

The impact of medulloblastoma-derived extracellular vesicles on the development of primary cortical neurones

Sophie McCann

Student ID Number - 20131577

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Assoc. Prof. Gareth Hathway
Assoc. Prof. Federico Dajas-Bailador

Abstract

Even though paediatric brain tumour survivors are more likely to be diagnosed with a neurodevelopmental or major psychiatric disorder later in life (Hsu et al., 2023), how tumour cells communicate with and alter the development of cortical neurones is poorly understood. One potential mechanism may be intercellular signalling mediated by the secretion of extracellular vesicles (EVs) by cancerous cells within the brain. EVs are small lipid vesicles that can contain proteins, lipids and RNA and have been shown to mediate intercellular signalling between peripheral cancer cells and neuronal models, promoting neurite outgrowth (Amit et al., 2020; Kovacs et al., 2020; Lucido et al., 2019; Madeo et al., 2018). However, it is unknown whether EVs derived from paediatric medulloblastoma cells have a similar effect on mouse primary cortical neurones.

To answer this question, EVs were isolated from two medulloblastoma cell lines (which were derived from a primary tumour and a recurrent tumour from the same patient) using size exclusion chromatography. Medulloblastoma was chosen for this study as it is a highly prevalent and highly survivable paediatric brain cancer. The EVs were then applied to mouse primary cortical neurones (C57BL/6J) after characterising the EVs using nanoparticle tracking analysis (NTA). The morphology of these neurones was then analysed at several time points using immunocytochemistry.

After 24 hours, EVs from both medulloblastoma cell lines accelerated the rate of neuritogenesis in the cortical neurones when compared to the vehicle treated group (One-way ANOVA, $p < 0.05$). EVs from the recurrent cell line increased axon length in the mouse primary cortical neurones after 24 hours (Kruskal-Wallis test, $p < 0.01$). The recurrent cell line released more EVs than the primary cell line, but this was not statistically significant (One-way ANOVA, $p > 0.05$). Importantly, the EV and protein fractions isolated from fresh medulloblastoma cell culture medium did not affect the growth or morphology of the cortical neurones (Kruskal-Wallis test, $p > 0.05$),

suggesting that these findings were not due to any bovine-derived contaminants in the medium.

The results presented show that EVs released from medulloblastoma cell lines can increase the proportion of mouse primary cortical neurones that had at least one neurite and also increase axon length. However, more work is needed to identify the mechanisms behind this and to establish whether this intercellular communication can contribute towards the established relationship between paediatric brain cancer and neurodevelopmental disorders.

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1. Introduction

1.1 The prevalence, economic impact and mortality of paediatric cancers in the UK

Every year in the UK, 375,000 people are diagnosed with cancer but less than 1% of these cases will be diagnosed in children. The most common cancers in children are leukaemia and other blood cancers, central nervous system (CNS) tumours and lymphomas (Cancer Research UK, 2022). The additional costs of caring for a child with cancer can put significant financial strain on families, with many having to spend hundreds of pounds per month on travel, accommodation, extra food costs and childcare to support their child (Young Lives vs Cancer & CLIC Sargent, 2017). Forty-two percent of parents stop working during their child's illness and 66% take unpaid leave, inevitability contributing to the financial burden put on families.

Approximately 250 children die every year from cancer in the UK but since the 1970s there has been a 68% decrease in paediatric cancer mortality rates due to the improved diagnosis and treatment of paediatric cancer (Cancer Research UK, 2022). As a result, more and more children are surviving their childhood cancer with 72% of patients diagnosed between 1997 and 2001 surviving for at least 20 years (National Cancer Registration and Analysis Service, 2021). Consequently, there is a need to better understand the long-term neurodevelopmental effects of paediatric cancer, in particular brain cancer, on survivors.

1.2 Medulloblastoma

One such brain cancer is medulloblastoma, a solid CNS cancer which arises in the cerebellum (Roussel & Hatten, 2011). It is much more prevalent in children than adults, with children between the ages of 1 and 9 being ten times more likely to be diagnosed with it compared to those over 19 years of age (Smoll & Drummond, 2012). As a result, the majority of medulloblastoma research focuses on paediatric medulloblastoma, with it being the most

common malignant paediatric brain tumour accounting for 20% of all brain tumours in children (Rossi et al., 2008). It is also more common in males than females (Ostrom et al., 2015).

1.2.1 Genetic mutations and altered signalling in medulloblastoma subgroups

Previously, medulloblastoma was classified by morphology but with the development of new sequencing technologies, medulloblastoma can now be subdivided into four distinct genetic groups: Wingless (Wnt), Sonic hedgehog (Shh), Group 3 and Group 4 (Pollack et al., 2019). Each group is characterised by different transcriptome profiles and chromosomal changes but also by their prognoses, cellular origin and demographic affected (Taylor et al., 2012). The idea of genetic subgroups within medulloblastoma first arose from the observation that there are several genetic conditions which are associated with increased risk for medulloblastoma (such as Gorlin and Turcot Syndrome) (Carta et al., 2020). However, it was the profiling of messenger RNA (mRNA) transcriptomes that enabled the definite classification of the four subgroups. Both the Wnt and Shh subgroups have clear changes in gene signalling. Wnt medulloblastoma is characterised by mutations in the APC and beta-catenin (CTNNB1) genes whereas Shh medulloblastoma is associated with mutations in genes which alter Shh signalling such as PTCH1, PTCH2, SUFU and SMO (Taylor et al., 2012). One study found that 15 out of 15 Wnt medulloblastoma cases had CTNNB1 mutations and 8 out of 22 Shh medulloblastoma cases had mutations in the PTCH1 gene. Importantly, these mutations were unique to each subgroup (Jones et al., 2012). Overactivation of these signalling pathways drives the formation of medulloblastoma (DeSouza et al., 2014). Less is known about which specific pathways are affected in Group 3 and Group 4 medulloblastoma as sequencing has not yet identified any one common gene mutation. However, pathway analysis has suggested that the pathways involved may include DNA repair, neuronal electrical activity, chromosome organisation and regulation of translation (Cavalli et al., 2017).

1.2.2 Chromosomal changes

Chromosomal changes are commonly reported in all 4 subgroups of medulloblastoma. The most distinct is the deletion of one copy of chromosome 6 in Wnt medulloblastoma. Monosomy 6 is found in around 85% of Wnt medulloblastoma cases (Jones et al., 2012). However, it has also been identified in a very small proportion of Shh and Group 3 medulloblastoma cases, so it is not completely restricted to the Wnt subgroup. Shh medulloblastoma is associated with the loss of chromosome 9q and Group 3 and Group 4 medulloblastoma are associated with the gain of chromosomes 7q, 7p and 17q (Kool et al., 2012). Again, no one chromosomal change is completely unique to one subgroup.

1.2.3 Cellular origin

Medulloblastoma is classified as an embryonal tumour, meaning that the cancer cells originate from cells that arose during embryonic development (McGovern et al., 2014). All four subgroups arise from cells which are present in the developing cerebellum (Figure 1). For example, Shh medulloblastoma originates from granule neurone precursor cells (GNPCs) (Oliver et al., 2005) which are located in the external granule cell layer of the developing cerebellum before they differentiate and migrate along the radial processes of Bergmann glial cells to the internal granule cell layer (Tamayo-Orrego & Charron, 2019). The other three subgroups (Wnt, Group 3 and Group 4) all originate from the rhombic lip which is present in the developing cerebellum (Haldipur et al., 2019). Wnt medulloblastoma arises from the ventricular zone whereas Group 3 and Group 4 are thought to originate from the subventricular zone (in particular from progenitors of unipolar brush cells) (Gibson et al., 2010; Hendrikse et al., 2022; Smith et al., 2022).

Figure 1

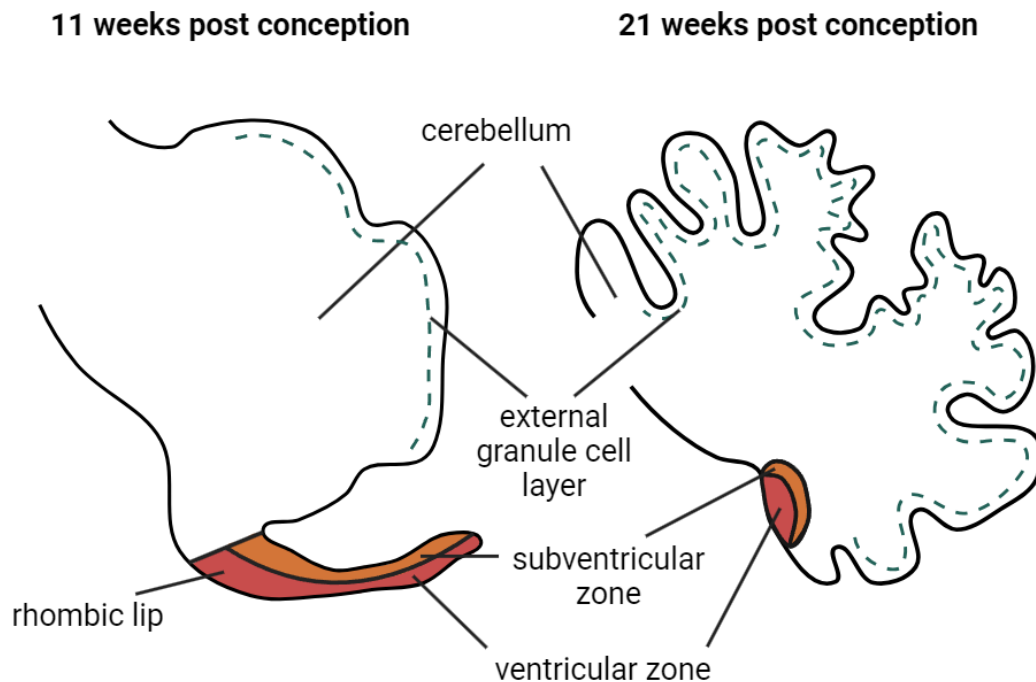


Figure 1. The cellular origin of medulloblastoma in humans.

Medulloblastoma originates in the cerebellum from embryonic cells. Sonic hedgehog medulloblastoma is thought to arise from granule neurone precursor cells in the external granule cell layer. Wingless medulloblastoma originates from the ventricular zone of the rhombic lip whereas Group 3 and Group 4 medulloblastoma arise from the subventricular zone. Diagram made using BioRender, based on Haldipur et al., 2019.

1.2.4 Demographic and prognosis

Interestingly, the four subgroups of medulloblastoma differ by age and sex affected. Shh medulloblastoma is most common in infants under 3 years old and teenagers over the age of 16. Conversely, Group 4 medulloblastoma is more common between the ages of 5 and 13. Group 3 medulloblastoma is more commonly found in infants and young children and Wnt medulloblastoma is more common in older children aged 10 to 12 (Cho et al., 2011; Kool et al., 2012; Northcott et al., 2011). Notably, Wnt medulloblastoma is rarely observed in young infants.

In general, medulloblastoma affects around 1.7 more males than females but again, this varies between subgroups (Juraschka & Taylor, 2019). Both Wnt and Shh medulloblastoma have an equal sex ratio (Kool et al., 2012) but Groups 3 and 4 affect significantly more males than females (T. Sharma et al., 2019). Some have suggested that the X chromosome may harbour tumour suppressor genes as up to 80% of females with Group 4 medulloblastoma show loss of one X chromosome (Gururangan & Schroeder, 2014; Northcott et al., 2011).

The genetic classification of medulloblastoma may also predict the prognosis and survival of the patient. For example, Wnt medulloblastoma has a 10-year survival rate of 95% in children aged 3 to 16, compared to only 50% of patients with Group 3 medulloblastoma (Kool et al., 2012). Wnt medulloblastoma and non-metastatic Group 4 (with chromosome 17 gain or chromosome 11 loss) are defined as low risk, metastatic Shh and Group 4 were defined as high risk and metastatic Group 3 and Shh TP53-mutation medulloblastoma have the worst prognosis (Ramaswamy et al., 2016). The presence of mutated TP53 has been identified as a critical risk factor for those with Shh medulloblastoma. One study found that overall survival at 5 years was only 41% for Shh TP53-mutant patients compared to 81% for patients with SSH TP53-wildtype medulloblastoma (Zhukova et al., 2013). As TP53 encodes a transcription factor which controls cell division and cell death (Aubrey et al., 2016), it is understandable that mutated versions of this gene are involved in oncogenic activity. Additionally, specific protein and metabolic signalling pathways which are associated with poorer prognoses have been identified in each subgroup (Park et al., 2019). For example, upregulation of NOTCH signalling in Group 4 medulloblastoma and glutamate metabolism in Shh medulloblastoma.

As medulloblastoma has an overall 5-year survival rate of around 70% (Johnston et al., 2018), many children survive into their teenage years or beyond. As our ability to diagnose and treat medulloblastoma improves, the number of survivors will continue to grow and so will the number of

individuals who are affected by the neurodevelopmental consequences of early life medulloblastoma.

1.3 Current treatment for medulloblastoma

Medulloblastoma treatment consists of resection of the tumour followed by chemotherapy and craniospinal irradiation (Choi, 2023). Two of the most commonly used chemotherapeutic agents are vincristine and cisplatin (ISPN, 2017). Other chemotherapeutic agents used are lomustine and cyclophosphamide but the exact regime depends on the individual tumour. Cisplatin causes DNA damage which triggers apoptosis whereas vincristine inhibits mitosis by binding to tubulin and preventing mitotic spindle formation (Dasari & Bernard Tchounwou, 2014; Martino et al., 2018). Vincristine is also known to be neurotoxic and can cause axonal degeneration (Gomez-Deza et al., 2023). In the early 1990s, several studies showed that chemotherapy combined with craniospinal irradiation improved outcomes, leading to the implementation of combined therapy as the standard treatment for medulloblastoma (Evans et al., 1990; Tait et al., 1990).

1.3.1 Personalised treatment plans

There are currently several ongoing clinical trials which are trying to determine whether personalised treatment plans based on medulloblastoma subgroup have better outcomes for patients. For example, one study attempted to treat Wnt medulloblastoma with chemotherapy and surgery only (without radiation treatment) but was suspended as two patients relapsed early into the study (NCT02212574). Another is treating Wnt medulloblastoma patients with reduced radiation and chemotherapy to try and minimise the side effects of treatment. However, at present, no results have been posted (NCT02724579). Several more personalised treatment clinical trials are ongoing but none have yet identified any treatment regimens that notably improve patient outcomes (Ghasemi et al., 2022; Thompson et al., 2020).

1.3.2 Caveats of genetic subgrouping

There are several caveats of genetic subgrouping which should be discussed. Firstly, Wnt and Shh signalling overlaps. One study identified that medulloblastoma mutant SUFU cannot reduce beta-catenin signalling unlike wildtype SUFU (Taylor et al., 2004). This results in an overactivation of both Shh and Wnt signalling. There is also evidence that Wnt signalling can reduce the proliferation of cancerous Shh medulloblastoma murine granule neurone precursor cells (Pöschl et al., 2014). If Wnt and Shh signalling are intertwined, then this may complicate the classification of Wnt and Shh MBs into distinct subgroups.

There is also significant debate around the true number of medulloblastoma subgroups. Although the four main subgroups are well established, how these groups are further divided into subtypes varies. For example, one study identified 12 subtypes (2 Wnt, 4 SSH, 3 Group 3 and 3 Group 4) (Cavalli et al., 2017), another identified 8 subtypes within Group 3 and Group 4 (T. Sharma et al., 2019) and another identified 9 subtypes in total (Schwalbe et al., 2017). One reason for the variation in the number of subtypes identified is the fact that different studies use samples from different treatment centres, introducing variability. The methods used to classify the different samples into subtypes vary as well. For example, Cavalli et al. used RNA sequence, DNA sequence and methylation profile in their classification method whereas Sharma et al. used a DNA methylation-based classifier.

Interestingly, one recent study has suggested that rather than being two distinct subgroups, Group 3 and Group 4 make up a spectrum on which transcriptome profile, DNA methylation, age, cellular origin and even prognosis can be placed (Williamson et al., 2022). This may explain why it has been so difficult to identify unique transcriptome profiles for Group 3 and Group 4 medulloblastoma and why automatic classifiers have struggled to distinguish between the two groups of medulloblastoma (Rathi et al., 2020).

Additionally, this may allow treatment to be individually personalised, based on where the tumour is located on the spectrum.

1.4 Cancer and neurodevelopment

Neurodevelopmental disorders are associated with impaired behavioural, cognitive and communication skills as a result of abnormal brain development (Mullin et al., 2013). These conditions usually arise in childhood but can continue to affect individuals all throughout their adult life. Common neurodevelopmental disorders include Attention-Deficit Hyperactivity Disorder (ADHD), Autism Spectrum Disorder (ASD) and other communication, learning and motor disorders (Francés et al., 2022). ADHD has an estimated global prevalence of 5.9% in young people (Faraone et al., 2021) and ASD has an estimated global prevalence of 1% (Zeidan et al., 2022). ADHD is commonly associated with hyperactivity and impulsivity but other lesser known symptoms can be observed such as emotional regulation difficulties and executive dysfunction (Groves et al., 2022). ASD is characterised by social communication deficits, poor nonverbal communication behaviours, sensory processing issues and by having special interests (Lauritsen, 2013).

Despite their contrasting nature, cancer and neurodevelopment share several underlying mechanisms which drive the growth, proliferation and migration of cells (Nussinov et al., 2022). There is a clear link between cancer and neurodevelopmental disorders as patients with ASD are more likely to be diagnosed with cancer between the ages of 15 and 19 compared to individuals of the same age who do not have a neurodevelopmental disorder (Chiang et al., 2015). Likewise, individuals with ADHD are at a greater risk of colorectal cancer compared to matched individuals who do not have ADHD.

Interestingly, the connection is also observed in the other direction. It is well established that paediatric brain tumour survivors (PBTs) have an increased incidence of symptoms of neurodevelopmental disorders (Hsu et al., 2023).

One study found that 23.8% of PBTS met the criteria of ADHD (Hardy et al., 2018), compared to between 2% and 7% of children globally (Sayal et al., 2018). For medulloblastoma in particular, 12% of the children had ADHD. It has also been observed that PBTS have poor facial recognition, social functioning and social attention, comparable with children with ASD (Brodsky et al., 2018; Hocking et al., 2020).

This connection is highlighted by the significant number of genes which are implicated in both cancer and neurodevelopmental disorders. For example, dysfunctional mTOR signalling is implicated in both paediatric brain tumours and ASD, as well as learning difficulties. This is not surprising as mTOR is highly involved in neuronal differentiation (D. Y. Lee, 2015). Additionally, mutations in the PTEN gene (a tumour suppressor gene that encodes a protein that acts upstream of mTOR) are found in individuals with paediatric brain tumours, ASD and ADHD (D. Y. Lee, 2015). PRUNE1 mutations are also implicated in the formation of medulloblastoma and neurodevelopmental disorders (Bibbò et al., 2021).

