the brain – the ventricular-subventricular zone (V-SVZ) in the subependymal region of the lateral ventricles and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus (Ming and Song, 2011). The V-SVZ NSCs generate neurons important for olfactory bulb maintenance and odour-reward memories in the rodent brain and perhaps the infant human brain (Alonso et al., 2019, Sanai et al., 2011). These NSCs also produce a substantial proportion of astrocytes and oligodendrocytes during the perinatal period which persists throughout life (Levison and Goldman 1993, Menn et al., 2006). The V-SVZ resides along the lateral ventricles, where cells in this niche are in close contact with circulating factors in the cerebrospinal fluid, blood vessels and the ependymal cells lining the ventricular surface (Tavazoie et al., 2008, Bond et al., 2015). These factors are important for the regulation of the NSC niche, and when qualitative or quantitative alterations in these environmental cues arise, this can result in perturbed NSC function (Lehtinen et al., 2011, Storer et al., 2018).

This led us to ask whether factors released from MB interact with the V-SVZ neurogenic niche, disrupting normal V-SVZ NSC function and maintenance. Recent work by a postdoctoral fellow in the lab, Dr. Alexander Gont, examined whether subcutaneous injection of human MB cells into the flanks of mice could influence brain function. He observed that mice bearing these tumours had an approximately 30% decrease in NSC number and 40% decrease in neural precursor cell (NPC) proliferation in the V-SVZ niche 7 weeks following tumour cell implantation (Fig. 3). These effects were observed using two MB cell lines from two different MB subgroups. Therefore, in these experiments, the effects seen on the forebrain neurogenic niche are not a result of the typical cerebellar location of the tumour but are likely due to tumour-specific mechanisms acting at a distance and perturbing NSC number and function.

In this present study we hypothesize that factors released by MB tumours influence NSC function in the developing V-SVZ. I predict that these factors diffuse through the brain to the V-SVZ or they circulate out of the parenchyma into the ventricular space, affecting the function of the V-SVZ NSCs and their downstream progenitors. We show that MB tumours grown in the flanks of mice perturb olfactory bulb neurogenesis, which is supported by differentiation defects observed when MB-conditioned medium is added to embryonic cortical cultures in vitro. We
show that the MB secretome results in a decrease in NPC proliferation one week after a single intraventricular administration of MB cell-conditioned medium. When assessed in vitro by the neurosphere assay, the MB secretome results in a decrease in V-SVZ NPC number and self-renewal ability. This work provides insight into the role that MB tumours, via their secretome, have on perturbing NSC function and potentially the late effects in pediatric brain cancer survivors.

Figure 3: Mice bearing human MB tumours, grown in their flanks, exhibit impaired V-SVZ neural stem cell (NSC) number and neural precursor cell (NPC) proliferation. A. Experimental timeline for the investigation of V-SVZ NSC number and NPC proliferation in a MB xenograft tumour model. B. Quantification of the proportion of GFAP;SOX2-double positive cells along the V-SVZ. *p=0.020; 4 animals per condition, 2 separate experiments. C. Quantification of the proportion of Ki67;SOX2-double positive cells along the V-SVZ. **p=0.0011; 5 animals per condition, 2 separate experiments. The mean and SEM are indicated in the above graphs. (Experiment performed by Dr. Alexander Gont).
3.2 Results

Medulloblastoma tumour bearing mice demonstrate impaired olfactory bulb neurogenesis

The MB xenograft model demonstrates that the tumour can affect V-SVZ NSC number and NPC proliferation at a distance. To ask whether these decreases in NSC number and NPC proliferation led to longer-term consequences, we next looked at the effect of the tumour on V-SVZ cell differentiation, specifically on olfactory bulb neurogenesis. Mice were injected with MB tumours, then were injected with bromodeoxyuridine (BrdU) once the tumours were of palpable size. Mice were injected with five rounds of BrdU to increase the number of BrdU-labelled, dividing cells (Lemasson et al., 2005) (Fig. 4A). The olfactory bulbs were harvested 21 days later – the approximate amount of time it takes for V-SVZ NSCs to migrate along the rostral migratory stream to the olfactory bulbs in the adult brain (Petreanu and Alvarez-Buylla, 2002). Olfactory bulb sections were immunostained for BrdU and the neuron-specific protein, NEUN (Fig. 4B). Olfactory bulb neurogenesis was impaired in ONS76 tumour bearing mice, with those mice having significantly fewer BrdU-labeled olfactory bulb neurons than controls (Fig. 4C). This work demonstrates that mice with MB grown in their flanks have perturbed V-SVZ NPC differentiation.

Figure 4: Mice bearing human MB tumours, grown in their flanks, exhibit impaired olfactory bulb neurogenesis. A. Experimental timeline for the investigation of olfactory bulb neurogenesis in a MB xenograft tumour model. B. Representative micrograph of olfactory bulb sections stained for NEUN (blue) and BrdU (magenta). Arrows denote double-positive cells.
Scale bar = 100μm. C. Quantification of the number of BrdU;NEUN-double positive cells in the olfactory bulb. *p=0.011; 4 animals per condition, 2 separate experiments. The mean and SEM are indicated in the above graphs.