Although there appears to be a genetic link between paediatric brain tumours and neurodevelopmental disorders, not every case of cancer, ADHD or ASD arises from mutations in these specific genes. Consequently, it can be suggested that other underlying factors might connect these conditions and that the tumour itself could contribute to the development of neurodevelopmental disorders. It can be argued that missed education and reduced socialisation, as a result of time spent in hospital during childhood, drives the emergence of neurodevelopmental disorders later in life. However, this does not appear to always be the case as different types of tumour are associated with different levels of risk for neurocognitive dysfunction later in life (Hsu et al., 2023). However, it should be noted that there is some evidence which suggests that childhood trauma (which is likely experienced by those diagnosed with paediatric cancer) can increase the risk of ADHD diagnosis later in life (Vrijssen et al., 2018).

One recent long-term longitudinal study observed that childhood brain cancer was one of two cancer types (the other being childhood lymphatic/haematopoietic cancer) with the highest number of major psychiatric disorder diagnoses (Hsu et al., 2023). Childhood brain cancer was associated with the highest risk of ADHD, Obsessive Compulsive Disorder and Post Traumatic Stress Disorder. This suggests that PBTS have a greater risk of developing neurodevelopmental disorders when compared to survivors of other childhood cancers. Additionally, compared to other childhood cancers, survivors of paediatric CNS tumours (including medulloblastoma) have fewer friends and interact with their friends less (Schulte et al., 2018). The differences between different types of childhood cancers suggests that these neurodevelopmental disorders are not simply a consequence of the social and neurobiological effects of experiencing cancer and long-term hospitalisation at a young age.

Medulloblastoma is also linked to broader cognitive deficits such as impaired verbal memory and reduced processing speed (Palmer, 2008). One potential confounding factor is the effect of cancer therapies on the developing brain as it is well established that radiation treatment contributes to cognitive impairments observed in PBTS (Pazzaglia et al., 2020). However, one study found that the prevalence of ADHD did not differ between children who had undergone craniospinal radiation for their paediatric brain tumour and those who did not (Hardy et al., 2018). Therefore it is important to investigate whether the tumour itself is able to contribute to the development of neurocognitive and social deficits independently of treatment.

1.5 Neuronal development, polarisation and maturation

In the developing brain, the production of mature cortical neurones occurs in four distinct stages: proliferation, migration, differentiation and polarisation. Radial glial progenitor cells generate immature cortical neurones via asymmetric proliferation (Hansen et al., 2010). These excitatory neurones can then migrate towards the developing cortex via somal translocation, or by migrating along radial glial cells through the ventricular and intermediate

zones and subplate to eventually form the cortical plate (Stiles & Jernigan, 2010). This occurs in an 'inside-out' manner where the neurones located in the deepest part of the cortex (layer VI) are generated first and then neurones generated later migrate past these cells to the outer layers of the cortex. This results in the formation of six cortical layers: the molecular (I), external granular (II), external pyramidal (III), internal granular (IV), internal pyramidal (V) and multiform (VI) layers (Cechedo & Topolovec, 2002; Cooper, 2008) (Figure 2A). Inhibitory neurones on the other hand migrate tangentially from other areas of the developing brain (Tanaka et al., 2009). Terminal differentiation then occurs in which the neurones increase in size and complexity (Fernández et al., 2016).

Polarisation occurs during terminal differentiation and refers to the process during which an immature neurone develops a single primary axon and dendrites which protrude from the soma (Sakakibara & Hatanaka, 2015). In vivo, the formation of synapses enables these mature neurones to form complex networks with other neurones and interneurones. Neuronal polarisation in vitro is comprised of five distinct stages which were first described in 1988: lamellipodia formation, the development of immature neurites, axon specification, axonal and dendritic growth and maturation (Dotti et al., 1988) (Figure 2B). Although first used to describe hippocampal neurones in culture, the same process of polarisation has been observed in cortical neurones (Hirai et al., 2011).

Figure 2

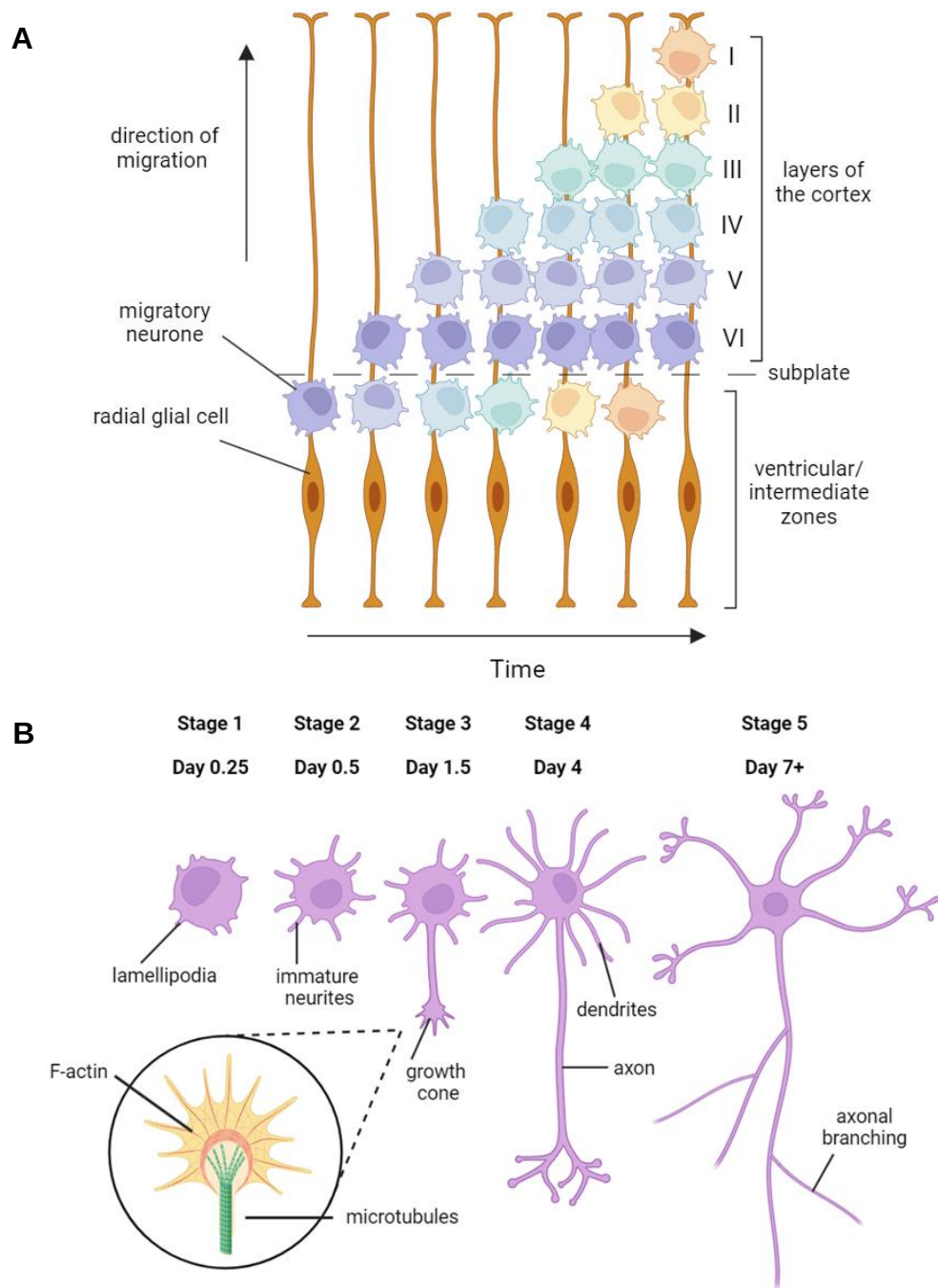


Figure 2. The process of neuronal development and polarisation. A schematic diagram depicting the migration of developing neurones along radial glial cells to form the cortical plate **(A)**. The cortex is formed ‘inside-out’ meaning that the oldest neurones remain in the deepest layers of the cortex. A schematic diagram showing the polarisation of neurones in vitro **(B)**. Once plated, primary neurones undergo polarisation resulting in the formation of a single primary axon. Stage 5 reflects a mature neurone in culture. Diagram made using BioRender, **(B)** based on Dotti et al., 1988.

However, the process of neuronal polarisation in vivo may differ from in vitro studies. Retinal ganglion cells (a type of neuronal cell located in the retina) do not develop immature neurites before the specification of an axon in vivo but rather the first neurite to emerge becomes the axon (Zolessi et al., 2006). Nevertheless, both in vivo and in culture, CNS neurones develop a single axon during the process of polarisation making in vitro cell culture a useful model for studying neuronal polarisation.

The emergence of a single elongated axon during polarisation is mediated by a range of intracellular signalling pathways and extracellular signalling molecules. For example, the small GTPase RhoA has been shown to restrict the growth of the axon during polarisation by promoting the formation of an actin arc. This encases the microtubule bundles, preventing the extension of the growth cone (Dupraz et al., 2019). Other intracellular signalling molecules involved in neuronal polarisation include PTEN and GSK3 (Hur & Zhou, 2010; Kath et al., 2018). Extracellular signalling molecules such as Wnt are also involved in polarisation. Wnt3a promotes neuronal polarisation as inhibiting Wnt3a signalling reduces the proportion of polarised neurones (Stanganello et al., 2019).

Within the cell, microRNAs (miRNAs) are able to control the translation of these proteins and modify neuronal polarisation and axon growth. A recent study from the Dajas-Bailador lab found that adding a miRNA-26a mimic to mouse primary cortical neurones increased axon length and promoted formation of multipolar neurones by inhibiting GSK3 activity (Lucci et al., 2020).

The process of neuronal polarisation appears to be very plastic in nature as cutting the axon at stage 3 or 4 causes one of the dendrites to elongate and form a new axon (Bradke & Dotti, 2000). It has been suggested that the length of the immature neurites is maintained by a balance of positive and negative signalling (Arimura & Kaibuchi, 2007).

1.6 Altered brain connectivity in neurodevelopmental disorders

Altered brain connectivity has been implicated in neurodevelopmental disorders including ADHD and ASD (Kana et al., 2014; Konrad & Eickhoff, 2010). Axonal abnormalities are present in both ADHD and ASD, with one study finding that neurones from ADHD and ASD rodent models have shorter axon initial segments (AIS) compared to wildtype animals (Usui et al., 2022). Neurodevelopmental disorders are also associated with dysregulated synapse formation and there is evidence of abnormal synaptogenesis in both ADHD and ASD (Guang et al., 2018; Washbourne, 2015).

As a paediatric cancer, medulloblastoma often emerges during an important time of neuronal growth and synaptogenesis (Bianchi et al., 2013; Gilmore et al., 2018). It may be that medulloblastoma is able to disrupt the balance of signalling mechanisms that control neuronal polarisation and axonal growth, contributing to the development of neurodevelopmental disorders in PBTS. Among many possible mechanisms to mediate this link, the secretion of extracellular vesicles (known to be actively secreted by cancer cells), has emerged as a potential mechanism for the delivery of signalling molecules which can affect neuronal development.

1.7 Extracellular vesicles

Extracellular vesicles (EVs) are small lipid vesicles that can contain proteins, lipids, mRNA and small non-coding RNA (van Niel et al., 2018). The first observation of EVs was recorded in 1983 when it was noted that immature red blood cells could release EVs via the fusing of multivesicular bodies (MVBs) to the plasma membrane, releasing EVs into the extracellular space (Harding et al., 1983; Pan & Johnstone, 1983). EVs were later divided into three subtypes: exosomes, microvesicles and apoptotic bodies (Doyle & Wang, 2019). Exosomes are the smallest of the EV subtypes with a diameter of 30 to 150nm, compared to a diameter of 100nm to 1µm for microvesicles. The biogenesis of exosomes and microvesicles also varies (Figure 3).

Figure 3

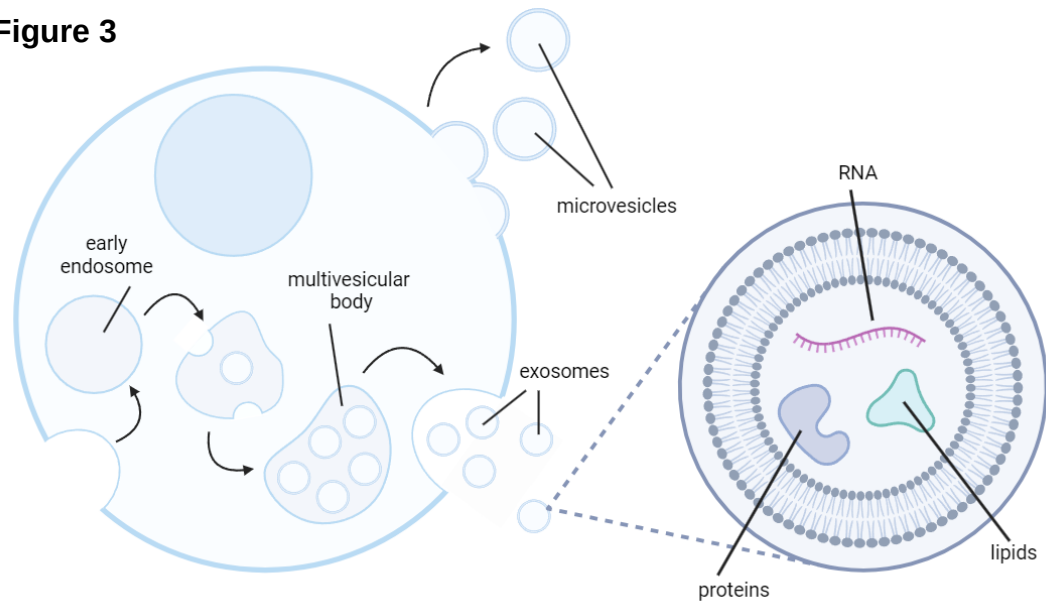


Figure 3. Extracellular vesicle biogenesis. Extracellular vesicles (EVs) can be subdivided into microvesicles and exosomes. Microvesicles are released by the budding of the plasma membrane whereas exosomes are released from multivesicular bodies which fuse with the plasma membrane, releasing them into the extracellular space. EVs can contain RNA (including mRNA and small non-coding RNA), proteins, small metabolites and lipids. Diagram made using BioRender.

Exosomes are released from MVBs which fuse with the plasma membrane, releasing the exosomes into the extracellular space (van Niel et al., 2018). By contrast, microvesicles are formed by the budding of the extracellular membrane and rely on the action of calcium-dependent enzymes such as calpain which enable the release of microvesicles from lipid rafts (Pasquet et al., 1996).

EVs mediate intercellular signalling via several different pathways. For example, EVs can fuse with the plasma membrane of the recipient cell enabling the transfer of signalling molecules or ligands embedded within the lipid bilayer of the EV can bind to receptors on the recipient cell (Buzás et al., 2018; Joshi et al., 2020). Alternatively, EVs are endocytosed by the recipient cell. For example, clathrin-mediated endocytosis prevents damage to the EV membrane as EVs are transported into the recipient cell inside clathrin-coated vesicles. Intact EVs can be observed inside the recipient cell using

transmission electron microscopy (Joshi et al., 2020). EVs can also interact with the extracellular matrix via integrin receptors (Altei et al., 2020). It has been shown that EVs are released by all cell types studied, including cancer cells (Kalluri & LeBleu, 2020). Cancer-derived EVs (also known as oncosomes) can transfer oncogenic receptors, alter the microenvironment surrounding the tumour, promote the innervation of tumours by nerves and even induce neuronal cytotoxicity (Al-Nedawi et al., 2008; Huang et al., 2022; Madeo et al., 2018; W. Zhou et al., 2022).

It is well established that EVs released from multiple cell types can significantly affect the growth and development of neurones. For example, glial cells in *Drosophila melanogaster* release EVs containing the miRNA miR-274 which can promote the growth of synaptic boutons in neurones (Tsai et al., 2019). Similarly, small EVs secreted by astrocytes promote synapse formation in primary cortical neurones (Patel & Weaver, 2021). Neuronal EVs can also affect the activity and growth of glial cells. EVs released from neurones can promote myelin maintenance in oligodendrocytes and promote synaptic pruning by microglia (Bahrini et al., 2015; Domingues et al., 2020). Neuronal EVs can also have an effect on other neurones, promoting neurogenesis and synaptogenesis (P. Sharma et al., 2019). These studies highlight the complex and diverse effects that EVs released by cells in the CNS can have on neurones.

1.7.1 Cancer-derived extracellular vesicles

As EVs can cross the blood-brain barrier (Banks et al., 2020), several studies have investigated whether EVs released from cancer cell lines can stimulate neurite outgrowth in PC12 cells (an immortal cell line which originates from a rat pheochromocytoma (Koike, 1983)). One study found that in vitro, EVs from high-grade serious ovarian cancer (HGSOC) cell lines can stimulate neurite growth when compared to EVs from control fallopian tube cell lines (Kovacs et al., 2020). The same result is observed when EVs from cervical cancer cell lines, a murine cell model of carcinoma or human head and neck cancer patients are used (Lucido et al., 2019; Madeo et al., 2018). Crucially,

these studies all used PC12 cells as their neuronal model rather than primary cell culture. Upon stimulation with nerve growth factor, PC12 cells differentiate into neurone-like cells. However, using PC12 cells as a neuronal model for the investigation of polarisation and neurite growth has significant limitations as they do not accurately replicate the morphology or function of genuine neurones (Wiatrak et al., 2020) and are normally only used to model peripheral sensory neurones (Convertino et al., 2018). Notably, one study, using EVs from oral cavity squamous cell carcinoma, used primary cell culture to assess neuronal morphology. However, they used mouse primary dorsal root ganglion (DRG) culture rather than primary cortical culture (Amit et al., 2020). Although somewhat relevant since DRG culture is used to investigate peripheral sensory neurones, these results cannot be directly applied to neurones of the CNS.

A more recent study has identified that EVs isolated from glioblastoma cell lines and human glioblastoma patients can induce neuronal cytotoxicity in human primary neurones (cultured for 8 days from P0) and embryonic mouse brain slices (W. Zhou et al., 2022). This appears to contradict the other studies which found that cancer-derived EVs promote neuronal growth. One possibility is that neuronal toxicity contributes to the highly fatal nature of glioblastoma which is reflected by its poor 5-year survival rate of only 7% (Miller et al., 2021). Although this study was investigating a type of brain cancer, glioblastoma and medulloblastoma differ in several important ways. Firstly, glioblastoma primarily affects adults (Miller et al., 2021) whereas medulloblastoma is a paediatric disease. This means that medulloblastoma has the potential to influence early brain development in patients whereas glioblastoma does not. Secondly, glioblastoma is highly fatal compared to medulloblastoma. Medulloblastoma has an overall 5-year survival rate of 70%, compared to only 7% for glioblastoma (Johnston et al., 2018; Miller et al., 2021). Thirdly, glioblastoma is glial in origin whereas medulloblastoma is an embryonal tumour (Kim et al., 2021). Equally, due to the heterogeneous nature of brain cancers, results relating to glioblastoma cannot be applied to medulloblastoma. As medulloblastoma has a high survival rate and the potential to alter brain development at a key developmental stage, it is

important to determine whether medulloblastoma EVs can alter cortical neurone development as more patients survive and live to see the potential neurodevelopmental consequences.

1.7.2 Chemotherapy modifies extracellular vesicle release

Treatment of cancer cells with a chemotherapeutic agent is known to increase the release of EVs compared to treatment with a vehicle control. One study found that treating human prostate cancer cells with Doxorubicin resulted in the production of up to 30 times more EVs over a 24 period (Aubertin et al., 2016). Additionally, cancer cells can expel chemotherapeutic molecules via EVs. Human ovarian carcinoma (HOC) cells which have developed resistance to cisplatin, secrete EVs which contain approximately 2.6 times as much cisplatin compared to HOC cells which are not cisplatin-resistant (Safaei et al., 2005). Consequently, it is possible that EVs released from drug-resistant medulloblastoma cell lines will also contain chemotherapeutic molecules, with the potential ability to affect neuronal growth and survival.

1.7.3 Isolating extracellular vesicles

There are numerous methods for the isolation of EVs from conditioned cell culture media or biofluids, all of which have their own advantages and limitations. Differential ultracentrifugation uses high powered centrifugation to isolate the EVs into a single pellet. First, the cell culture medium or biofluid is spun at 1500 x g for 10 minutes to pellet and remove any cell debris, followed by centrifugation of the supernatant at 10,000 to 20,000 x g for up to 30 minutes. Finally the supernatant is spun again at 100,000 x g for at least 70 minutes (Momen-Heravi et al., 2013). The pellet (which contains the EVs) is then resuspended in buffer ready for EV characterisation or functional studies (Momen-Heravi, 2017).

Size exclusion chromatography (SEC) is a more recent method that enables the isolation of particles (including EVs) based on their size from conditioned

medium or biofluid. SEC uses columns which are filled with porous beads. The sample is loaded at the top of the column (followed by buffer) and as the sample moves through the column smaller particles enter the pores of the beads, slowing their passage. The void fraction is eluted first followed by several EV fractions and then smaller particles (including proteins) (Böing et al., 2014). SEC can now be performed using an automated fraction collector (AFC) which ensures precise collection of fractions and reduces the time needed to isolate EVs.

Another method for EV isolation is ExoQuick which utilises the precipitation method of EV isolation. Briefly, the conditioned medium or biofluid is incubated overnight with the ExoQuick solution (which contains a unique polymer that precipitates the EVs) and is then spun at 3,000 x g for 30 minutes to produce a pellet containing the EVs (Veerman et al., 2021).

All of these methods have their limitations. Ultracentrifugation can result in contamination of the EV sample by protein aggregates, damage the EVs and often produces a low yield of EVs (Lamparski et al., 2002). It can also be quite time consuming due to the multiple spin steps and requires an ultracentrifuge. Although SEC has been found to produce EV fractions which have greater levels of the EV marker CD81 when compared to ultracentrifugation, it may also result in more contamination by lipoproteins and greater protein contamination in the later EV fractions (Takov et al., 2019). Unlike differential ultracentrifugation, the low spin speed used for ExoQuick means that damage to EVs is less likely to occur. However, ExoQuick produces less useful miRNA sequencing data compared to SEC, despite producing a greater yield of EVs (Veerman et al., 2021; Yang et al., 2021). This is because SEC produces purer isolates and the smallest amount of non-specific miRNA. This suggests that SEC may be the optimal method of EV isolation for generating miRNA data especially as automated SEC ensures reproducibility between laboratories. All of these isolation methods are routinely used in EV research and as suggested by one study which compared five different methods of EV isolation, the best isolation

method may differ between researchers depending on their sample type and research aims (Veerman et al., 2021).

1.7.4 Storing extracellular vesicles

Although there are several well-established methods for EV isolation, a way to store EVs (particularly in the long term) that does not result in changes in EV size, concentration or cargo has not been established. One study demonstrated that storage of EVs at 4°C, -20°C or -80°C for up to 4 weeks maintains the size distribution of the EVs but that the total EV number decreases by more than 40% in a time-dependent manner. -80°C was recommended for the long-term storage of EVs. Additionally, this study found that after 4 weeks of storage the cellular uptake of EVs is reduced (Wu et al., 2021). However, others have shown that EV concentration does not change when EVs are stored for 4 weeks at 4°C or at -80°C (Deville et al., 2021). The reason for the contrasting nature of these two studies is unclear as both used differential ultracentrifugation to isolate the EVs and nanoparticle tracking analysis (NTA) to quantify the concentration of EVs in the sample. However, even if EVs can be successfully stored for up to 4 weeks it appears that beyond that changes to EV characteristics are much more difficult to prevent. One study clearly demonstrated this by testing 8 different storage conditions but they found no way to prevent the decrease in EV sample quality after 6 months (Gelibter et al., 2022).

The use of cryoprotectants may improve the storage of EVs. For example, one study found that simply storing EVs at -80°C in a 5% sucrose solution, rather than phosphate buffered saline, maintained the concentration of EVs in the sample for 9 weeks. CD63 and CD81 (commonly used markers of EVs) were also enriched in the sucrose-stored samples (Walker et al., 2022). Trehalose has also been used to preserve frozen EVs. By adding 25 mM of trehalose to the EV storage buffer the EV concentration remained stable after 4 freeze-thaw cycles (a process which is known to reduce the stability of the sample) (Bosch et al., 2016).

1.7.5 Characterising the physical properties of extracellular vesicles

After the isolation of EVs from conditioned cell culture medium or biofluid the characterisation of EVs is an important step to determine their morphology, abundance and the presence of established markers. One of the most commonly used techniques to quantify the concentration and size of EV samples is NTA. NTA uses a laser and camera to calculate the size of individual particles based on their Brownian motion. By injecting a small sample of EVs (0.5 ml) the user can quickly determine the size distribution, concentration and colloidal stability (by using Zeta-potential to measure the surface potential of the EVs) of the EVs within the sample (Filipe et al., 2010; Midekessa et al., 2020). NTA can also be used in conjunction with fluorescent dyes or antibodies to tag EVs and reduce the likelihood that contaminants in the sample are recognised as particles (Thane et al., 2019). However, NTA cannot be used to determine the morphology of EVs.

Cryo-electron microscopy (Cryo-EM) is often used to visualise the morphology of EVs. Cryo-EM works by flash-freezing a sample to form a layer of ice as thick as a single molecule (Milne et al., 2013). This enables the visualisation of subcellular particles, such as EVs. One drawback of cryo-EM is that the images produced have very low contrast, but it remains an indispensable tool for visualising EVs. Using cryo-EM it has been shown that EVs have a diverse morphology including ovals, tubules and double EVs (where a smaller EV is located within a larger one) (Zabeo et al., 2017). Notably, the 'Minimal Information for Studies of Extracellular Vesicles – 2018' (MISEV2018) published by the International Society of Extracellular Vesicles (ISEV) recommends cryo-EM as the gold-standard for the visualisation of single vesicles (Théry et al., 2018).

Advanced flow cytometry techniques are now being used in order to better visualise and characterise EVs. High-sensitivity flow cytometry (HS-FCM) can be used to quantify the size of individual EVs (Deville et al., 2021; Stoner et al., 2016). Imaging flow cytometry can also be used to visualise individual EVs within a sample by using fluorescent dyes or antibodies which either

label all EVs or specific EV markers (such as the tetraspanins) (Lannigan & Erdbruegger, 2017; Tertel et al., 2020). It can also identify whether an EV is double-positive for two specified markers (Woud et al., 2022). Another new technique is tunable resistive pulse sensing (tRPS) which measures the size and zeta-potential of individual EVs as they pass through a nanopore by recording the change in current caused by ionic flow (Maas et al., 2017). Izon have recently developed a commercially available tRPS system (EXOID from Izon Science) which aims to provide a standardised approach to EV measurement. Importantly, tRPS appears to be better able to distinguish between subgroups of uniform polystyrene beads of different sizes when compared to NTA (Vogel et al., 2021).

Membrane dyes such as MemBright can also be used to visualise isolated EVs as they emit a fluorescent signal when they come into contact with a plasma membrane (Collot et al., 2019). This dye has enabled the visualisation and spatiotemporal tracking of EVs which have been isolated and incubated with MemBright within living zebrafish embryos, allowing the authors to follow the path of EVs through the bloodstream and determine which cell types the EVs are taken up by using fluorescent microscopy (Hyenne et al., 2019). It can also be used to visualise the uptake of EVs by primary mouse hippocampal and cortical neurones in culture (Antoniou et al., 2023).

1.7.6 Characterising the proteomics, cargo and RNA profiles of extracellular vesicles

Known protein markers of EVs can be useful in determining the presence of EVs within a sample. MISEV2018 (Théry et al., 2018) categorises these markers into 5 categories, 3 of which should be reported by any study which claims to show the presence of EVs (Table 1). Western blotting is often used to identify the presence of these markers within a sample (Kowal et al., 2017). Western blotting uses both gel electrophoresis and immunocytochemistry principles to visualise the presence of proteins and can be used to estimate the abundance of the protein within the sample.

After lysis and centrifugation of the EV sample, proteins are loaded onto a gel and subjected to gel electrophoresis before being transferred to a membrane. The membrane is then blocked and incubated in the primary antibodies needed to visualise the chosen EV markers. After washing the membrane is incubated in a solution of secondary antibodies which are conjugated to a reporter enzyme or a fluorescent dye which enables the visualisation of proteins on the membrane (Iavello et al., 2016).

Table 1. The three essential categories of extracellular vesicle and non-extracellular vesicle markers as required by MISEV2018.	
Category	Examples of markers
Transmembrane proteins which are found within the bilayer membrane of extracellular vesicles	CD63 CD81 CD82 Transferrin receptor (TFR2) Sonic Hedgehog (SHH) Acetylcholinesterase (found in neuronal extracellular vesicles)
Proteins known to be found in the cytosol of extracellular vesicles	ESCRT-I ESCRT-II ESCRT-III ALIX FLOT1 FLOT2
Proteins associated with common contaminants of extracellular vesicle samples such as lipoproteins and albumin. This helps to determine the purity of the sample	Albumin (ALB) Apolipoprotein A1 Apolipoprotein A2

Mass spectrometry can be used to provide a more detailed characterisation of the protein, lipid and metabolite contents of EVs (Jalaludin et al., 2023). Mass spectrometry has been used to identify potential cancer-specific EV biomarkers for a range of cancers including bladder, colon, breast and ovarian (Bandu et al., 2019). Mass spectrometry works by deflecting vaporised ionised molecules using a magnetic field. The deflection recorded enables the characterisation of the molecules within the sample (Olshina & Sharon, 2016). However, mass spectrometry cannot characterise single EVs, something which can now be achieved with single EV sequencing-based protein analysis. This method may help us to better understand the heterogenous nature of EVs. By tagging individual EVs with unique antibody-DNA tags it is possible to perform next-generation sequencing on individual EVs to determine the expression profiles of different proteins across a sample (Ko et al., 2021).

Quantitative reverse transcription polymerase chain reaction (RT-qPCR) is commonly used to determine the levels of different mRNAs in a sample of EVs but can also be used to assess levels of different miRNAs (H. Lee et al., 2020). To perform RT-qPCR, the isolated RNA is converted into complementary DNA (cDNA) using primers and reverse transcriptase. This is followed by amplification of the cDNA by DNA polymerase which is monitored by a fluorescent probe. The number of cycles it takes for the cDNA fluorescence to reach a set threshold reflects the original quantity of cDNA, and therefore mRNA, in the sample (Adams, 2020). However, it should be noted that increased mRNA expression does not always directly translate to increased protein level (Koussounadis et al., 2015). Although, it should be noted that RT-qPCR is much more sensitive than western blotting so a protein that is not identified by western blot may still be present within the EV if the mRNA is detected by RT-qPCR (O'Brien et al., 2020).

1.7.7 New techniques for extracellular vesicle isolation and characterisation

Despite the numerous techniques for EV isolation and characterisation, several new techniques are being developed which attempt to streamline this process. One such technique is electric field-induced release and measurement, or EFIRM. Magnetic beads conjugated to an anti-CD63 antibody were used to capture EVs and then a non-uniform electric field was applied to disrupt the membrane of the EVs and release RNA and proteins from within the EV. These RNA and proteins then bind to detector electrodes which can quantify the amount present in the sample (Wei et al., 2013). This technique could be used to rapidly check for EV tumour biomarkers present in both blood and saliva.

Increasingly, microfluidic devices are being used to isolate EVs. One study combined immuno-affinity and a microfluidic device called the OncoBean to capture EVs within the device, which could later be released for downstream analysis or functional studies. Using antibodies against the common EV markers CD9, CD63 and CD81 (which were conjugated to desthiobiotin) they could coat the OncoBean with these antibodies via desthiobiotin-avidin binding. After injecting the sample and immobilising the EVs via the desthiobiotin-conjugated antibodies, the EVs could then be released by adding biotin which binds more strongly to avidin, displacing the desthiobiotin-conjugated antibodies (Lo et al., 2020). This method enables the isolation of EVs and reduces the risk of contamination by similarly sized molecules or protein contaminants. Similarly, the exosome total isolation chip (exoTIC) utilises a microfluidic device to isolate EVs. The exoTIC device contains several nanoporous membranes to filter and isolate EVs from conditioned medium or biofluid. As the device contains several membranes with pores of different sizes, different size populations of EVs can be isolated individually. It also produces a much greater yield of EVs compared to traditional ultracentrifugation (Liu et al., 2017).

However, these newer techniques are limited by their availability and cost. When compared to more typical EV isolation and characterisation methods it may be that, at present, the cost of these methods outweighs the advantages that they have over more traditional techniques.

1.8 Using medulloblastoma cell lines and primary neuronal culture

When studying medulloblastoma, many researchers use medulloblastoma cell lines which are derived from human tumours and maintained as continuous cell lines. Continuous cell lines emerge when a random genetic mutation occurs, enabling the continuous cell division of a particular cell. Although there are many medulloblastoma cell lines, a comprehensive 2016 review identified that out of 44 established medulloblastoma cell lines, only 18 have been classified into subgroups (Ivanov et al., 2016). Two of these cell lines (D425 and D458) are classified as Group 3 medulloblastoma and originate from the same 6-year-old male patient. However, one cell line is derived from the original medulloblastoma tumour (D425) and the other from a recurrent tumour (D458) (He et al., 1991). The primary tumour was located in the posterior fossa and the recurrent tumour cells were isolated from the cerebrospinal fluid 6 months after diagnosis (after chemotherapy and radiation treatment were unsuccessful). This makes these cell lines particularly useful as it may be possible to use them to investigate the differences between primary and recurrent medulloblastoma. Cell lines have the advantage of being more readily available than human tumour tissue and in principle, ensure that experiments are reproducible as there should be little variation between cultures from the same line. However, using different cell culture media, different coating agents and different plating densities can produce variation within a single cell line (Hirsch & Schildknecht, 2019).

Cross-species application of human EVs to rodent neuronal models is well established, including the use of rodent primary culture (Amit et al., 2020; Kovacs et al., 2020; Lucido et al., 2019). Using mouse primary cortical neurones enables the study of neurones in their earliest stages of development (Lesuisse & Martin, 2002). Mouse cortical neurones are a well-established model in neuroscience research and are similar in morphology and gene expression to human neurones (Hodge et al., 2019). Although human and mouse neurones are not identical (for example, human dendrites are longer and thicker (Ofer et al., 2022)), primary mouse neurones are an

invaluable model which can be used to investigate a wide range of biological mechanisms.

1.9 Aims and hypotheses

It has been identified that EVs from both peripheral and central tumours can alter neuronal development, either by stimulating neurite outgrowth or by inducing neuronal cytotoxicity (Kovacs et al., 2020; Lucido et al., 2019; Madeo et al., 2018; X. Zhou et al., 2020). Although these studies demonstrate that EVs derived from cancer cell lines and patient samples can alter neuronal morphology, it has not been established whether EVs from medulloblastoma cell lines can alter neuronal growth and development in the same way (despite evidence that PBTS are more likely to develop neurodevelopmental disorders than healthy children (Hsu et al., 2023)).

The normal development of mouse primary cortical neurones in culture was characterised by quantifying axon length, mean branch length and the proportion of polarised neurones. EVs were collected from the primary and recurrent medulloblastoma cell lines and the size distribution and size of the particles was measured using NTA to ascertain any differences in EV production between the two cell lines. MemBright, a fluorescent membrane probe (Collot et al., 2019), was used in an attempt to visualise the EVs. The primary hypothesis was that EVs derived from medulloblastoma cell lines would affect neuronal development and axonal growth in mouse primary cortical neurones. To test this, EVs from the medulloblastoma cell lines were added to the cortical neurones and a well-established immunocytochemistry protocol was used to quantify any changes in neuronal development (axon length, mean branch length, neuronal polarisation and neuritogenesis) after 24, 72 and 120 hours. It was also necessary to confirm that molecules found within the fresh medulloblastoma cell culture medium (which may be inadvertently isolated with the medulloblastoma EVs) were not responsible for any observed effects.

2. Methods

2.1 Animals

C57BL/6J mice (Charles River, UK) were housed in the Biomedical Services Unit at the University of Nottingham and treated according to the ethics and welfare regulations of the Animals (Scientific Procedures) Act 1986.

2.2 Culture of mouse primary cortical neurones

Required media, solutions and reagents:

- Poly-l-ornithine (PLO) (P4638-100mg, Sigma-Aldrich). Stock solution 1mg/ml in dH₂O. Stored at -20°C. Working solution 50µg/ml in dH₂O – filter sterilised before use. Stored at 4°C
- Hanks' Balanced Salt Solution (HBSS) (H9394, Sigma-Aldrich). Stored at 4°C
- Dulbecco's phosphate-buffered saline (DPBS) (D8537-500ml, Sigma). Stored at 4°C
- Trypsin (T9201, Sigma-Aldrich). 10mg/ml in DPBS – filter sterilised before use. Stored at -20°C
- HyPure™ Particle-free water (Cytiva). Stored at room temperature
- Deoxyribonuclease (DNase) (D5025-15KU, Sigma-Aldrich). 5mg/ml in HyPure™ Particle-free water (Cytiva). Stored at -80°C
- Trypsin inhibitor (T9003, Sigma). 10mg/ml in DPBS – filter sterilise before use. Stored at -20°C
- Neurobasal medium (21103-049, Gibco). Stored at 4°C
- Supplemented neurobasal medium. Glutamax (35050-061, Gibco) 1% (v/v) and B27 supplement (50x) (17504-044, Gibco) 2% (v/v) in neurobasal medium. Stored at 4°C
- Chemgene™ (Medimark International™)
- 70% ethanol in dH₂O

Before each use, the class II biological safety cabinet (BSC) was wiped down with Chemgene (5%) and sprayed with 70% ethanol. Aseptic techniques were adhered to throughout the dissection and cell culture process.