**MB cell-conditioned medium causes a decrease in NPC proliferation in the dorsal V-SVZ one week following a single intraventricular administration.**

The MB xenograft model demonstrates that the tumour can affect V-SVZ NSCs and their downstream progeny at a distance. Next, I was interested in whether the MB secretome directly affects the neurogenic niche. Therefore, to examine the local effects of the MB secretome on the developing V-SVZ, intracerebroventricular (ICV) injections of concentrated conditioned medium (CM) from MB cell lines were performed. A separate flask of the same media that MB cells were grown in was concentrated using the same protocol as for the MB-CM and was used as control media. Conditioned medium from the ONS76 MB cell line or control medium was injected into each lateral ventricle of P0-1 neonatal mice and the brains were harvested one week later (Fig. 5A). I also investigated regional effects of the MB secretome by analyzing the striatal and dorsal V-SVZ regions separately (Fig. 5B). To assess the effect on NPC proliferation, I immunostained V-SVZ coronal sections for the pan-neural precursor marker SOX2 and the proliferation marker Ki67. While there was no effect on NPC proliferation along the striatal V-SVZ (Fig. 5C), proliferation decreased with a single injection of ONS76-CM in the dorsal region (Fig. 5D). There was no change in the total amount of SOX2-expressing cells (Fig. 5E,F). This analysis indicates that the MB secretome may locally affect V-SVZ precursor cell function, and in particular, proliferation.
Figure 5: Late effects of a single injection of MB-conditioned medium (CM) result in a decrease in NPC proliferation in the dorsal V-SVZ. A. Experimental timeline for the ICV injection of medulloblastoma-conditioned medium. B. Schematic of the V-SVZ. C. Quantification of the proportion of Ki67;SOX2-double positive cells along the lateral ventricle. \( p = 0.85 \); 4 animals per condition, 1 experiment. D. Quantification of the proportion of Ki67;SOX2-double positive cells along the dorsal V-SVZ. \( *p = 0.017 \); 4 animals per condition, 1 experiment. E. Quantification of the SOX2-positive cells along the dorsal V-SVZ, with each point representing a single section quantified. F. Quantification of the total number of SOX2-positive cells along the dorsal region. \( p = 0.19 \); 4 animals per condition, 1 experiment. The mean and SEM are indicated in the above graphs. C = control medium; O = ONS76-CM.

V-SVZ cell number and function is unaffected following short-term exposure to MB-CM.

A single injection of MB-CM appears to dysregulate NPC proliferation in the dorsal V-SVZ one week later. This led me to investigate how long after injection does the MB-CM affect V-SVZ
cell proliferation. To do this, I examined the acute effects of the MB-CM on NSCs and NPCs in the V-SVZ 24 hours after ICV injection (Fig. 6A). To assess the effects of ONS76-CM on NSC proliferation, I immunostained V-SVZ sections for V-SVZ NSC marker VCAM1 and proliferation marker Ki67 (Hu et al., 2017, Kokovay et al., 2012). VCAM1;Ki67-double positive cells were quantified along the lateral ventricle (Fig. 6B). 24 hours following injection of ONS76-CM, NSC proliferation along the striatal V-SVZ appears to be unaffected (Fig. 6C). There was also no change in NSC proliferation in the dorsal V-SVZ (Fig. 6D).

Figure 6: Neural stem cell proliferation along the V-SVZ is unaffected 24 hours after injection of ONS76-CM. A. Experimental timeline for the ICV injection of MB-CM and schematic of the V-SVZ. B. Representative micrograph of V-SVZ sections stained for VCAM1 (magenta) and counterstained with Hoechst 33258 (blue). Scale bar = 360μm. C. The proportion of VCAM1;Ki67-double positive cells along the striatal V-SVZ. p=0.74; 3 animals per condition, 1 experiment. D. Quantification of the proportion of VCAM1;Ki67-double positive
cells along the dorsal V-SVZ. p=0.075; 3 animals per condition, 1 experiment. The mean and SEM are indicated in the above graphs. LV = lateral ventricle.

Since there was a decrease in NPC proliferation in the dorsal V-SVZ one week following injection (Fig. 5D), I next examined whether there was also a decrease in NPC proliferation at an earlier time point following injection. Specifically, coronal V-SVZ sections from 24 hours after injection were immunostained for SOX2 and Ki67 (Fig. 7A). Short-term exposure to ONS76-CM in the neonatal brain did not affect V-SVZ NPC number (Fig. 7B,C) or proliferation 24 hours after injection (Fig. 7D,E). I next asked whether there was an effect on the NPC progeny, the transit amplifying (TA) cells. To do this, I stained V-SVZ sections for the TA cell marker MASH1 in combination with SOX2 and Ki67 (Fig. 7F). Short-term V-SVZ exposure to ONS76-CM did not result in a change in TA cell number or proliferation along the striatal V-SVZ (Fig. 7G,H), nor in the dorsal V-SVZ (Fig. 7I,J). It is possible that 24 hours after injection may be too early for the MB-conditioned medium to have any noticeable, local effects on the V-SVZ niche.

Figure 7: Short-term exposure to ONS76-CM in the neonatal brain does not affect NPC and transit amplifying cell (TA) number and proliferation in the V-SVZ. A. Representative
micrograph of V-SVZ sections stained for Ki67 (green) and SOX2 (blue). Arrows denote double-positive cells. Scale bar = 20μm. B. Quantification of the total number of SOX2-positive cells along the striatal V-SVZ. p=0.37; 3 controls, 4 ONS76-CM-injected pups from 2 experiments. C. Quantification of the total number of SOX2-positive cells along the dorsal region. p=0.86; 5 controls, 4 ONS76-CM-injected pups from 2 experiments. D. Quantification of the proportion of Ki67;SOX2-double positive cells along the striatal V-SVZ. p=0.38; n = 3 control, 4 ONS76-CM-injected from 2 experiments. E. The proportion of Ki67;SOX2-double positive cells along the dorsal V-SVZ. p=0.62; 3 animals per conditions from 2 experiments. F. Representative micrograph of V-SVZ sections stained for MASH1 (green) and SOX2 (magenta). Arrows denote double-positive cells. Scale bar = 11μm. G. Quantification of the proportion of MASH1;SOX2-double positive cells along the striatal V-SVZ. p=0.66; 5 animals per condition from 2 experiments. H. The proportion of MASH1;SOX2;Ki67-triple positive cells along the striatal V-SVZ. p=0.57; 5 animals per condition from 2 experiments. I. Quantification of the proportion of MASH1;SOX2-double positive cells along the dorsal V-SVZ. p=0.62; 3 animals per condition from 2 experiments. J. Quantification of the proportion of MASH1;SOX2;Ki67-triple positive cells along the dorsal V-SVZ. p=0.45; 3 animals per condition from 2 experiments. The mean and SEM are indicated in the above graphs. LV = lateral ventricle.