2.2.1 Coating of plates and coverslips

The day before embryonic tissue was harvested, 19mm round glass coverslips were placed into a petri dish containing 70% ethanol and left for 30 seconds to sterilise. Using a pair of forceps, the coverslips were transferred to a 12-well plate and left to dry. 0.5ml of the Poly-L-ornithine (PLO) working solution (0.05mg/ml in dH₂O) was added to each well and the plates were placed into an incubator and left overnight (37°C, 5% CO₂). On the morning of tissue collection, in the BSC, the PLO was removed from the plates and each well was rinsed twice with sterile dH₂O. The plates were left to dry in the BSC until the primary cortical neurones were plated.

2.2.2 Dissection of the mouse cortices

Schedule 1 killing of the pregnant adult female mouse was performed by a trained individual. The abdomen was sprayed with 70% ethanol in preparation for the removal of the uterus. The skin was cut using scissors and forceps were used to expose the uterus. The uterus was cut away from the abdominal cavity and placed into a 90mm petri dish filled with approximately 20ml of HBSS. Using two pairs of forceps, a trained individual then carefully removed the embryos from the uterus and amniotic sac and immediately decapitated them using a pair of scissors. Each head was transferred to an empty petri dish where the brain was removed and immediately transferred to another petri dish containing HBSS. To remove the brain, a pair of forceps were used to hold the head in place whilst spring scissors were used to cut through the skin and cartilage from posterior to anterior along the midline. The brain was then gently removed from each embryo using a curved Iris tool and placed into another petri dish containing HBSS.

Under a dissection microscope, the cortices were isolated using two pairs of forceps to split the cortices along the superior sagittal sinus and pull them away from the surrounding brain areas. The meninges were gently pulled away from the cortices and up to 6 individual cortices were placed into a sterile nunc containing 2ml of HBSS.

2.2.3 Dissociation of the mouse cortices

Back in the BSC, the cortices were dissociated into small fragments using two pairs of forceps and then 200µl of trypsin (final concentration – 1mg/ml) and 20µl of DNase (final concentration - 50µg/ml) were added to each nunc. Each nunc was gently agitated and then placed in the incubator for 30 minutes.

After 30 minutes, 100µl of trypsin inhibitor (final concentration – 5% vol) was added to each nunc before the contents were transferred to separate 50ml falcon tubes containing 6ml of neurobasal medium. If the tissue sank to the bottom of the tube, most of the medium was carefully removing using a 10ml stripette, leaving around 1ml of medium in the tube. 10µl of DNase was added and the tube agitated gently. If the tissue did not sink to the bottom of the tube, the same volume of DNase was added without any medium being removed. 5ml of neurobasal medium was then added to each tube and again, most of the medium was removed leaving approximately only 1.5ml of medium. Another 10µl of DNase was added before the tissue was triturated using a 2ml stripette to gently draw up and expel the tissue around 10 times until no visible pieces of tissue were left. Next, each tube was topped up to a total volume of 10ml with neurobasal medium and spun in a centrifuge (Eppendorf 5810, D462 rotor) for 5 minutes at 250 x g to pellet the cells. The supernatant was carefully removed without disturbing the pellet and 1ml of supplemented neurobasal medium was added per brain and the pellet resuspended to create the stock cell suspension. A cell count was performed using a TC20™ Automated Cell Counter (Bio-Rad) and then the stock suspension was diluted if necessary to produce a cell stock of 10,000,000 cells per ml.

2.2.4 Plating of mouse primary cortical neurones

For low density 12-well plates, the stock suspension was diluted 1:12 in supplemented neurobasal medium and then this was diluted again at a concentration of 1:12 to create the final cell suspension (1:144). 1ml of the final cell suspension was then added to each well before the plate was placed back in the incubator.

2.3 Medulloblastoma cell lines

Required media, solutions and reagents:

- Dulbecco's Modified Eagle Medium (DMEM) (41966029, Gibco™, ThermoFisher Scientific). Supplemented with 10% Bovine growth serum (BGS) to produce complete medium. Stored at 4°C
- HyClone bovine growth serum (BGS) (SH30541.03, ThermoFisher Scientific). Stored at -20°C until use in complete medium
- Trypsin-EDTA solution (T4171, Sigma-Aldrich). Stored at -20°C until use and then stored at 4°C. 5ml trypsin-EDTA added to 45ml of HBSS to produce x1 trypsin-EDTA solution (v/v)
- HBSS (24020117, Gibco™, Fisher Scientific). Stored at 4°C until opened and then stored at room temperature
- EV-depleted medium (ultracentrifuge 50ml of BGS at 100,000 x g for 18 hours at 4°C (Optima™ LE-80K ultracentrifuge, Beckman Coulter, SW41TI rotor) and carefully remove the supernatant leaving at least 1cm at the bottom of the tube. Filter sterilise twice before adding to DMEM to produce 10% EV-depleted BGS in DMEM)
- Trypan Blue (25-900-CI, Corning). Stored at room temperature

2.3.1 Maintenance of cells

The D458 and D425 cell lines were passaged twice a week (Monday and Thursday) with the Monday split being a 1:8 split and the Thursday being 1:10 to make up for the slight differences in time between them. This

ensured that the cells were approximately 70% confluent whenever they were split. The medium was removed from each flask and added to a 50ml falcon tube. The T75 flask was then washed with 5ml HBSS by gently rocking the flask side to side and this was then added to the same falcon tube. This ensured that all of the non-adherent cells were collected. 3ml of trypsin was added to each flask and the flasks were placed back into the incubator (37°C, 5% CO₂) for 4 minutes. After 4 minutes, the flasks were removed and gently tapped to ensure that all the cells had lifted off from the base of the flask (this was checked under a microscope and the flasks were placed back into the incubator for another minute if necessary). 3ml of complete medium was then added to each flask to inactive the trypsin before the back of the flask was washed with this complete medium/trypsin solution to ensure that all of the cells were detached. All the liquid was transferred to the falcon tube and then spun in a centrifuge (Sigma 3-16 centrifuge, 11180 rotor) at 220 x g for 5 minutes at room temperature to pellet the cells. During this time, new T75 flasks were prepared by adding 9.5ml of DMEM (BGS 10%) to each one and labelling them with the cell line, date, passage number and split ratio. The supernatant was carefully removed from the pelleted cells and discarded into a waste bucket before the cells were resuspended in 5ml (for a 1:10 split) or 4ml (for a 1:8 split). 0.5ml of the cell stock was then added to each flask to make a total volume of 10ml. On the Monday, each cell line was split into 3 flasks to provide the 2 T75 flasks needed for EV isolation and one flask to keep the cell line going.

2.3.2 Collection of conditioned medium

24 hours after splitting, the cells were approximately 30% confluent and ready to have their medium changed to EV-depleted medium in preparation for EV collection. The medium was removed from each flask and added to a 50ml falcon tube (one tube for each cell line). The flasks were then washed twice with 5ml HBSS to ensure that any free-floating bovine-derived EVs found in the BGS had been removed. The medium and washes were then spun at 220 x g for 5 minutes to pellet the cells that were non-adherent. 5ml of EV-depleted medium was added to each flask to prevent the cells from

drying out. The supernatant was then carefully removed and the cell pellet was resuspended in 10ml EV-depleted medium (as the pellet contained two flasks worth of cells). 5ml of these cells were then added back to each flask containing the adherent cells to produce a final volume of 10ml.

48 hours later, the medium was removed and then spun at 110 x g for 5 minutes to pellet the cells. The medium was then transferred to a new tube which was spun at 1500 x g for 10 minutes to pellet any cell debris. This medium containing the EVs was then transferred to another tube and was stored in the fridge at 4°C until EV isolation. The cell pellet from the first spin was resuspended in 10ml HBSS and combined with the cells from the flasks following trypsinisation. 10µl of these cells were added to 10µl of Trypan Blue before a cell count and cell viability check was performed using an automated cell counter (CellDrop™ Automated Cell Counter, DeNovix) with the focus set to 645 on the Trypan Blue setting.

2.4 EV isolation

Required solutions and reagents:

- Dulbecco's phosphate-buffered saline (DPBS) (D8537-500ml, Sigma). Stored at room temperature once opened and only opened in a BSC (sterile)
- NaOH (S5881-500g, Sigma-Aldrich) 0.5M in dH₂O. Stored at 4°C
- Sodium Azide (71920, Aldrich). Stored at room temperature
- 70% ethanol in dH₂O

To concentrate the 20ml of collected medium for EV isolation, each sample was transferred to a protein concentrator (10kDa PES MWCO 5-20ml, Pierce). This was undertaken in a BSC to prevent contamination of the samples. The concentrators were spun at 3600 x g for approximately 45 minutes, or until the sample had been concentrated to 0.5ml (GT4R centrifuge, Fisherbrand). In the BSC, the 0.5ml of concentrated medium was

transferred to a 1.5ml Eppendorf tube and then stored at 4°C for no more than 2 weeks before use.

EV isolation was performed using the Izon Automatic Fraction Collector (AFC) with Legacy 70nm columns (which have an optimum isolation range of 70nm to 1000nm). The columns were stored at 4°C until use when they were left at room temperature for 15 minutes to equilibrate. To start, the AFC carousel was aligned and calibrated as per the manufacturer's instructions. The upper and lower caps were removed and the column was mounted to the AFC. The AFC was set to collect 6 fractions, each 0.5ml in volume with the default buffer volume (2.7ml). This enabled the collection of 3 EV fractions (EV1, EV2 and EV3) and 3 protein fractions (P1, P2 and P3). To clean the column before the first sample collection, it was flushed with 10ml of 0.5M NaOH followed by 20ml of DPBS. Six sterile 1.5ml Eppendorf tubes were loaded into the carousel as instructed by the AFC with the lids facing inwards. The column was then primed with approximately 5ml of sterile DPBS and 0.5ml of the sample was loaded onto the top of the column using a filtered tip (to reduce the risk of contamination). Once the sample had been completely drawn into the column, 6ml of DPBS was added. The AFC then collected the 6 fractions in order as the sample moved through the column. Once complete, the Eppendorf tubes were labelled (EV1 – P3), removed and placed into the fridge at 4°C. Between samples, the column was cleaned as described above. After the final clean, 12ml of sodium azide in DPBS (0.05% w/v) was flushed through the column until only 2ml remained above the frit. The column was then removed from the AFC, the caps were replaced and the column was placed back into the fridge at 4°C. Each column was used a maximum of 5 times as per the manufacturer's instructions. The AFC valve and nozzle were cleaned using 5ml of distilled water followed by 5ml of 70% ethanol to prevent the formation of salt crystals.

2.5 Nanoparticle Tracking Analysis

Required solutions and reagents:

- HyPure™ Particle-free water (Cytiva). Stored at room temperature

- DPBS (D8537-500ml, Sigma). Stored at room temperature once opened
- Alignment suspension polystyrene beads (70325C, Applied Microspheres). Stored at 4°C

Nanoparticle tracking analysis was performed using the ZetaView (Analytik Ltd.). The ZetaView cell was rinsed with particle-free water from the reservoir for 60 seconds whilst 20ml of particle-free water was injected into the front port using a 20ml syringe. When the rinse was finished the ZetaView performed a cell quality check to ensure that the cell was clear of debris and ready for experiments. The alignment suspension was then prepared using alignment suspension polystyrene beads and particle-free water. To make a final dilution of 1:250,000, 10µl of the alignment suspension polystyrene beads were added to 10ml of particle-free water in a tube. The tube was rocked gently to ensure that the beads were evenly distributed without introducing microbubbles. 80µl of this solution was added to 20ml of particle-free water in another tube. Again, the tube was rocked gently to mix the solution. 2ml of this final dilution was then injected into the front port using a syringe before the ZetaView performed focus optimisation and calibration. The temperature was then set to 25°C and the cell was flushed with DPBS (both using the pump and injecting 20ml of DPBS into the front port using a 20ml syringe). Flushing with the same buffer that the sample is suspended in prevents ionic drift once the sample is loaded. Approximately 750µl of sample was then injected into the front port using a syringe before video acquisition was run. A standard operating procedure was used with 11 positions and 3 cycles selected (camera sensitivity - 80, frame rate – 30 frames per second, shutter speed - 100). Video acquisition was then run to count the number of particles per ml and measure the size of each particle. This was repeated for all three EV fractions isolated from each sample. Between each sample, the ZetaView was flushed with DPBS to prevent contamination from the previous sample. Once the final measurement was complete, the ZetaView was flushed with particle-free water from the reservoir whilst at least 3 x 1ml of particle-free water was injected through

the front port using a syringe. This was then immediately followed by 4 x 20ml syringes of air to dry out the cell.

2.6 Dosing mouse primary cortical neurones with medulloblastoma-derived EVs

To assess the effect of medulloblastoma-derived EVs on cortical neurone development, cells were dosed on Day 2 (24 hours after seeding) with either 100µl of D458 EV2, D425 EV2, D458 mixed protein fractions, D425 mixed protein fractions, DPBS (as the vehicle solution) or left as a control well. Two wells were used for each condition to produce two technical replicates.

2.6.1 Preparation of the EV samples used

For the mixed protein fractions, 150µl of each protein fraction was added to a single Eppendorf and gently mixed to produce a mixed protein fraction that could be added to the cells. 100µl of the mixed protein fraction was added to each well. EV2 was used to dose the cells and was diluted using sterile DPBS to ensure a consistent number of EVs between experiments. For the D425 cell line EV2 was diluted down to 1.2×10^{10} EVs per ml and for the D458 cell line the EV2 fraction was diluted down to 4×10^{10} EVs per ml when possible. 100µl was then added to each well. Where dilution was not possible (due to a lower number of EVs) 100µl of the isolated EV2 fraction was added.

For the medium control experiments, EVs and protein fractions were isolated from 20ml of fresh 10% BGS in DMEM and 20ml of fresh 10% EV-depleted BGS in DMEM. As described above, cells were dosed on Day 2 with either 100µl of 10% BGS/DMEM EV2, 10% EV-depleted BGS/DMEM EV2, 10% BGS/DMEM mixed protein fractions, 10% EV-depleted BGS/DMEM mixed protein fractions, DPBS (as the vehicle solution) or left as a control well.

2.7 Cell fixation and immunocytochemistry

Required solutions and reagents:

- Paraformaldehyde (P/0840/53, FisherChemical) 4% (w/v) in 1x PBS and 2.7% (w/v) sucrose. Stored at 4°C and can be kept for up to 1 week
- PBS (BR0014G, Oxoid). Made up to 1x PBS in dH₂O and then autoclaved before use. Stored at room temperature
- Triton X-100 (Sigma). Stored at room temperature
- Glycine (G8898-1kg, Sigma). Stored at room temperature
- Sucrose (S7903-1kg, Sigma). Stored at room temperature
- Bovine serum albumin (BSA) (A7906-100g, Sigma-Aldrich) 3% (w/v) in PBS. Stored at 4°C
- Acetylated tubulin primary antibody (T7451, Sigma-Aldrich). Stored at -20°C
- Goat anti-mouse IgG (488) (A11001, AlexaFluor-488, Invitrogen). Stored at 4°C
- DAPI (D9542-10mg, Sigma). Stored at -20°C
- Phalloidin (P1951-1mg, Sigma). Stored at -20°C
- VectaShield mounting media (Vector). Stored at 4°C

- Buffer 1: 10mM glycine in PBS. Stored at 4°C
- Buffer 2: 10mM glycine and 0.2% Triton X-100 in PBS. Stored at 4°C
- Buffer 3: 0.1% Triton X-100 in PBS. Stored at room temperature

Cells were fixed using PFA either 24, 72 or 120 hours after dosing, depending on the experiment. The medium was removed from the cells and PBS was gently added using a plastic pipette and then removed using a vacuum pump to wash the cells. Just enough 4% PFA (at room temperature) was added to each well to submerge the coverslip and after 30 minutes the PFA was removed. The coverslips were then washed 3 x 5 minutes with PBS, 3 x 5 minutes with buffer 1 and then incubated in buffer 2 for 30

minutes at room temperature to permeabilise the membranes of the cells. Next, the coverslips were incubated in 3% (w/v) BSA at room temperature for 30 minutes. This process of blocking prevents non-specific binding of the primary or secondary antibody as the BSA binds to non-specific binding sites within the cells. The primary antibody (anti-acetylated tubulin) was then prepared in 3% BSA at a concentration of 1:300 with a total volume of approximately 30µl per coverslip. Within a humidified opaque incubation chamber, approximately 25µl of the primary antibody solution was pipetted onto parafilm with one droplet for each coverslip. With a pair of forceps, each coverslip was then gently touched to absorbent paper roll to remove excess BSA and then transferred and placed cell side down on the parafilm on top of the antibody solution. The lid was then replaced on the incubation chamber and then it was placed in the fridge overnight at 4°C for approximately 18 hours.

The next day, the coverslips were transferred into 12-well plates from the incubator chamber cell side up. The coverslips were then washed with buffer 3 for 3 x 5 minutes. The following secondary antibody and stains were prepared in 3% BSA (again with a total volume of 30µl per coverslip):

- Goat anti-mouse IgG (488) (1:300)
- DAPI (1:300)
- Phalloidin (1:200)

As described before, approximately 25µl per coverslip of the secondary antibody solution was pipetted onto a piece of parafilm within the incubator chamber, being careful to keep the lid on the incubation chamber to prevent the fluorophores from photobleaching. The coverslips were then placed cell side down on top of the droplets and incubated for 90 minutes at room temperature. After 90 minutes, the coverslips were returned to the 12-well plate cell side up, washed for 3 x 5 minutes with buffer 3 and then the wells were filled with 1ml of PBS. To mount the coverslips, 22µl of VectaShield mounting media was pipetted onto a glass slide and the coverslips were touched to absorbent paper roll to remove any excess PBS before being

placed gently onto the mounting media. Two coverslips were mounted onto 1 glass slide. The slides were left to dry in a dark box at room temperature overnight before being transferred to a 4°C fridge for storage until imaging.

2.8 MemBright Staining

Required solutions and reagents:

- BioTracker™ MemBright 560 Live Cell Dye (SCT084, Sigma-Aldrich). Supplied at 200µM in DMSO. Stored at -20°C
- DPBS (D8537-500ml, Sigma). Stored at room temperature once opened

To visualise and confirm the presence of medulloblastoma EVs, the fluorescent membrane probe MemBright was used (Collot et al., 2019). After isolation, the three EV fractions were concentrated down to between 100µl and 500µl using a 500µL protein concentrator (Vivaspin 500 - 10kDa MWCO, Cytiva) at 4,000 x g to 5,000 x g (Eppendorf 5415R microcentrifuge). MemBright was added to the concentrated EV fractions to produce a final concentration of 200nM and the EVs were left to incubate with the dye for 30 minutes at room temperature (Hyenne et al., 2019). After 30 minutes, 15ml of DPBS was added to rinse the EVs and an ultracentrifuge (Optima™ LE-80K, Beckman Coulter, SW55TI rotor) was used to pellet the EVs (100,000 x g for 70 minutes at 4°C) (Mary et al., 2020). The excess buffer was then removed and the pellets resuspended in sterile DPBS. As a negative control, the same concentration of MemBright was incubated with just DBPS, ultracentrifuged and resuspended in the same way as the EV sample. To visualise the dyed EVs, an imaging spacer (Grace Bio-Labs, Secure Seal™ Imaging Spacer 13mm x 0.12mm) was stuck onto a microscope slide that had been sterilised with 70% ethanol to create a chamber for the MemBright-EVs. 20µl of the MemBright-EV solution was pipetted into the centre (after gentle vortexing to break up any large clumps of EVs) and a 22mm square glass coverslip was placed on top to seal the chamber. The EVs and control sample were visualised using a fluorescent microscope with an LED light source (Axio

Vert.A1, Zeiss™) with a x63 oil immersion lens. A sample of just DPBS was also imaged.

2.9 Microscopy and quantification of neuronal morphology

For each condition there were two technical replicates per experiment where possible (in some cases one technical replicate was lost due to contamination of the well). For each technical replicate, 10 images were taken (except for EV dosing at day 5 and day 7 where only 5 images were taken due to time constraints) at x20 magnification on a fluorescent microscope with an LED light source (Axio Vert.A1, Zeiss™). For x63 images an oil-immersion lens was used.

Fiji ImageJ version 1.53 and the neuroanatomy SNT plugin (Arshadi et al., 2021) were used to produce traces of individual axons. SNT enables partially automated tracing of axons to produce accurate traces which can be saved and measured. An axon was defined as a neurite which was at least 3 times the length of any other immature neurites protruding from the soma (Lucci et al., 2020). Each axon was traced by hand (assisted by the automated tracing function) and then the SNT programme was used to measure the length of each axon (defined as the longest path) and the mean length of all the branches. To quantify the proportion of polarised neurones for each image, the total number of neurones and the number of polarised neurones was counted (i.e. any neurone with an axon at least three times the length of any other immature neurites protruding from the soma (Lucci et al., 2020)). This enabled the calculation of the proportion of polarised neurones:

$$\frac{\text{Number of polarised neurones}}{\text{Total number of neurones}} = \text{Proportion of neurones polarised}$$

To assess neuritogenesis, neurones were counted if they had at least one neurite which was at least as long as the diameter of the soma (Gertz et al., 2010). A similar calculation to the one above was used to calculate the proportion of neurones with immature neurites. Neuritogenesis was not

analysed past 24 hours after the addition of medulloblastoma EVs as it is a useful measure to capture only the earliest stages in the process of polarisation. To assess dendrite length, dendrites protruding from the soma of polarised neurones were measured. The quantification of neuronal morphology was performed blind where possible to experimental group, cell line and timepoint to reduce bias. To trace some of the axons, two adjacent images had to be stitched together in ImageJ. To achieve this, the Pairwise Stitching plugin within ImageJ was used.

2.10 Statistical analysis

N refers to the number of biological replicates (between 3 and 5 depending on the experiment). To assess normality, the Kolmogorov-Smirnov test was used. If data were considered normal, an ordinary one-way ANOVA was used to determine differences between experimental conditions followed by Bonferroni's test for post-hoc analysis. For non-parametric data, the Kruskal-Wallis test was used followed by Dunn's post hoc test for multiple comparisons. If only two groups were being compared, a Mann-Whitney test was used as the data were non-parametric. In the case of two independent variables, a two-way ANOVA followed by Bonferroni's post-hoc test was used. p was considered significant when less than 0.05 and all tests were two-tailed. Data was analysed using GraphPad PRISM 9.5.1.

3. Results

3.1 Mouse primary cortical neurones undergo neuronal polarisation in vitro

In order to establish how mouse primary cortical neurones grow and develop over time in vitro, mouse primary cortical neurones (harvested at E16.5) were cultured on PLO-coated coverslips in 12-well plates and fixed at several timepoints (Figure 4A) to observe the normal growth and development of these neurones without any intervention or treatment over the course of a week. Immunocytochemistry was performed to visualise the neurones and analyse their morphological development (Figure 4B). An antibody that detects acetylated tubulin was used to visualise the neurones as acetylated tubulin is highly expressed in the axon (Baas et al., 2016). Additionally, a fluorescently-labelled version of the phallotoxin phalloidin (which selectively binds to F-actin structures) was used to visualise actin filaments within the neurones. Using these two fluorescent probes in conjunction provides a detailed look at the structure of the primary cortical neurones. As DAPI binds to DNA, it was used to visualise the nucleus of each cell and confirm the presence and location of the soma.

To analyse the changes in morphology of the neurones, three parameters were measured – axon length, mean branch length and the proportion of polarised neurones. A neurone was considered to be polarised if it possessed a single primary axon i.e., a process that was at least three times the length of any other neurites protruding from the soma (Lucci et al., 2020) (Figure 5A). If the neurone was polarised, the length of the axon and any branches extending from it were measured. The first images were taken on Day 2 to allow the neurones to adhere to the coverslips and to begin to extend projections. All the values were normalised to the mean of the values at Day 2 from the same culture to account for variability between biological replicates, which normally arises as a result of slight differences in seeding densities and fixing times. All three parameters measured increased from

Day 2 to Day 7 (Figure 5B, C & D). The typical stages of neuronal polarisation were observed (Figure 5E).

Figure 4

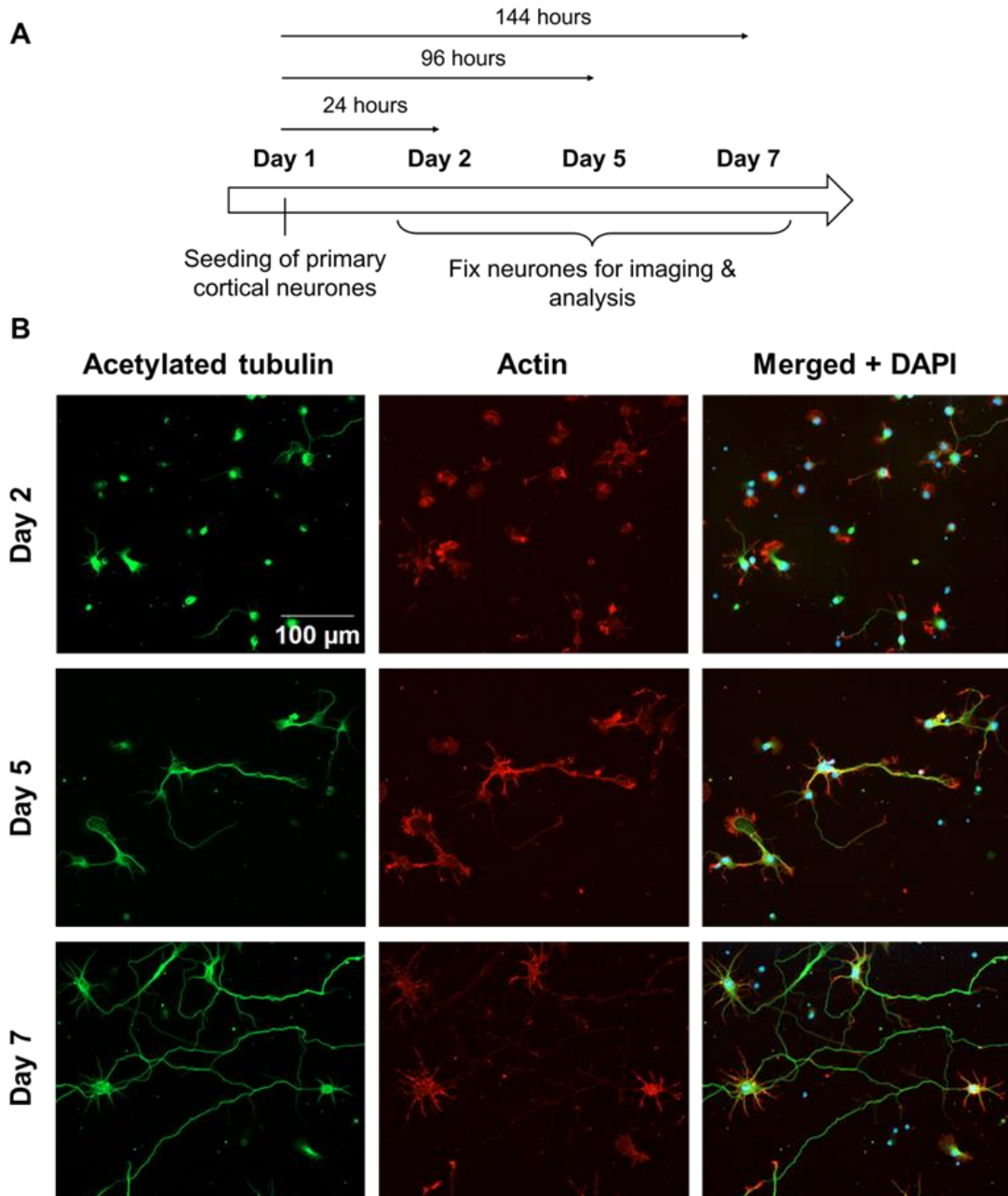
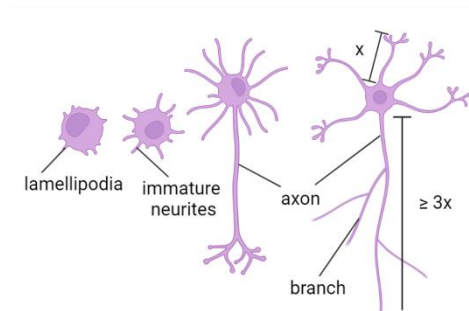


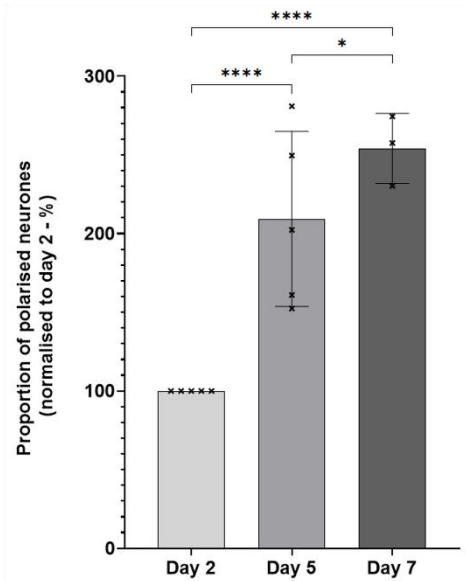
Figure 4. The development of mouse primary cortical neurones over one week. Cortical neurones were cultured for 7 days in standard conditions (see Methods 2.2) **(A)**. To visualise and characterise changes in morphology over 1 week, immunocytochemistry was used to visualise the cortical neurones at different time points **(B)**. Green – acetylated tubulin, red – actin and blue – DAPI. Images taken using a CCD camera at x20 magnification on a fluorescent microscope with a LED light source.

Figure 5

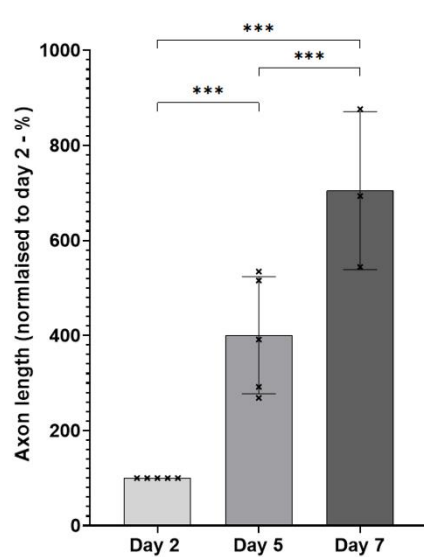
A



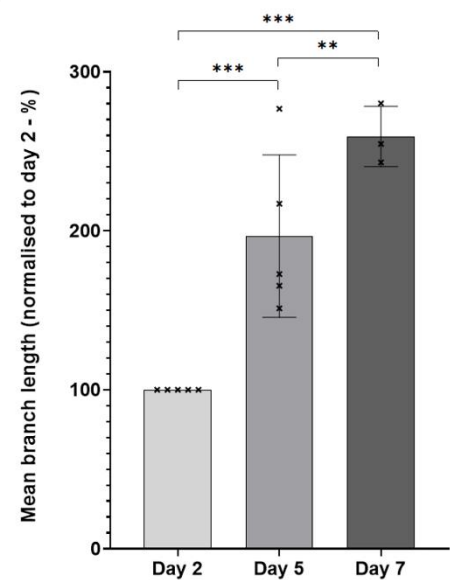
B



C



D



E

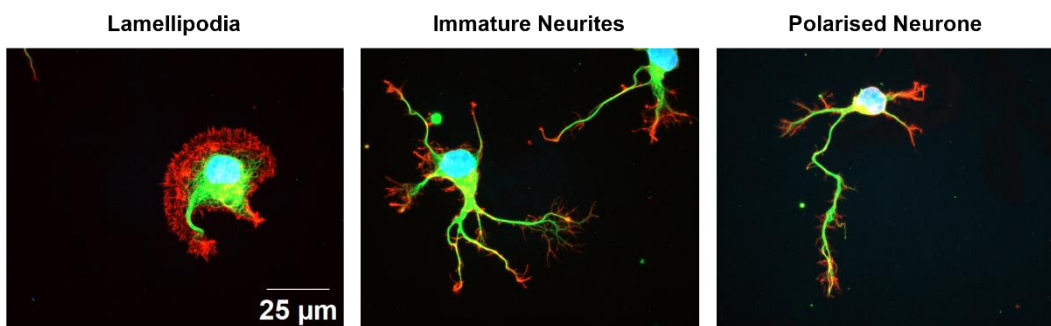


Figure 5. Mouse primary cortical neurones undergo typical polarisation in vitro. Mouse primary cortical neurones were grown in culture for 7 days and fixed at various timepoints. Immunocytochemistry was performed to visualise the neurones (see Methods 2.2 and 2.7). A schematic illustration of the process of neuronal polarisation **(A)**. ImageJ was used to quantify neuronal morphology including the proportion of polarised neurones **(B)**, axon length **(C)** and mean branch length **(D)**. Mouse primary cortical neurones in culture show typical neuronal morphology and polarisation **(E)**. Green – acetylated tubulin, red – actin and blue – DAPI. For **(E)**, images were taken using a CCD camera at x63 magnification on a fluorescent microscope with a LED light source. Significant differences were calculated using a Kruskal-Wallis test followed by Dunn's post hoc test for multiple comparisons (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ **** = $p \leq 0.0001$). Data are the normalised mean of between 3 and 5 independent biological replicates with error bars indicating mean $\pm$ SEM.

3.2 Medulloblastoma cell lines release particles which are consistent with the size distribution of extracellular vesicles

Two medulloblastoma cell lines were cultured for EV collection and isolation – the primary tumour cell line D425 and the recurrent tumour cell line D458. EVs were collected once per week as described in Methods 2.3.

Nanoparticle tracking analysis (NTA) was used to quantify the number of EVs found in each EV fraction (EV1 to EV3). Although the D458 cell line released more EVs (Figure 6A & B), a two-way ANOVA revealed that there were no significant differences in the number of EVs in each fraction between the D425 and D458 cell lines ($F(2,39) = 0.3785$, $p = 0.6874$). The particle size distribution of both cell lines is consistent with the expected profile of EVs, with EVs from both cell lines found to have a peak mode diameter of 105nm (Figure 6C). To further confirm the presence of EVs, the fluorescent plasma membrane probe MemBright was used to stain EVs released from the D458 cell line which were then visualised using a fluorescent microscope with an LED light source and a x63 oil-immersion lens (Figure 6D). In the sample containing D458 EVs, a considerable number of small particles (likely EVs) were observed that were not present in the

DPBS/MemBright or untreated DPBS. Large clumps were observed in both the EV sample and the DPBS/MemBright suggesting that they are clumps of unbound dye that were not removed in the washing process.