**ICV-injection of MB-conditioned medium after one week affects V-SVZ NPC number and function as assessed in vitro.**

The experiments of Fig. 5D suggest that MB-CM may perturb NPC proliferation one week following a single injection. To extend these findings, I performed a neurosphere assay following exposure to MB-CM as a biological readout for NSC/NPC proliferative and self-renewal activity. NPCs can be isolated from the V-SVZ and grown in vitro in a serum-free culture system as non-adherent spheres (Reynolds and Weiss, 1992). One neurosphere represents a single stem or progenitor cell that has undergone multiple rounds of cell division, and the number of neurospheres represents the number of NPCs isolated from the neurogenic niche. The potential of these cells to self-renew is assessed by the ability of a single cell dissociated from a primary neurosphere to form a second neurosphere. Specifically, spheres from primary neurospheres are mechanically dissociated into single cell suspension and replated. The number of secondary
spheres is a surrogate measure of the ability of primary spheres to self-renew (Reynolds and Weiss, 1996).

To determine whether the MB-CM affects V-SVZ NPC number (primary sphere assay) and self-renewal (replating to secondary spheres), neonatal mice (P0-P1) were injected with MB-CM and 24 hours later the V-SVZ was dissected and cultured as neurospheres (Fig. 8A). One week later, the number of primary neurospheres were counted as a surrogate measure of NPCs in the V-SVZ. Injection of ONS76-CM resulted in a decrease in the number of primary neurospheres relative to pups injected with control medium, indicating that the ONS76 cancer secretome reduces the NPC pool (Fig. 8B). To assess whether a similar phenotype is seen across MB cell lines, conditioned medium from a second cell line, DAOY, was also injected into the lateral ventricle of neonatal mice and V-SVZ NPCs were cultured as neurospheres. There was no significant difference in primary sphere number when DAOY-CM was injected into the lateral ventricle compared to the control media (Fig. 8C). To assess the affect on NPC self-renewal, primary spheres were passaged and secondary spheres were counted 6 days later. While no change was exhibited in the number of secondary neurospheres with ONS76-CM (Fig. 8D), there was a significant decrease in the number of secondary neurospheres with DAOY-CM (Fig. 8E). This data suggests that the MB secretome alters V-SVZ NPC behaviour. Specifically, one week following a single injection of ONS76-CM, there is a decrease number of V-SVZ NPCs, while DAOY-CM results in a decrease in NPC self-renewal.
Figure 8: One week following MB-CM injection, ONS76-CM results in a decrease in the number of V-SVZ NPCs, while DAOY-CM results in a decrease in NPC self-renew. A. Experimental timeline for the ICV injection of MB-CM and neurosphere assay. B. Quantification of primary neurospheres from ONS76-CM- or control media-injected pups. Each point represents a single animal, as an average of 4 technical replicates. *p=0.048; n = 6 animals per condition, 3 experiments. C. The number of primary neurospheres from DAOY-CM- or control media-injected pups. Each point represents a single animal, as an average of 4 technical replicates. p=0.058; n = 10 animals per condition, 5 experiments. D. Quantification of secondary neurospheres generated from V-SVZ primary neurospheres from ONS76-CM-injected pups with each point representing a single animal. p=0.11; n = 4 animals per condition, 2 experiments. E. The number of secondary neurospheres generated from V-SVZ primary neurospheres from DAOY-CM-injected pups with each point representing a single animal, as an average of 4 technical replicates. **p=0.0096; n = 6 animals per condition, 3 experiments. Error bars represent SEM. (See Appendix 2).
MB-conditioned media causes a differentiation defect in vitro when added to embryonic cortical cultures.

Local injection of MB-CM into the lateral ventricles suggests that the MB secretome does affect normal V-SVZ NPC function. To expand on this, I next wanted to investigate what effect the MB secretome has on the differentiation of V-SVZ cells, in particular on gliogenesis, since there are white matter deficits in MB patients (Liu et al., 2015). To do so, I used the embryonic cortical culture system, a well-established culture system in our laboratory. Cortical precursor cultures temporally mimic the in vivo differentiation pattern, first generating neurons, followed by glial cells (Barnabe-Heider et al, 2005). To test the effects of the MB-CM on gliogenesis, cortical precursor cells were harvested from E13.5 embryonic cortices, triturated and plated on coverslips pre-coated with laminin and poly-D-lysine. The following day, conditioned media collected from MB cell lines was added 1:1 with fresh medium to the cultures. After 5 days in vitro (DIV), cells were fixed, permeabilized and immunostained for markers of differentiation (Fig. 9A). To assess the effects of MB-CM on astrocytes, cultures were immunostained for astrocyte marker glial fibrillary acidic protein (GFAP) (Fig. 9B). I compared all the technical replicates between each condition (Fig. 9C), as well as the average of the technical replicates from each separate experiment (Fig. 9D) to see whether there were differences between individual experiments performed. The proportion of GFAP-positive cells increased when ONS76- and DAOY-CM were added to cortical cultures (Fig. 9C). Further analysis showed that the DAOY-CM had a significant increase in GFAP-positive cells when the averages from three separate experiments were compared, while the ONS76-CM did not (Fig. 9D). To investigate whether the MB secretome affects the proportion of mature oligodendrocytes generated from NPCs in vitro, I immunostained the cultures for the oligodendrocyte marker myelin basic protein (MBP) (Fig. 9E). There was no significant difference in the proportion of MBP-positive oligodendrocytes in either condition compared to the control media, however, there were hardly any MBP+ cells when ONS76-CM was added to cultures (Fig. 9F,G). Furthermore, the ONS76-CM resulted in a significant decrease in the proportion of mature oligodendrocytes compared to the DAOY-CM when technical replicates were analyzed (Fig. 9F). DAOY-CM did not appear to affect cell number; however, ONS76-CM cultures had an increased number of Hoechst-positive cells compared to the control media and DAOY-CM (Fig. 9E,H).
Figure 9: Addition of MB-conditioned media to embryonic cortical cultures induces differentiation in vitro. A. Experimental timeline for the addition of MB-conditioned medium onto embryonic cortical cultures. B. Representative images of cortical cultures with the indicated conditioned medium added and immunostained for GFAP (magenta). Scale bar = 50μm. C. Quantification of the proportion of GFAP-positive astrocytes. Each point represents a single technical replicate from a randomly chosen field of view. ***p<0.001. D. Data from Figure C. with each point representing an average of 3 technical replicates from a single experiment.
*p<0.05, n=3 per condition. **E.** Representative images of cortical cultures with the indicated conditioned medium added. Cultures were immunostained for MBP (red) and counterstained with DAPI (blue). Scale bar = 20μm. **F.** Quantification of the proportion of MBP-positive mature oligodendrocytes with each point representing a technical replicate. **p<0.01. **G.** Data from Figure F. with each point representing an average of 3 technical replicates. **H.** Quantification of the number of Hoechst-positive cells with each point representing an average of 3 technical replicates from a single experiment. *p<0.05, **p<0.01, n=3 per condition. Error bars represent SEM.