Figure 6

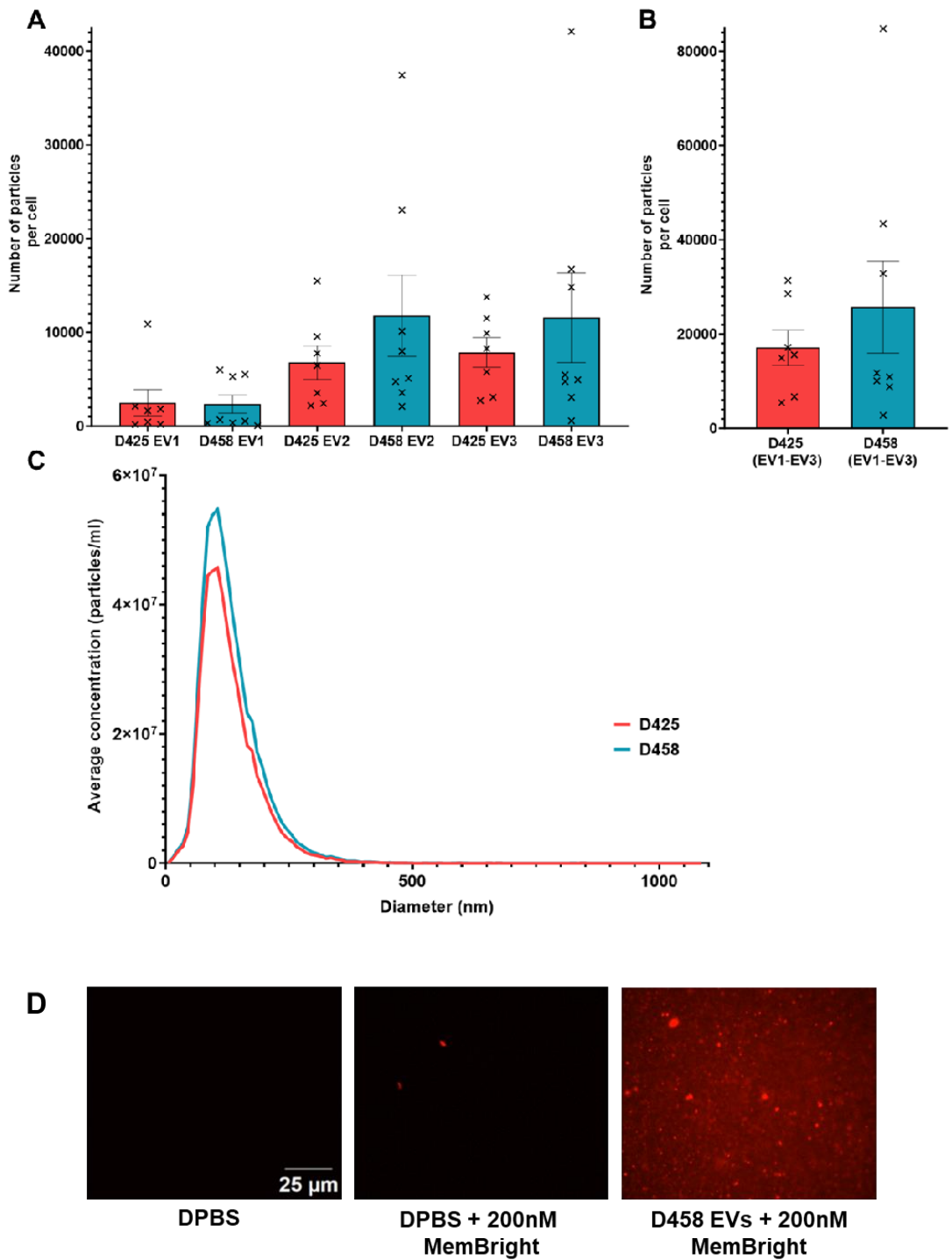


Figure 6. D425 and D458 cell lines release extracellular vesicles (EVs) which can be quantified using nanoparticle tracking analysis (NTA). D425 and D458 cells were grown in culture and EVs were isolated from the conditioned medium using size exclusion chromatography (see methods 2.3 and 2.4). NTA was used to quantify the concentration of EVs in the samples isolated from the two medulloblastoma cell lines. The number of particles released per cell was calculated for each fraction (EV1-EV3) **(A)** and for the fractions combined **(B)**. NTA was also used to generate the size distribution of both cell lines **(C)** and MemBright was used to confirm the presence of lipid bioparticles **(D)**. For **(D)**, images were taken using a CCD camera at x63 magnification on a fluorescent microscope with a LED light source. Red – MemBright. A two-way ANOVA was used to test for significant differences between the EV fractions with Bonferroni's post hoc test. For the combined EV fractions a Mann-Whitney test was used. Data are the mean values from 3 ZetaView cycles for 7 or 8 EV collections from different cell passages where **(B)** is the sum of the values in **(A)**. Error bars indicate mean $\pm$ SEM. Size distribution data is derived from the average of 3 independent samples which contain EV1, EV2 and EV3.

3.3 Medulloblastoma EVs from both primary and recurrent tumour cells increase neuritogenesis in primary cortical neurones after 24 hours

EVs, the mixed protein fraction or DPBS (vehicle) were added to mouse primary cortical neurones on Day 2 of culture and the cells were fixed 24 hours later (see methods). All conditions appeared to have similar normal neuronal morphology (Figure 7A). Analysis of neuronal morphology revealed that addition of EVs from both the primary (D425) and recurrent (D458) medulloblastoma cell lines increased the proportion of neurones which had developed at least one neurite (independent of whether it was an immature neurite or an axon) when compared to the vehicle (DPBS) treated neurones (Figure 7B). To assess neuritogenesis rather than axon length or polarisation, a neurite was defined as a process which was at least as long as the diameter of the soma (Gertz et al., 2010). Notably, this effect was not observed in the mixed protein fractions isolated from the conditioned cell culture medium ($p > 0.05$).

Figure 7

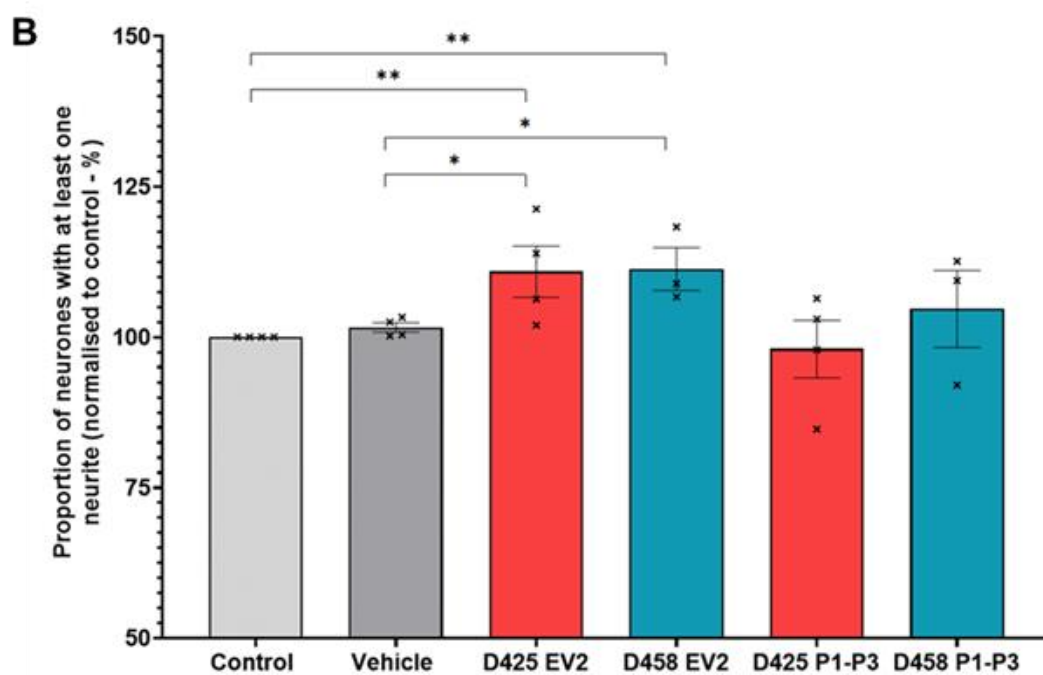
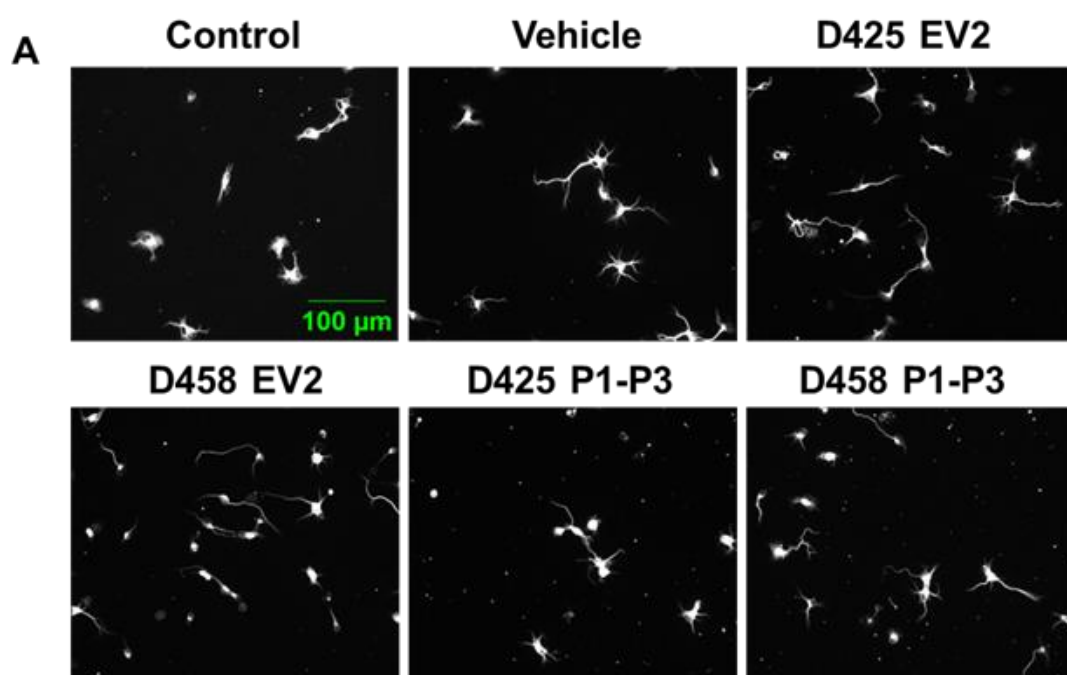


Figure 7. Extracellular vesicles (EVs) released from medulloblastoma cells increase neuritogenesis in mouse primary cortical neurones after 24 hours.

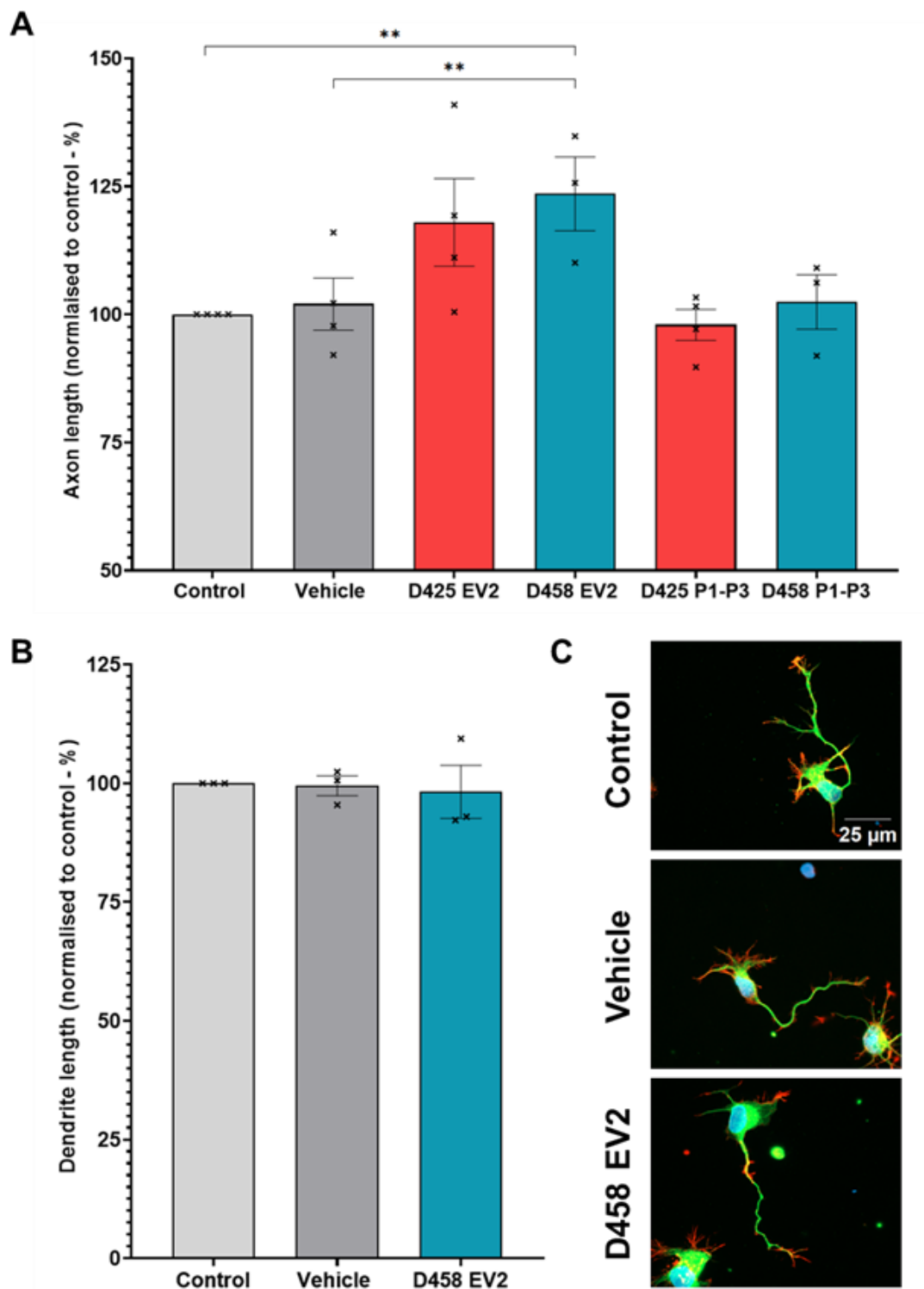
Mouse primary cortical neurones were grown in culture for 24 hours before being treated with either vehicle (DPBS), EVs (EV2) or the mixed protein fraction (P1-P3) isolated from D425 and D458 cells (see Methods 2.6). Twenty-four hours later the cells were fixed, and immunocytochemistry was used to visualise the neurones **(A)**. White – acetylated tubulin. Images taken using a CCD camera at x20 magnification on a fluorescent microscope with a LED light source. The proportion of neurones with at least one neurite was calculated for each condition and normalised to the untreated control **(B)**. A neurite was defined as any projection that was at least as long as the diameter of the soma. Significant differences were calculated using a one-way ANOVA with Bonferroni's post hoc test (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$). Data are the normalised mean of 3 or 4 independent biological replicates with error bars indicating mean $\pm$ SEM.

3.4 Medulloblastoma EVs from recurrent tumour cells increase axon length, but not dendrite length, in primary cortical neurones after 24 hours

EVs derived from the D458 recurrent cell line increased axon length in the primary cortical neurones after 24 hours when compared to the vehicle treated neurones (Figure 8A). This was not observed in neurones treated with EVs from the primary cell line (D425) or in either of the groups treated with the mixed protein fractions ($p > 0.05$). In order to identify whether the increase in axon length was specific to the axon or part of a more globalised increase in the length of neuronal processes, the mature dendrites protruding from the soma of polarised neurones were measured. Despite increasing axon length in the cortical neurones, EVs derived from the D458 cell line did not alter dendrite length as no significant difference was identified between the vehicle treated and D458 EV treated groups ($p > 0.05$) (Figure 8B). Additionally across these three conditions, there did not appear to be any changes in neuronal morphology (Figure 8C). There was no difference in the proportion of polarised neurones between any of the groups treated with either EVs or mixed protein fractions when compared to the vehicle treated cells ($p > 0.05$) (Figure 8D). Although a Kruskal-Wallis test suggested that

there was a difference in mean branch length between the different treatment conditions ($p = 0.0274$), post-hoc testing revealed that there was no difference in mean branch length between any of the conditions ($p > 0.05$) (Figure 8E).

Figure 8



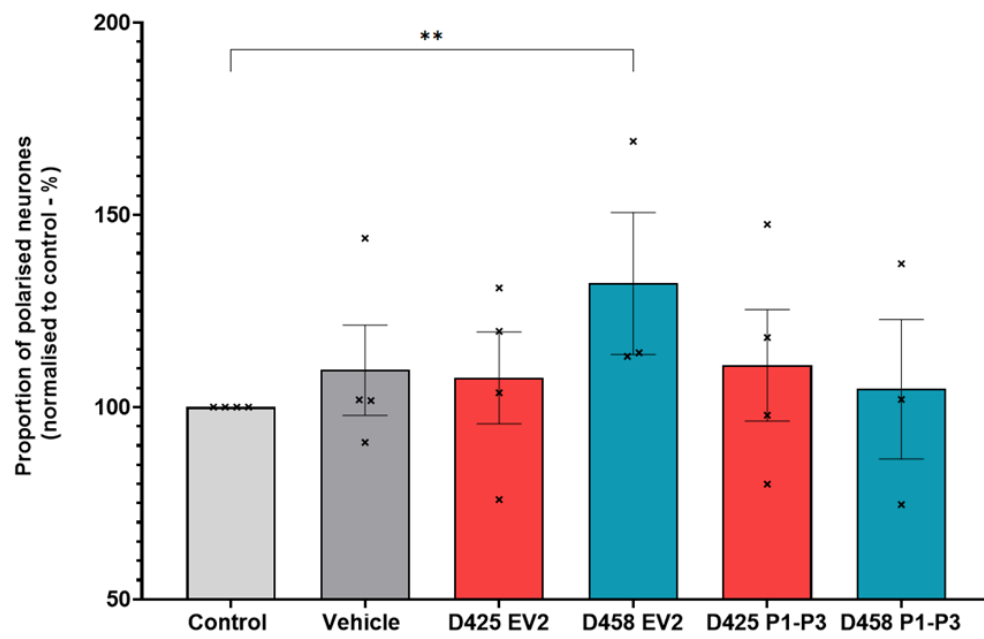
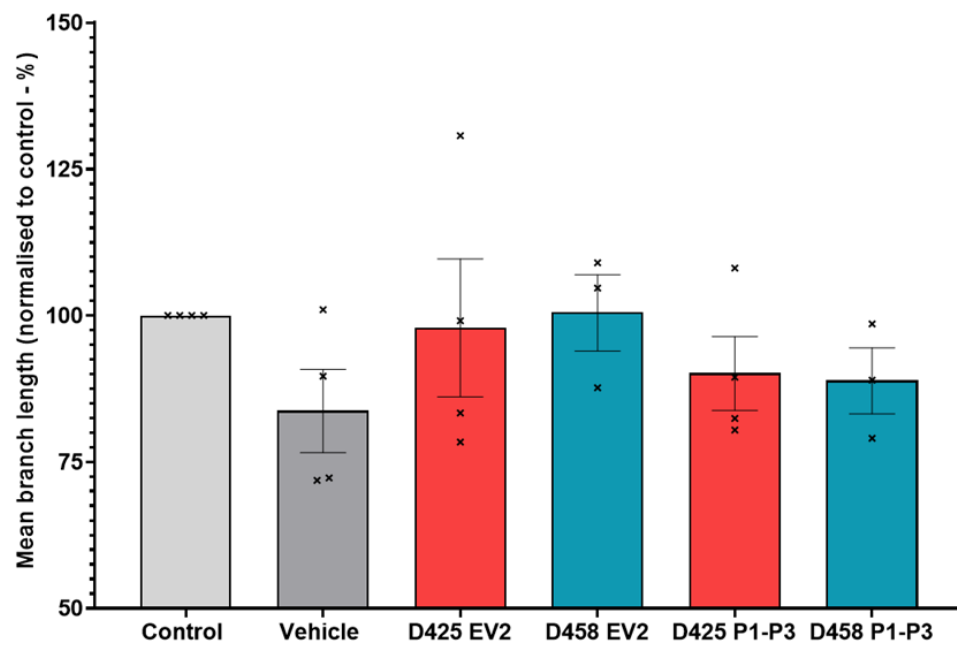
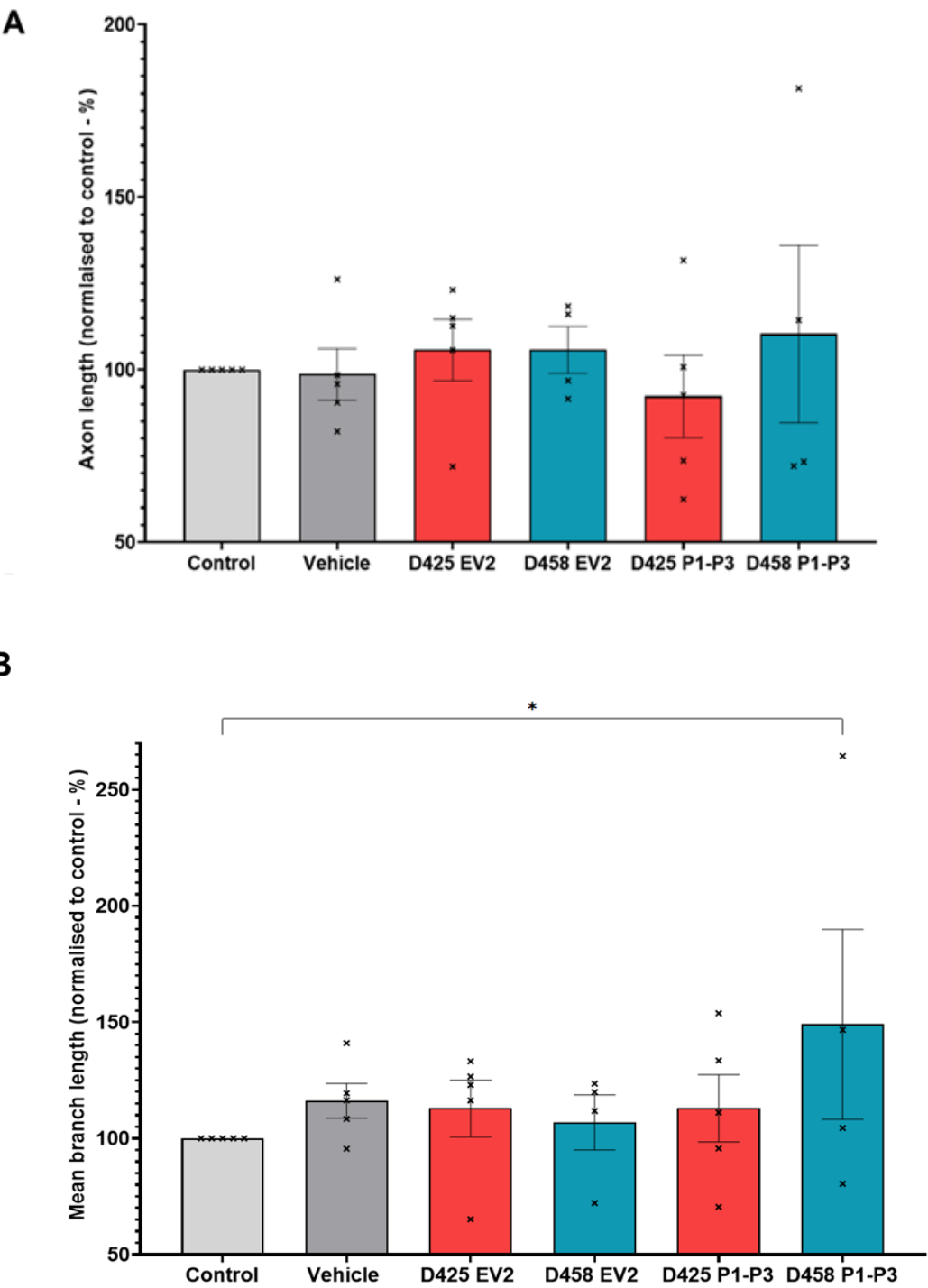
D**E**