### 3.3 Discussion

Survivors of MB and other pediatric brain tumours can exhibit neurocognitive deficits that persist throughout life, whether these patients had undergone radiation therapy or not. While these poor cognitive outcomes have been correlated with white matter compromise, the late effects of MB on stem cells in the developing brain have not been previously investigated. Here we demonstrate that MB tumours have long-range effects on the V-SVZ niche in our MB xenograft model. We also provide evidence that the MB secretome has direct effects on neural precursor cells in the developing brain. I have shown that MB cell-conditioned medium, representative of the cancer secretome, results in a decrease in NPC proliferation one week after a single intraventricular administration. MB-conditioned medium also decreases V-SVZ NPC number and self-renewal ability in vitro as assessed by the neurosphere assay. Lastly, MB-conditioned medium results in a dysregulation of V-SVZ precursor differentiation in vitro.

Our xenograft model demonstrates that the MB tumour itself can affect the neurogenic niche at a distance. This data suggests that the tumour could be acting through the activation of the host immune system or that tumour-secreted factors cross the BBB and perturb cells along the V-SVZ. We show that intraventricular injection of MB-CM affects NPC function, which supports that hypothesis that bioactive factors released from the tumour contribute to the effects seen on the V-SVZ niche. Furthermore, in our MB xenograft model, we see a decrease in olfactory bulb neurogenesis seven weeks after grafting. This could be a result of the tumour-secreted factors inhibiting the V-SVZ NSCs from differentiating into downstream progenitors. Another possibility is that the tumour-secreted factors or conditioned medium increases
NSC/NPC or progeny cell death. However, my embryonic cortical culture experiments do not support this hypothesis as the ONS76-CM cultures had an increase number of Hoechst+ cells (Fig. 9H) and there were no notable changes in the number of condensed nuclei between conditions (Fig. 9E). To confirm this, I would need to stain these cultures for an apoptotic marker such as cleaved caspase 3 (CC3), as well as stain V-SVZ sections from MB xenograft mice for CC3 to see if there are any changes in apoptosis in the V-SVZ of tumour bearing mice compared to control mice. Lastly, this phenotype could also be explained by premature differentiation, resulting in NSC/NPC exhaustion in the long-term. While we observed a decrease in neurogenesis, perhaps if we harvested the brains at an earlier timepoint, we may have noticed an earlier increase in neurogenesis.

The embryonic cortical cultures demonstrate that there is a differentiation defect in vitro. Specifically, we see an increase in astrocytes with both MB-CMs. I also see a decreasing trend in mature oligodendrocytes with ONS76-CM, suggesting that the ONS76 tumour secretome may prevent precursor cells from making oligodendrocytes. However, there are several limitations with the above experiments. Firstly, although we have the control medium as our negative control, it would be important to add a positive control to this assay. An appropriate positive control to assess astrogenesis would be to add cardiotrophin-1 directly to cultures (Barnabe-Heider et al., 2005), and neuregulin-1 as a positive control for the production of oligodendrocytes (Calaora et al., 2001). Furthermore, immunostaining these cultures for a neuronal marker such as βIII-tubulin, would help to resolve whether MB-CM results in a general perturbed differentiation pattern or biased differentiation (ex. towards astrocytic differentiation) (Menezes and Luskin, 1994). Since gliogenesis begins in culture around 5-6 DIV, by fixing these cultures after 6 DIV, I may be testing too early and missing much of the differentiation that would have occurred (Qian et al., 2000). Additionally, oligodendrogenesis in cortical cultures peaks even later than astrogenesis, which explains why the percentage of MBP-positive cells was so low. To better assess the effects of the MB-CM on mature oligodendrocytes, I would wait longer, at 10 DIV for example, to fix and analyze these cultures. Alternatively, I could use earlier-appearing cell markers such as OLIG2 and PDGFRα to assess oligodendrocyte precursor cell (OPC) numbers. Lastly, an alternate method to characterize these effects would be to perform clonal analysis using the piggyBac (PB) transposon system which allows for the
permanent genomic incorporation of eGFP into a single precursor (Voronova et al., 2017). Specifically, embryonic precursors are transfected with the PB eGFP reporter and PB transposase under conditions where less than 1% of precursors are labelled. The transfected cultures can be immunostained for downstream cell markers (ex. gliogenic markers) to examine the effects of MB-CM on a single precursor and their progeny. This may help reduce technical challenges such as quantification errors or plating-density issues that may occur with other techniques.