Figure 8. Extracellular vesicles (EVs) from the recurrent medulloblastoma cell line increase axon length but not dendrite length. EV fractions from two medulloblastoma cell lines (D425 and D458) were isolated using size exclusion chromatography and added to mouse primary cortical neurones. Neurones were treated with either vehicle (DPBS), EVs (EV2) or the mixed protein fraction (P1-P3) (see Methods 2.6). Immunocytochemistry followed by image analysis (see Methods 2.9) was used to assess neuronal morphology after 24 hours. **(A)** shows that only EVs from the D458 cell line had a significant effect on axon length in the primary cortical neurones after 24 hours. However, dendrite length did not change **(B)**. Similar neuronal morphology was observed in all tested conditions **(C)**. Green – acetylated tubulin, red – actin and blue – DAPI. Images taken using a CCD camera at x63 magnification on a fluorescent microscope with a LED light source. There was no significant difference in the proportion of polarised neurones between the D458 EV treated neurones and the vehicle treated neurones **(D)**. Additionally, there was no significant difference in mean branch length **(E)** between any of the conditions. Significant differences were calculated using a Kruskal-Wallis test followed by Dunn's post hoc test (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ **** = $p \leq 0.0001$). Data are the normalised mean of 3 or 4 independent biological replicates with error bars indicating mean $\pm$ SEM.

3.5 The addition of medulloblastoma-derived EVs had no effect on axon length or mean branch length after 72 hours

Despite the increase in axon length in the D458 EV treated group compared with the vehicle treated group after 24 hours, this effect was not observed after 72 hours ($p > 0.05$) (Figure 9A). Similarly, there was no difference in mean branch length in either of the EV treated groups when compared to the control and vehicle treated groups ($p > 0.05$) (Figure 9B). Although the D458 mixed protein fraction did increase the mean branch length of the neurones after 72 hours when compared to the control neurones, this was not significantly different from the vehicle treated group ($p > 0.05$). Regarding the proportion of polarised cells, there were no significant differences between any of the conditions ($p > 0.05$) (Figure 9C).

Figure 9



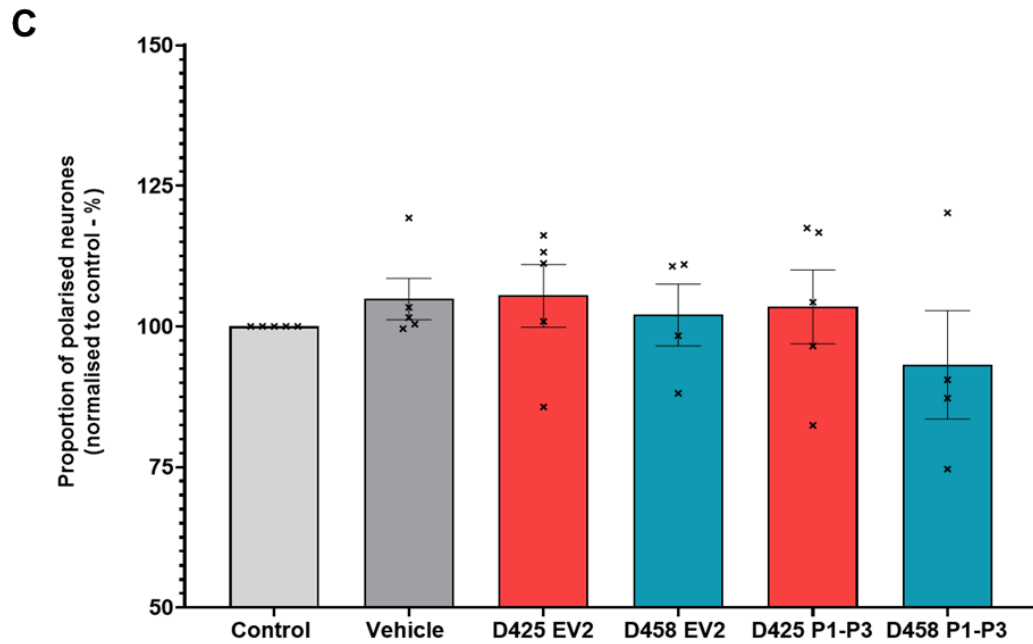


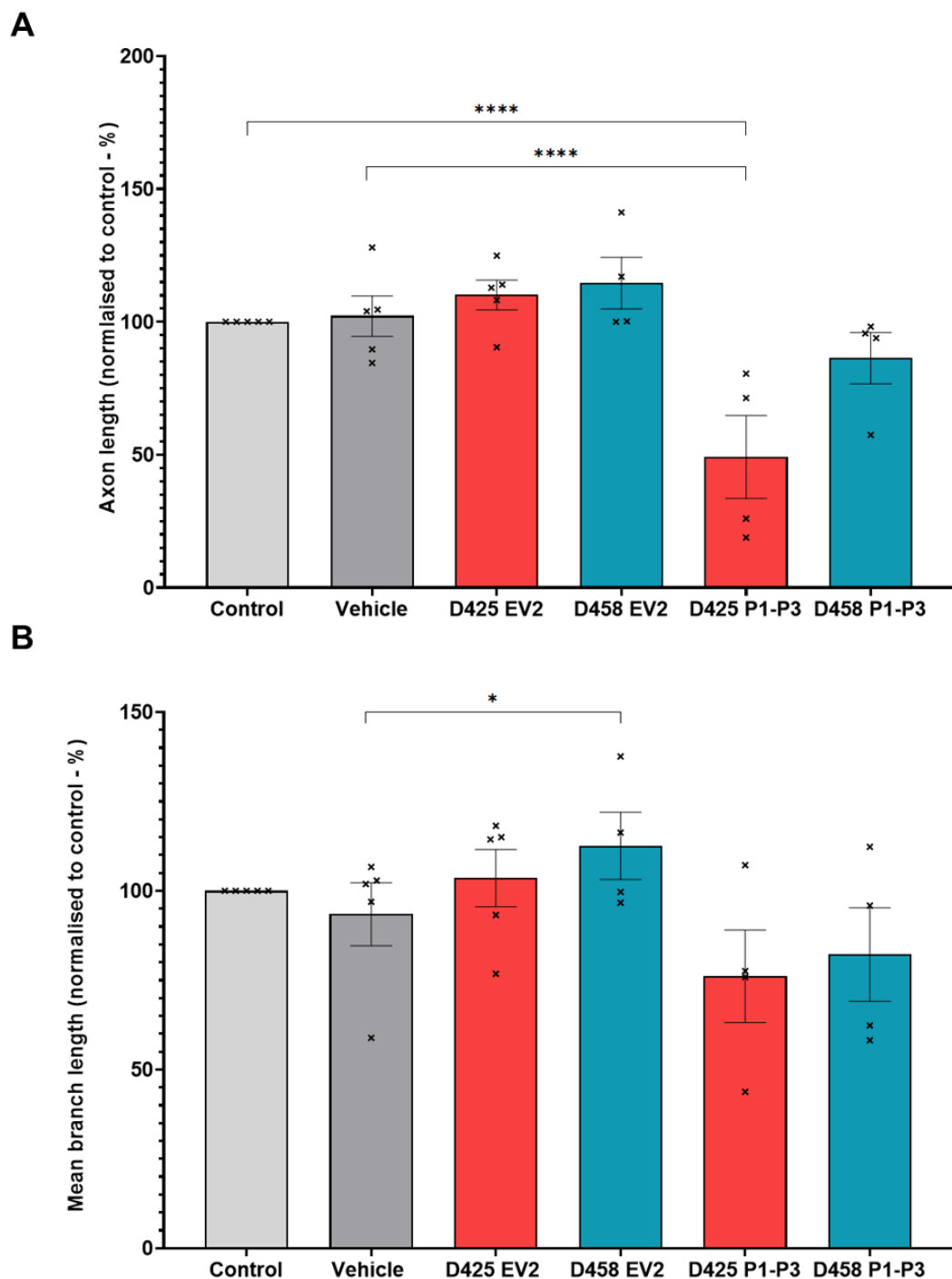
Figure 9. Medulloblastoma-derived EVs have no effect on axon length or mean branch length after 72 hours. Mouse primary cortical neurones were grown in culture for 24 hours before being treated with either vehicle (DPBS), EVs (EV2) or the mixed protein fraction (P1-P3) isolated from D425 and D458 cells (see Methods 2.6). Seventy-two hours after dosing, there was no significant difference in axon length between any of the conditions **(A)**. There was no significant difference in mean branch length between the D458 P1-P3 treated neurones and the vehicle treated neurones **(B)**. Additionally, there was no significant difference in the proportion of polarised neurones between the conditions **(C)**. Significant differences were calculated using a Kruskal-Wallis test followed by Dunn's post hoc test (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ **** = $p \leq 0.0001$). Data are the normalised mean of between 3 and 5 independent biological replicates with error bars indicating mean $\pm$ SEM.

3.6 The addition of medulloblastoma-derived EVs had no effect on axon length or mean branch length after 120 hours

After 120 hours, no difference in axon length was observed in either of the EV treated groups when compared to the control or vehicle treated groups ($p > 0.05$) but a significant decrease in axon length was observed in the neurones treated with the D425 mixed protein fraction ($p < 0.0001$) (Figure 10A).

Additionally, there was a significant increase in mean branch length in the D458 EV treated neurones after 120 hours when compared to the vehicle treated neurones ($p = 0.0462$), but not the control neurones ($p > 0.05$) (Figure 10B). Also, the neurones treated with D425 EVs had a greater proportion of polarised neurones when compared to the control neurones, but not the vehicle treated neurones ($p > 0.05$) (Figure 10C).

Figure 10



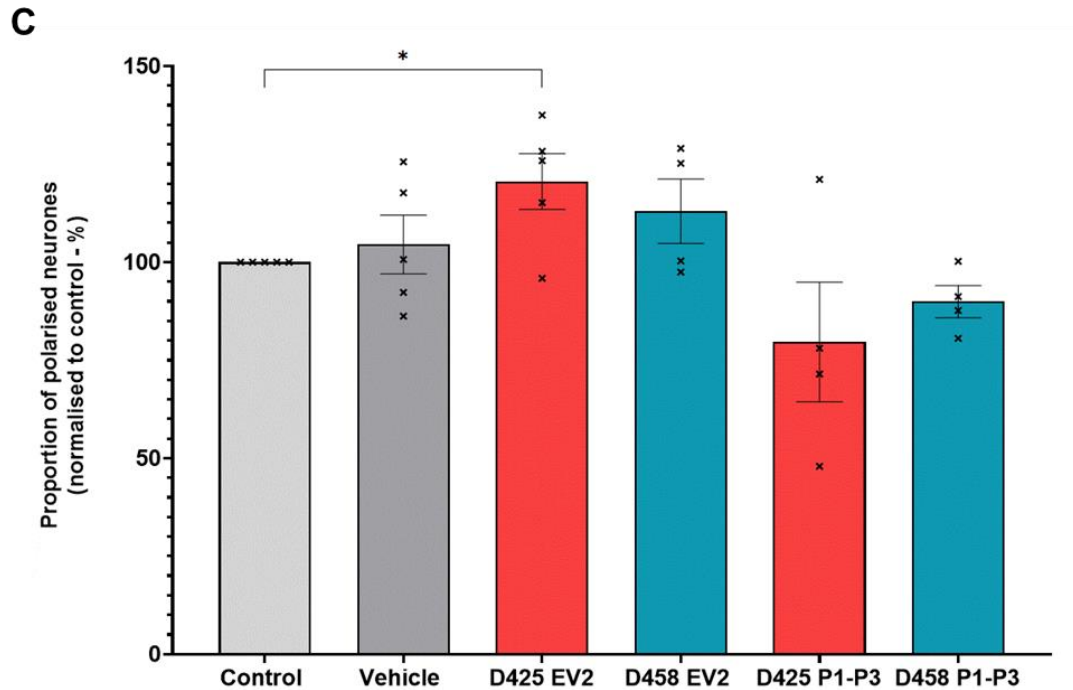


Figure 10. There was no effect of the medulloblastoma-derived extracellular vesicles (EVs) on axon length after 120 hours. Mouse primary cortical neurones were grown in culture for 24 hours before being treated with either vehicle (DPBS), EVs (EV2) or the mixed protein fraction (P1-P3) isolated from D425 and D458 cells (see Methods 2.6). One hundred and twenty hours after dosing, only the mixed protein fraction from the D425 cells had a significant effect on axon length **(A)**. There was no significant difference in mean branch length between the D458 EV treated neurones and the untreated control neurones **(B)**. Similarly, there was no significant difference in the proportion of polarised neurones between the D425 EV treated neurones and vehicle treated neurones **(C)**. Significant differences were calculated using a Kruskal-Wallis test followed by Dunn's post hoc test **(A & B)** or a one-way ANOVA with Bonferroni's post hoc test **(C)** (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$). Data are the normalised mean of between 3 and 5 independent biological replicates with error bars indicating mean $\pm$ SEM.

3.7 EVs isolated from 10% EV-depleted medium and 10% bovine growth serum (BGS) supplemented medium have no effect on neuritogenesis or axon length

To ensure that the increase in neuritogenesis and axon length observed after 24 hours was being driven by EVs released from the medulloblastoma cells

lines and not by EVs or other bovine-derived contaminants from the 10% EV-depleted bovine growth serum (BGS) medium, a similar experiment was performed with the isolated EV and protein fractions from 20ml of fresh 10% BGS in DMEM and 20ml of fresh 10% EV-depleted BGS in DMEM. There was no significant difference in the proportion of neurones which had developed at least one neurite between any of the treatment conditions ($p > 0.05$) (Figure 11A). All conditions appeared to have similar neuronal morphology (Figure 11B).