Following one week of a single ICV injection of MB-CM, I observed a decrease in NPC proliferation in the dorsal V-SVZ. However, ongoing experiments include repeating these quantifications several more times to confirm these suggestive results. Specifically, one of the control samples had a very high number of SOX2+ cells compared to the other control samples (Figure 5E,F). This outlier makes it difficult to draw conclusions from this experiment without repetition. If these trends do persist in consecutive experiments, this phenotype may be explained by region-specific effects. Firstly, these two regions arise from different embryonic origins (Fuentealba et al., 2015). As well, the dorsal region has been shown to be enriched in FGFR1 and FGFR2 (Azim et al., 2012). Therefore, it is possible that these and other receptors are more responsive to the factors in the conditioned medium injected, compared to on the striatal side and thus, respond differently to the MB-CM. Alternatively, these differences may be explained by the fact that the dorsal region has greater exposure to the lateral ventricle and to the conditioned medium injected based on the way injections are performed. Furthermore, although there was no significant change, a decreasing trend in NSC proliferation was observed in the dorsal V-SVZ, 24 hours following conditioned medium injection (Fig. 6). Perhaps if this experiment was performed on more animals, this trend would reach significance, further supporting that these MB-CM injections have region-specific effects.

While the decreasing trend in NSC proliferation 24 hours after injection does provide a hint that the MB-CM may acutely act on the V-SVZ cells, no changes were observed when the NPCs and TA cells were assessed at this timepoint (Fig. 7). The reason we may not be seeing effects from the MB-CM on the V-SVZ NSCs and their progeny is that this may be too early to see changes. It is possible that the effects of the conditioned medium are being masked at this early neonatal timepoint, which is a very active time during postnatal forebrain development,
specifically for gliogenesis (Burns et al., 2009). If I waited a few days, for instance 3 days following injection to analyze the V-SVZ, we may observe changes in the number of NPCs and TA cells in the V-SVZ. Alternatively, administering multiple injections of MB-CM may be necessary to see a more drastic effect on the V-SVZ niche. This would also be more representative of what happens in medulloblastoma patients since the tumour is constantly secreting factors.

I show that MB-conditioned medium alters normal V-SVZ NPC number and self-renewal ability in vitro, by the neurosphere assay. To complement these findings, when Dr. Gont added ONS76-CM and DAOY-CM directly onto cultured V-SVZ cells, he saw a significant decrease in the number of spheres formed one week later (Appendix 1). This suggests that the effects I observed on neurosphere-forming cells following ICV injections are direct. While I observed that the trends are relatively consistent between the ONS76-CM and DAOY-CM (Fig. 8), the number of primary spheres significantly decreased only with ONS76-CM, and secondary sphere number significantly decreased with DAOY-CM. The reason these trends may have not reached significance is that there may have been some slight differences in plating technique between experiments, thus some experiments may have had more V-SVZ NPCs to start and thus the significance is lost when the experiments are pooled (Appendix 2).

The neurosphere assay is a useful tool to support and further explain in vivo observations, however, there are some limitations to this assay. For example, it has been shown that both NSCs and TA cells can generate neurospheres (Doetsch et al., 2002). As well, neurospheres are grown in media that is not representative of an in vivo environment. Therefore, it is important that the neurosphere data is done in parallel with other experiments as we have done here with our MB xenograft model and ICV injections of MB-CM.

To identify the factors in MB-conditioned medium, Dr. Gont performed cytokine antibody arrays of conditioned media from MB cells. While the ONS76 and DAOY cell lines both express IL-6 family ligands (IL11 in ONS76 and both IL-11 and IL-6 in DAOY), their secretory profiles do present some qualitative and quantitative differences. This may explain why the CM from these cell lines have different phenotypes in the neurosphere assay (Fig. 8) and in the embryonic cortical culture experiments (Fig. 9). Important next steps would be to compare the effects of the
different condition media by histology, looking at various cell types in the V-SVZ such as the OPCs.

This is the first study, to our knowledge, that has examined the role of MB tumour-secreted factors and their effect on V-SVZ NSCs and their progeny. Together, this data demonstrates that the MB secretome acts on the V-SVZ NSCs and perturbs normal cell function. This dysregulation in stem cell function may explain the changes seen in the brain structure of MB patients and the long-term cognitive deficits they experience.
Chapter 4
Discussion and Future Directions

4 Concluding Chapter – Discussion and Future Directions

4.1 Summary

Here I asked whether the MB secretome can act on the V-SVZ NSCs to perturb their function. To accomplish this, I investigated whether the MB secretome has direct effects on V-SVZ NPC in the perinatal mouse brain. By intraventricular administration of MB-conditioned medium, I observed a decrease in V-SVZ NPC number and self-renewal ability in vitro by the neurosphere assay. MB-conditioned medium also resulted in a dysregulation of V-SVZ precursor differentiation in vitro. Lastly, MB tumours were shown to have long-range effects on the V-SVZ niche and olfactory bulb neurogenesis in our MB xenograft model.

Previous work has shown that radiotherapy causes brain structural changes and perturbs V-SVZ neurogenesis in mice (Beera et al., 2018). Other reasons for these perturbations are chemotherapy treatment or surgical intervention. In contrast, I found tumour-mediated changes in the neurogenic niche in the absence of treatment. In the following sections, I will describe the strengths and weaknesses of the approaches I have taken to come to the conclusion that tumour-specific mechanisms may also influence V-SVZ stem cell function.

4.2 Strengths and weaknesses of my approaches

Medulloblastoma cell lines

In these experiments, we used the ONS76 cell line and conditioned media from ONS76 and DAOY cells. We have compared the transcriptome of the cell lines we used to the transcriptome of patient tumours and found that the patient tumours and the ONS76 cell lines both expressed VEGFA, HDGF and NOV. As well, candidate factors from the ligand-receptor modelling of the MB cell lines and tumours were shown to be secreted from MB cell lines by cytokine antibody arrays. For instance, ONS76-CM was shown to secrete PDGFA, MDK and NOV. Therefore, the MB cell lines used in our study secrete ligands present in patient tumours. Furthermore, established cell lines consist of a relatively uniform population of cells, which is beneficial for
reproducing experimental findings. Patient cell lines on the other hand, are often a heterogeneous mix of cells which often leads to increased variability and makes repeating experiments difficult to do (Ivanov et al., 2016). However, there are a few issues with using these established cell lines. MB is recognized for being a heterogeneous tumour, therefore, by using only two cell lines, we do not have a full representation of the disease (Cavalli et al., 2017). Specifically, we selected cell lines that are both classified as SHH subgroup tumours. It is important that this work be expanded using cell lines from other subgroups to get a broader analysis on the effect of tumour-specific factors on endogenous forebrain progenitors. In addition, these cell lines are grown over several passages and have adapted to the artificial mix of nutrients they are grown in, which results in selection biases and phenotypic and genetic drift (Wenger et al., 2004). While we did identify ligands that were the same among the cell lines and patient tumour samples, there are still many tumour-secreted factors that are missing from our cell lines, such as GDF11, VEGFB and TGFB3. An alternative method would be to use primary patient-derived cell lines, which are harvested directly from the patient. Since they have a low passage number, they undergo less genetic modifications compared to the established cell lines and thus more closely resemble MB tumours (Ivanov et al., 2016). As well, they present with more cellular heterogeneity, compared to established cell lines (Cavalli et al., 2017).

**MB xenograft model:**

We used a MB xenograft model to examine how MB affects the adult V-SVZ niche at a distance (Fig. 3,4). This technique allows us to examine the effects of the MB secretome over a long period, like in MB patients where the tumour is constantly secreting factors. This also provides an advantage over intracranial injection of MB cells, where there is risk of tissue damage which can compromise the experimental findings. However, there are some limitations to this system. Firstly, the tumour cells injected were cultured over several passages in media not representative of the tumour environment, which alters their molecular and genetic identity. Another weakness of the xenograft model is that we are using human MB tumour cells in a mouse. Fundamental differences in immune function exist between species, such as the lack of a functional Toll-Like Receptor 10 (TLR10) in mice and mice express TLR11, TLR12, and TLR13 which are not present in the human genome (Mestas and Hughes, 2004, Hasan et al., 2005). There are humanized models of the immune system, such as the XactMice, which by expanding human
hematopoietic stem and progenitor cells, these mice produce immune cells that help replicate the human immune system and the human response to tumour formation (Morton et al., 2016). Despite this, many of the ligands that are expressed in the human tumour do interact with mouse V-SVZ cells, as seen when I added human recombinant IL-6 to V-SVZ neurospheres, resulting in a decreased number of spheres (Appendix 3). These MB xenograft experiments were repeated using another MB cell line, DAOY, which showed similar trends as seen with ONS76 mice, where there was a decrease in NSC number, NPC proliferation and olfactory bulb neurogenesis. However, to better address this limitation, a more clinically-relevant model would be to use primary patient MB cell lines. Another issue is that since we are injecting the tumour cells into the flank of the mouse, we cannot differentiate whether the tumour-secreted factors are acting directly on the V-SVZ or indirectly via the immune system. Therefore, we performed intraventricular injection of MB cell-conditioned media to determine whether the MB secretome directly affects the V-SVZ. Additionally, in the xenograft model, tumour cells are injected into the flank of the mouse, which is not representative of the typical location of the tumour in the posterior fossa. As well, grafting of tumour cells does not portray the malignancy and changes in the microenvironment that occurs in situ. To resolve this, ongoing experiments include using a MB in situ model – the Math1-Cre/Ptcfl/fl transgenic mouse line (Yang et al., 2008). patched (ptc) is an antagonist of the Shh signaling pathway, and when deleted, this repression is removed and there is an increase in cell proliferation. When Math1-Cre mice are crossed to Ptcfl/fl mice, this allows the deletion of ptc in Math1-positive granule neuron precursor cells. This causes hyperplasia in the cerebellum by P21 and these mice are symptomatic by about 8 weeks of age (Yang et al., 2008). With this model, we can repeat similar analysis to what was performed in the MB xenograft mice.

**Intracerebroventricular injections**

To resolve many of the issues addressed with the flank model, and to better test our hypothesis, we turned to intracerebroventricular injections of MB-CM. This is a useful technique to examine the direct effects on the niche as similar to factors secreted from MB in patients, the MB-CM does not have to cross the BBB. Additionally, injecting into the brain is more representative of the tumour location. MB can arise during adulthood; however, most cases occur in the pediatric population. The intracerebroventricular injections in neonatal pups is more representative of the majority
of MB patients, while our MB xenograft model is more representative of the smaller proportion of adult cases. Although this method can provide a rapid readout on whether the MB secretome affects the V-SVZ niche, one downside to this technique is that this is an artificial system. The conditioned medium is bathed in basal DMEM media and the MB-CM may not contain the exact factors or concentration of factors typically present in the MB tumour secretome. Another limitation to these experiments is that we administered only a single dose of the conditioned medium, whereas the tumour would constantly secrete factors.

**Neurosphere experiments**

I used the neurosphere cultures as a surrogate measure of NPC number and self-renewal. This technique provides us with a quick estimate of the number of precursor cells in the niche in an isolated system. Additionally, this method enables us to observe changes in NPC number and self-renewal, even when the NPCs are no longer exposed to the secretome. Changes in secondary sphere growth or self-renewal, for instance, can suggest whether the MB-CM causes cell-intrinsic changes that persist even when they are out of the V-SVZ and no longer exposed to MB-secreted factors. However, a weakness of this method includes the artificial environment the cells are grown in. Neurospheres are grown in a serum-free medium not representative of the in vivo environment. TA cells can also form neurospheres, thus, we cannot identify the effect of the condition medium solely on NSCs (Doetsch et al., 2002). Furthermore, different regions of the V-SVZ have been shown to contribute more than others to neurosphere formation, with approximately 70% of neurosphere-forming cells originating from the striatal V-SVZ. (Young et al., 2007). My observations, one week following MB-CM administration, suggest region-specific effects (Fig. 5D). It is possible that generating neurospheres following dissection of the entire V-SVZ may be diluting the effects of the MB-CM on NPC number and self-renewal in the neurosphere assay. Perhaps if I dissected and cultured different regions separately, there may be significant changes with the administration of MB-CM, especially in the dorsal region where I saw a decrease in NPC proliferation with ONS76-CM.

**Embryonic cortical cultures**

The embryonic cortical culture system was used to investigate the differentiation potential of MB-CM on NPCs. Factors can be added to assess their ability to promote neurogenesis,
gliogenesis and oligodendrogenesis that occurs in these cultures sequentially and in a readily quantifiable manner (Qian et al., 2000, Barnabe-Heider et al, 2005). While this technique can provide insight into the effects of the MB secretome on NPC differentiation, the developmental timepoint of these cultures is not representative of the pediatric brain when MB is most prevalent. Thus, these cultures are not clinically relevant and need to be complemented with other experiments, such as ICV injections of MB-CM and the MB xenograft model.

4.3 Remaining questions

I have demonstrated that the MB secretome can alter V-SVZ NSC function. To further characterize this phenotype and tease apart what exactly is causing these effects in the niche, I would next identify ligands released by medulloblastoma tumours that influence NPC function. Our lab has done extensive analysis on identifying important ligands that may be involved. Specifically, we compared tumour tissue microarray data from 100 randomly-selected MB tumours (Cavalli et al., 2017) to cell surface proteomic data of early postnatal V-SVZ NSC/NPCs (Yuzwa et al., 2016) to predict ligands expressed by MB cells that could bind receptors on NSC or NPCs. A number of these ligands were validated using postnatal NSC/NPC-derived neurospheres in cultures, including VEGF and PDGF-AA. In particular, I found that ligands predicted from SHH-group tumours, such as IL-6, when added to secondary neurospheres, resulted in fewer spheres, suggesting that there was a decrease in NPC self-renewal (Appendix 3).

To further validate and expand this analysis, it would need to be determined whether these factors directly influence forebrain NPC function in vivo. This can be done by testing individual candidate ligands or a cocktail of ligands by ICV-injection into the neonatal brain and characterizing the effects on V-SVZ NSC and NPC number and function by histology. Specifically, V-SVZ sections can be immunostained for markers of NSCs, SOX2 and GFAP, as well as proliferation marker, Ki67. Progeny such as OPCs could also be examined by immunostaining for OLIG2 and PDGFRα and astrocytes by quantifying the number of GFAP-positive and SOX2-negative cells. V-SVZ neurospheres can also be cultured following the injection of these factors and the number of primary and secondary spheres quantified to assess NPC number and self-renewal. I would next determine whether factors that affect V-SVZ NSC
function are necessary to cause this phenotype. This can be done by systematically depleting validated factors of interest from the medulloblastoma-conditioned media. Specifically, individual factors or a combination can be immunodepleted from MB-CM and injected into the lateral ventricles of neonatal pups. Alternatively, chemical inhibitors or function-blocking antibodies can be used to inhibit the function of a receptor for a ligand of interest. For instance, an entire pathway can be inhibited, such as the IL-6 family compounds by using a gp130 inhibitor, bazedoxifene, or a neutralizing antibody (Wu et al., 2016, Burger et al., 2017). Similar analysis can be performed as previously mentioned to assess the impact on the V-SVZ – by histology and the neurosphere assay. I expect that immunodepleting factors that are essential for perturbing V-SVZ NSC function will reverse these effects.

We see decreased NPC proliferation one week following injection, however, there were no significant effects 24 hours after injection. This begs the question: how early do we see these effects? It is possible that 24 hours is too soon to see changes in NSC or NPC function, especially in the progeny, such as in the TA cells. Furthermore, administration frequency is another factor that is likely important in examining the effects of the MB secretome. It is possible that in order to see a strong enough outcome, MB-CM must be administered on multiple occasions, even daily. However, this can be technically challenging as the mice age and the skull begins to harden, which would increase the risk of brain damage to these mice. Another solution would be to have the MB-CM administered constantly using an osmotic minipump. This method would allow for the constant and regular delivery of the MB-CM into the lateral ventricle, which is more comparable to MB patients that are continuously being exposed to the tumour secretome (Gaviro et al., 2013).

To assess the effects on V-SVZ NSC differentiation in the MB xenograft model, we assessed olfactory bulb neurogenesis by quantifying the number of BrdU;NeuN-positive cells at 7 weeks after tumour grafting. To further analyze how olfactory neurogenesis is affected, we could ask whether one type of interneuron is affected more than the other, for instance, if we observe a decrease in tyrosine-hydroxylase-positive PGCs, but no change in calbindin-positive number (Fuentealba et al., 2015). We could also examine whether the correct types of neurons are made in the appropriate location within the olfactory bulb. Lastly, performance on odour
discrimination tasks could be assessed as a measure of functional impact of the MB secretome on V-SVZ neurogenesis (Lazarini et al., 2009).

For this project, I focused on the effect of the MB secretome on the V-SVZ because of its close contact with circulating factors in the CSF in the lateral ventricle. However, the SGZ also receives signals from the vasculature, neighbouring cells and components of the extracellular matrix. There are many reasons why investigating the effects on the hippocampal neurogenic niche would be a critical future direction. Firstly, SGZ neurogenesis is important for memory formation and cognition throughout life (Leutgeb et al., 2007, Gu et al., 2012). Perturbation of hippocampal neurogenesis leads to deficits in some forms of memory in rodents and has been associated with neuropsychiatric disorders, such as schizophrenia and depression (Arruda-Carvalho, Duan et al., 2007, Boldrini et al., 2013). Furthermore, hippocampal volume has been shown to decrease in pediatric brain tumour survivors, including MB (Riggs et al., 2014, Decker et al., 2017). Although these decreases in hippocampal volume were largely attributed to the treatments these patients received, it would be important to investigate whether tumour-specific mechanisms act and perturb the NSCs in the SGZ neurogenic niche. Since we do see these structural and behavioural changes in these patients, I do expect that we would see an effect on SGZ NPC function. On the other hand, previous work in our lab has shown that transient maternal IL-6 increases the V-SVZ NPC pools and perturbs olfactory neurogenesis in adult progeny. However, this transient exposure of embryos to IL-6 did not affect hippocampal NPC pools (Gallagher et al., 2013). It is possible that the SGZ has better repair mechanisms for changes in cytokine levels, and perhaps this niche will be more resilient when exposed to the tumour secretome.

To investigate the effects on the SGZ niche, I would first analyze changes in NSC and NPC number and function, as well as neurogenesis by histology in the MB xenograft model. The SGZ niche can also be examined following ICV injection of MB-CM. Hippocampal volume could also be measured by MRI to examine whether MB treatment or tumour-secreted factors alone cause changes in hippocampal structure (Winocur et al., 2018). If MB tumour bearing mice or MB-CM injected mice demonstrate structural and histological defects in the hippocampus, it would be important to next examine behavioural changes such as memory-related tasks. One test that can be performed is the Morris water maze, which assesses spatial memory (Morris et al.,
1982). Mice are placed in a pool filled with opaque water and must find the hidden platform using spatial cues around the room. After 6 days of training, the platform is removed and the amount of time the mice spend in the area the platform used to be in, is quantified. The more time mice spend in the region where the platform once was is associated with better spatial memory. I expect that if tumour-associated factors cause hippocampal deficits, these mice will spend less time in the platform region, associated with poor spatial memory function.

Whole brain and local radiation to the anterior commissure, olfactory bulbs and V-SVZ regions resulted in a decrease in V-SVZ in mice (Beera et al., 2018). If we immunodeplete MB-secreted factors and see improved V-SVZ NSC function and neurogenesis, what would the outcome be if these mice were also exposed to radiation on V-SVZ NSC function and neurogenesis? Perhaps the effect of radiation in MB patients is exacerbated by these factors, and the elimination of these factors would also reduce the impact of radiotherapy. It would also be intriguing to examine the effect on brain structure volume, specifically in the V-SVZ and white matter.

From our work thus far, our data suggests that there is some interaction between these MB secreted factors and the V-SVZ niche cells. Following additional validation and characterization of these findings as described above, the next step is to investigate how we can disrupt the communication between tumour secreted factors and cells in the V-SVZ. One strategy could include using CRISPR/Cas9 technology to ablate candidate factors from MB cells. These cells with the ablated ligand can then be injected into the flank of NOD/SCID mice to observe the late effects of the tumour on the V-SVZ. I expect that if this ligand is necessary for perturbing normal V-SVZ NSC function, the injection of these cells should not affect the function of V-SVZ NSCs and their progeny. One issue that may arise is that the tumour may not grow as efficiently if these ligands are required for tumour growth. Thus, it will be essential to measure tumour volumes as we have done in our experiments, to make sure the tumours are forming. Alternatively, the tumour conditioned medium from these manipulated cells can be injected into the lateral ventricle and histological analysis can be performed to determine the effects on the V-SVZ niche.
If the above experiments identify specific ligands in the MB secretome important for dysregulating V-SVZ cell function, the question then remains, how can we prevent long-term outcomes in MB patients? Cytokine antagonists have been used for cancer patients diagnosed with cytokine-induced sickness behaviour caused by cancer and cancer treatment and have been shown to prevent some features of sickness behaviour (Monk et al., 2006, Laye et al., 2000). Antagonists against our ligand of interest or its receptor may be used to reduce or prevent the late effects of cancer in MB patients. Inhibitors for candidate factors that are already used clinically can also be tested for potential therapies. This would involve screening FDA-approved compounds that inhibit ligand production or prevent the proper function of downstream targets. Similar strategies have been used previously in our lab, where clinically-approved drugs were repurposed for skin repair (Naska et al., 2016), axon degeneration (Feinberg et al., 2017) and neurogenesis using metformin (Wang et al., 2012). The timing in which these therapies are administered, whether it be during treatment or immediately at diagnosis, will need to be considered.

By the time of diagnosis, MB survivors may already have changes in the neurogenic niche, brain structure and cognition (Margelisch et al., 2015). It is also possible that we may only be able to eliminate the presence of one of these secreted factors of many that may affect V-SVZ function, and these children may therefore still be left with poor intellectual outcomes. Therefore, if we cannot prevent these long-term cognitive effects, how can we manage these survivors if damage has already been done? Our lab has shown that metformin can increase hippocampal neurogenesis, resulting in enhanced spatial memory in mice (Wang et al., 2012). These findings were translated into the clinic, where metformin was shown to improve memory encoding and white matter structure in pediatric brain tumour survivors who had undergone radiotherapy (Ayoub et al., 2018). Another strategy involves using exercise for neural recovery following cranial radiation. Specifically, exercise training was shown to improve white matter and hippocampal structure and increase reaction time in pediatric brain tumour survivors – many of which had MB (Riggs et al., 2017). At the Hospital for Sick Children, exercise therapy and metformin are currently being combined in the hopes of improving learning and cognitive deficits in brain tumour survivors.
In conclusion, determining whether the MB secretome directly affects endogenous forebrain NSCs and their progeny will lead to the identification of individual factors involved in these effects, with the long-term goal of identifying therapies that can preserve neurocognitive function in MB survivors.
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Appendices

Appendix 1: MB-CM causes an exhaustion of NPCs *in vitro*. A. Experimental timeline for the addition of MB-CM onto dissociated V-SVZ neurospheres. B. Quantification of the number of secondary neurospheres. ***p<0.0001. Error bars represent SEM. (Experiment performed by Dr. Gont).
Appendix 2: Data from Figure 8 graphed as individual experiments. A. Quantification of the number of primary neurospheres from ONS76-CM- or control media-injected pups, showing each separate experiment. In scatterplots, individual technical replicates are shown as points. B. The number of primary neurospheres from DAOY-CM- or control media-injected pups. Each point represents an individual technical replicate. C. Quantification of secondary neurospheres generated from V-SVZ primary neurospheres from ONS76-CM-injected pups, showing each separate experiment. Each point represents a technical replicate. D. The number of secondary
neurospheres generated from primary neurospheres from DAOY-CM-injected pups with each point representing an individual technical replicate. Error bars represent SEM.

Appendix 3: IL-6 causes an exhaustion of NPCs in vitro. Quantification of the number of secondary neurospheres. **p=0.0062. Error bars represent SEM.