Figure 11

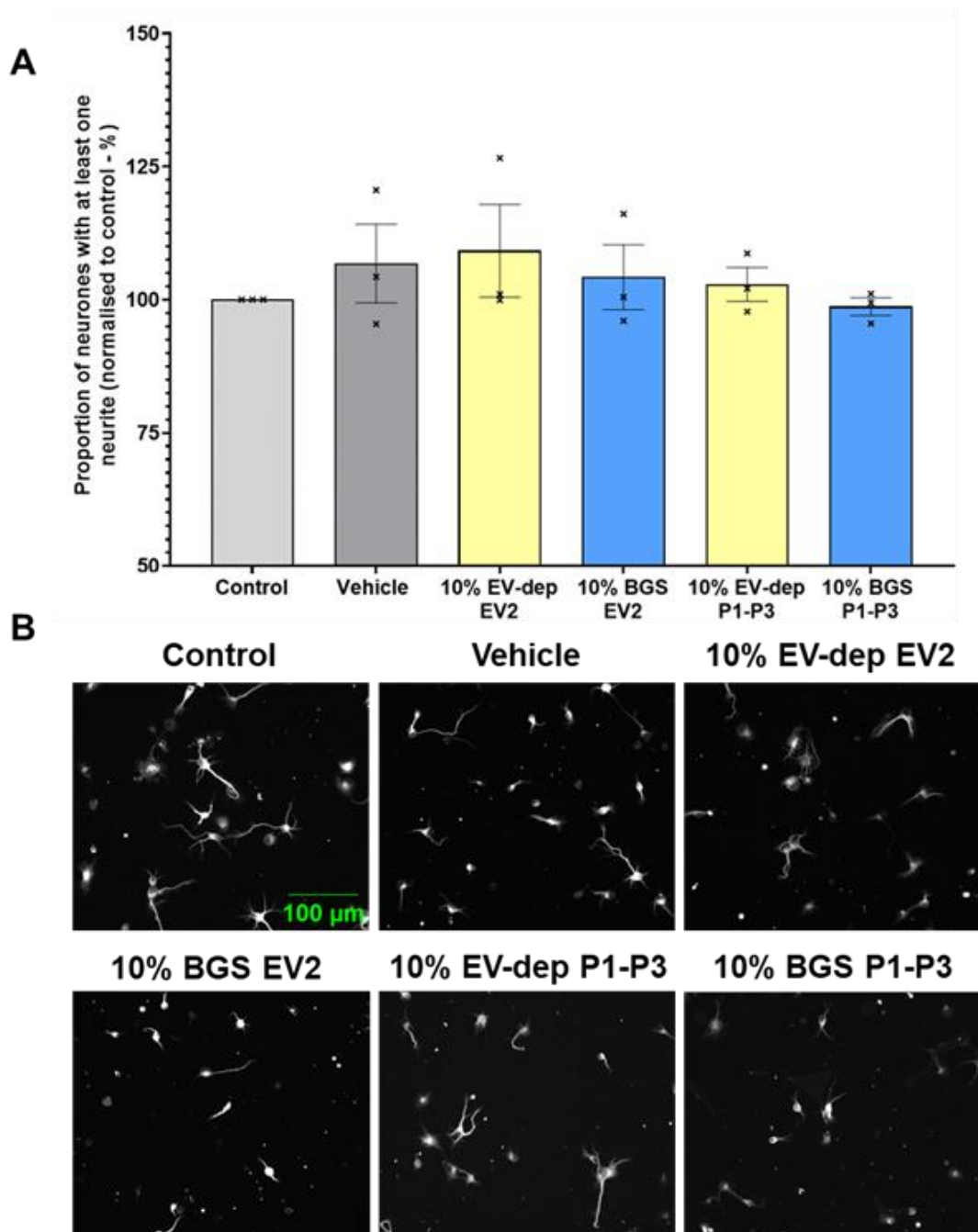
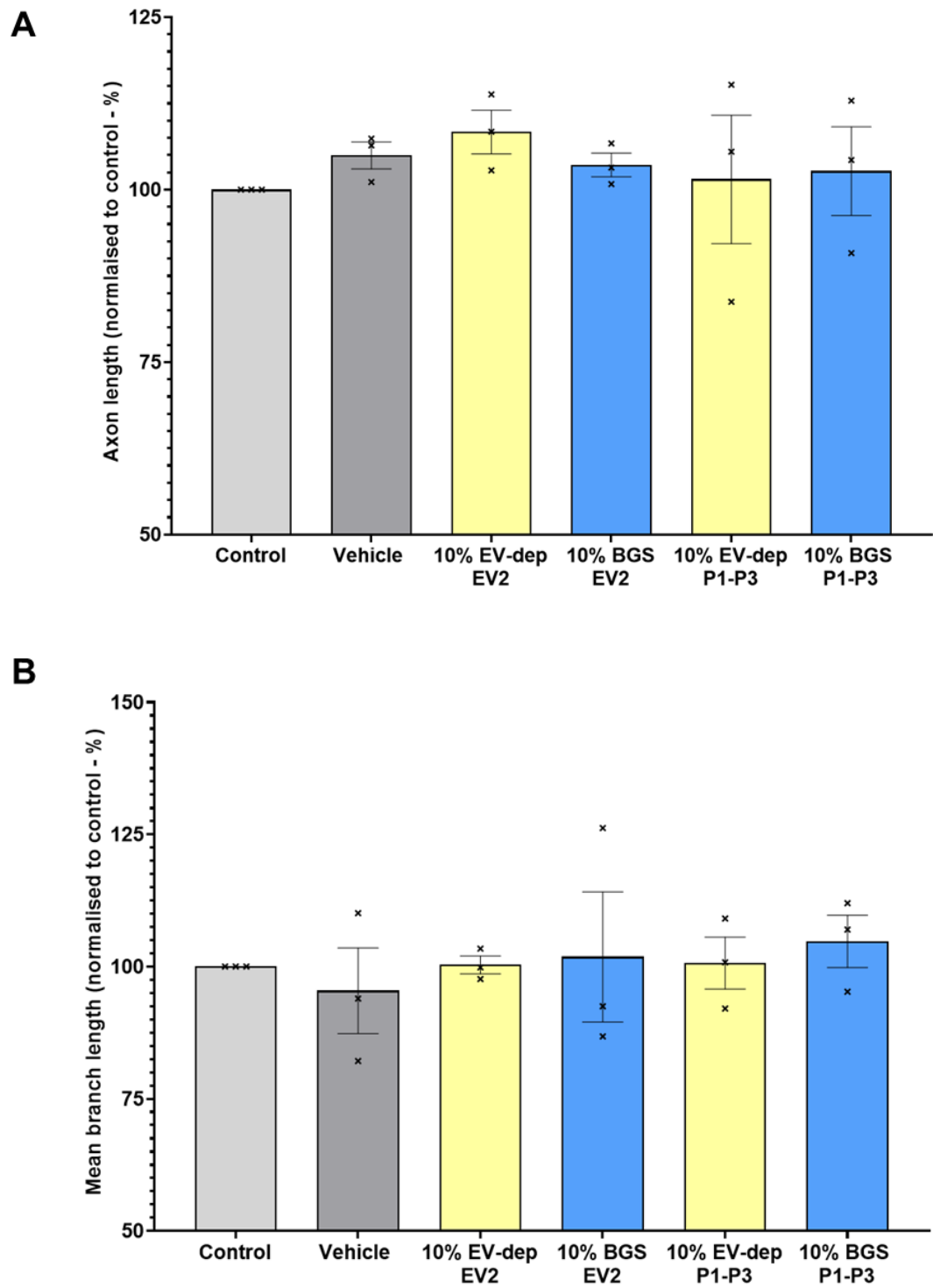


Figure 11. Extracellular vesicles (EVs) isolated from fresh DMEM supplemented with 10% EV-depleted bovine growth serum (BGS) or 10% BGS have no effect on neuritogenesis after 24 hours. EV and protein fractions were isolated from fresh cell culture medium containing either 10% EV-depleted BGS or normal BGS. Mouse primary cortical neurones were grown in culture for 24 hours before being treated with either vehicle (DPBS), EVs (EV2) or the mixed protein fraction (P1-P3) isolated from the cell culture media. Twenty-four hours later, the neurones were fixed and immunocytochemistry was performed to assess neuronal growth and development. There were no significant differences in neuritogenesis between the conditions **(A)**. Immunocytochemistry was used to stain for acetylated tubulin **(B)** and similar neuronal morphology was observed in all tested conditions. Images taken using a CCD camera at x20 magnification on a fluorescent microscope with a LED light source. White – acetylated tubulin. Significant differences were calculated using a one-way ANOVA with Bonferroni's post hoc test (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ **** = $p \leq 0.0001$). Data are the normalised mean of 3 independent biological replicates with error bars indicating mean $\pm$ SEM.

Likewise, there was no significant difference in axon length (Figure 12A), mean branch length (Figure 12B) or the proportion of polarised neurones (Figure 12C) between any of the conditions ($p > 0.05$).

Figure 12



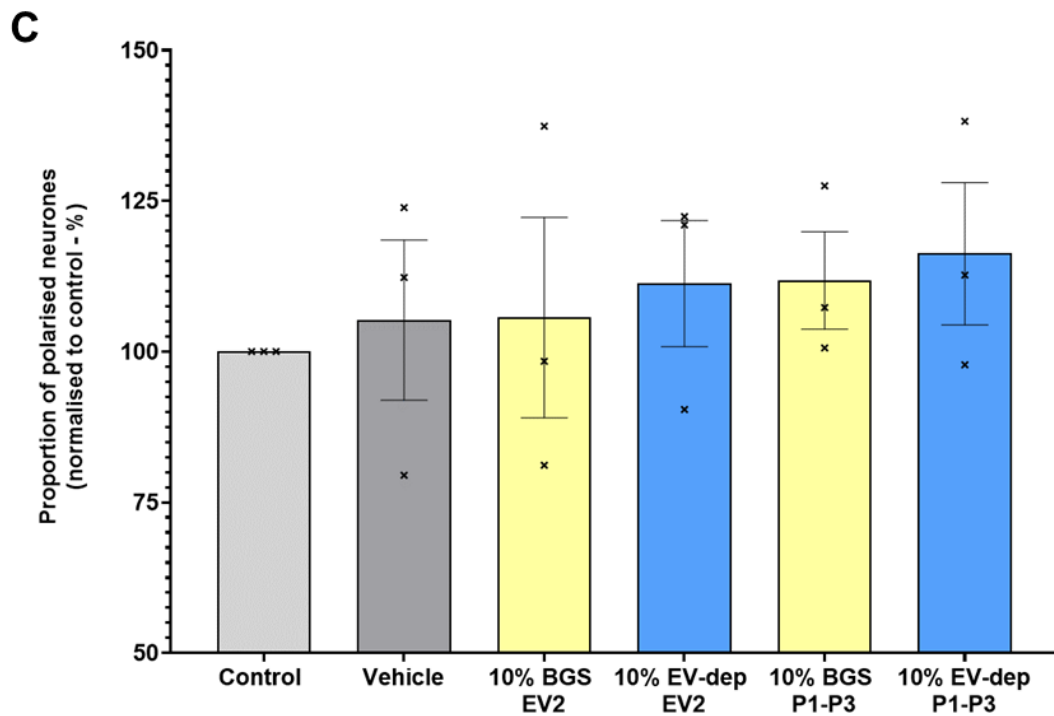


Figure 12. Extracellular vesicles (EVs) isolated from fresh DMEM supplemented with 10% EV-depleted bovine growth serum (BGS) or 10% BGS have no effect on axon length or mean branch length after 24 hours. EV and protein fractions were isolated from fresh cell culture medium containing either 10% EV-depleted BGS or normal BGS. Mouse primary cortical neurones were grown in culture for 24 hours before being treated with either vehicle (DPBS), EVs (EV2) or the mixed protein fraction (P1-P3) isolated from the cell culture media. Twenty-four hours later, the neurones were fixed and immunocytochemistry was performed to assess neuronal morphology (see Methods 2.7). There were no significant differences in axon length **(A)**, mean branch length **(B)** or the proportion of polarised neurones **(C)** between any of the groups. Significant differences were calculated using a Kruskal-Wallis test followed by Dunn's post hoc test (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ **** = $p \leq 0.0001$). Data are the normalised mean of between 3 independent biological replicates with error bars indicating mean $\pm$ SEM.

4. Discussion

Survivors of paediatric brain tumours are more likely to develop symptoms of neurodevelopmental disorders later in life than the rest of the population (Hardy et al., 2018; Hsu et al., 2023). To explore the neurobiological basis of this connection, EVs were isolated from two medulloblastoma cell lines and it was investigated whether they could alter the development and morphology of mouse primary cortical neurones in vitro. Here it was demonstrated that medulloblastoma-derived EVs promote neuritogenesis and increase axon length in mouse cortical neurones, supporting the original hypothesis that medulloblastoma EVs can alter neuronal development in a manner similar to other cancers studied. However, there was no meaningful change (where the neurones treated with EVs were significantly different from both the control and vehicle treated groups) observed in the proportion of polarised cortical neurones or in the mean branch length of the axons.

4.1 Key findings

4.1.1 Characterisation of D425 and D458 extracellular vesicles

For this study EVs from two well characterised medulloblastoma cell lines were used, D425 cells which originate from a primary tumour in the cerebellum and D458 cells which originate from a recurrent tumour in the cerebrospinal fluid (CSF) from the same patient. Both of the cell lines released particles which matched the established size distribution of EVs (approximately 30 to 1000nm) (van der Pol et al., 2012). Other studies which have investigated medulloblastoma-derived EVs using NTA have identified the peak of the size distribution to be 126nm or between approximately 50 and 200nm (Epple et al., 2012; Jackson et al., 2023). This is relatively similar to the peak of 105nm identified in this study. Slight differences in size distribution peaks may be a result of differences in isolation methods and cell lines used. A recent study found that D458 cells produce approximately 55% more EVs than D425 cells (Jackson et al., 2023). Although this study observed a similar increase of 66%, this was not statistically significant. One

possibility is that contaminants within the buffers and samples used for NTA (such as salts, microbubbles and protein aggregates) were recognised as particles by the NTA scatter mode and skewed the data (Filipe et al., 2010). Despite this, small lipid structures were visualised within the D458 EV sample using MemBright (a fluorescent plasma membrane probe). This suggests that EVs were present within the EV samples as MemBright has been successfully used in previous studies to visualise EVs (Hyenne et al., 2019; Mary et al., 2020).

4.1.2 The effect of medulloblastoma EVs on mouse primary cortical neurone development

First, the normal development of mouse primary cortical neurones in vitro was established over a period of 7 days. An increase in axon length, mean branch length and the proportion of polarised neurones was observed - as expected. The distinct stages of neuronal polarisation were also observed including the formation of lamellipodia, the growth of immature neurites and the formation of a distinct axon. The neuronal development and morphology observed was similar to other studies which have used mouse primary cortical neurones (Dajas-Bailador et al., 2008; Lesuisse & Martin, 2002; Lucci et al., 2020; Parker et al., 2018).

The next step was to establish whether adding medulloblastoma-derived EVs to mouse primary cortical neurones would alter the development of the neurones. Twenty-four hours after the addition of EVs from both cell lines, there was an increase in neuritogenesis when compared to the control and vehicle treated groups. To quantify neuritogenesis, the proportion of neurones which had at least one neurite (i.e., an immature neurite or an axon) was calculated. By quantifying neuritogenesis in this way, it was possible to capture the earliest stages of the process of polarisation by identifying the proportion of cells which had developed past the lamellipodia stage.

The finding that medulloblastoma-derived EVs can promote neuritogenesis after 24 hours is consistent with the results of work with EVs derived from head and neck squamous cell carcinoma, cervical cancer and ovarian cancer using PC12 cells as a neuronal model (Kovacs et al., 2020; Lucido et al., 2019; Madeo et al., 2018). This suggests that the increase in neuritogenesis observed in the mouse primary cortical neurones is not limited to one particular cancer and may be a more general property of cancer-derived extracellular vesicles.

EVs from the D458 cell line increased axon length after 24 hours when compared to the control and vehicle treated groups but did not have any effect on dendrite length, suggesting an axonal-specific response rather than a globalised increase in the growth of cell processes. However, by 72 hours there was no significant difference in axon length between any of the conditions.

There was no significant difference in axon length after 72 hours and as previous studies did not look beyond 48 hours (Kovacs et al., 2020; Lucido et al., 2019; Madeo et al., 2018), it is difficult to compare the effect of medulloblastoma EVs to other cancer EVs in this case. However, it is possible that the effect would have been sustained if the cortical neurones had been continuously dosed or exposed to medulloblastoma EVs – as would happen in vivo.

The fact that the increase in axon length at 24 hours was only observed in the cortical neurones treated with EVs derived from the D458 cell line suggests that the recurrent cell line produces EVs that have a different and more growth promoting effect than EVs from the primary cell line. This may be due to differences in EV cargo, and therefore function, between the two cell lines. For example, it has been shown that exosomes released by the D458 cell line express greater levels of EMMPRIN than exosomes released from the D425 cell line (Jackson et al., 2023). EMMPRIN, an extracellular matrix remodelling endopeptidase (Kaushik et al., 2015), is involved in

promoting cancer invasion and migration which might help to explain the growth promoting effects.

When investigating polarisation of the neurones, there were no significant differences in the proportion of polarised cells between the EV and vehicle treated neurones at any of the time points investigated. After 120 hours the D458 EVs increased the mean branch length of the cortical neurones when compared to the vehicle treated neurones, but not the completely untouched control neurones.

Unexpectedly, the D425 mixed protein fraction reduced axon length after 120 hours. Medulloblastoma cells are known to secrete proteins, including proteins which can induce oxidative stress in neural stem cell progenitors (N. Sharma et al., 2016). However, although this finding might prompt greater understanding of the proteins released from these cells, the physiological relevance of this is questionable as it is unclear if proteins secreted by medulloblastoma cells in the cerebellum could travel undisrupted to the cortex, unlike EVs which are known to travel long distances in the CSF (Muraoka et al., 2020). Despite this, this finding was significant and reproducible so may warrant more investigation.

The effect of brain cancer-derived EVs on primary neurones has been investigated in the context of glioblastoma but these EVs promoted neuronal apoptosis and necrosis rather than growth (W. Zhou et al., 2022). This is likely a reflection of the very different natures of medulloblastoma and glioblastoma. Whereas medulloblastoma is a highly survivable paediatric brain cancer, glioblastoma predominantly affects adults and is one of the deadliest cancers with only 5% of patients surviving for more than 5 years (Tamini & Juweid, 2017). In vitro it has been demonstrated that glioblastoma EVs induce neuronal death by promoting oxidative stress (Genc et al., 2022).

Unlike previous studies on the effect of EVs from other cancers on neurone-like cells, this study was able to quantify neuronal morphology in a more comprehensive way by using mouse primary cortical neurones rather than a

non-polarising cell model. For example, previous studies have demonstrated that EVs derived from head and neck squamous cell carcinoma, cervical cancer and ovarian cancer can promote neuritogenesis (Kovacs et al., 2020; Lucido et al., 2019; Madeo et al., 2018). These studies used PC12 cells as their neuronal model which originate from a rat pheochromocytoma (a neuroendocrine tumour) and can differentiate into neurone-like cells which extend neurites but do not undergo axon development (Votin et al., 2005). Consequently, these cells are morphologically distinct from cortical neurones and this previous analysis of neurone-like morphology was not based on polarised cells. Likewise, studies which have used mouse dorsal root ganglion (DRG) neurones as their neuronal model (Amit et al., 2020) have not been able to investigate changes in polarisation as DRG neurones are multipolar when cultured in vitro (Clark et al., 1993). Therefore, this study is one of the first to investigate the effects of cancer-derived EVs on neuronal morphology using a cell model which more accurately reflects the development of cortical neurones in vivo.

Overall, these results support the hypothesis that medulloblastoma cells release EVs which are able to alter the development of cortical neurones, potentially contributing to the increased incidence of neurodevelopmental disorders seen in PBTS by changing the network structure of the cortex at a neuronal level.

4.1.3 The effect of extracellular vesicle and protein fractions isolated from fresh medulloblastoma cell culture medium on mouse primary cortical neurones

Importantly, this study has shown that EVs isolated from fresh DMEM supplemented with 10% BGS or 10% EV-depleted BGS do not have any effect on neuritogenesis, polarisation, axon length or mean branch length after 24 hours. This confirms that bovine contaminants in the medium (such as proteins, lipids and EVs) are not responsible for the differences in neuronal morphology and growth that were observed 24 hours after adding the medulloblastoma EVs. However, as these experiments were conducted

independently from those using medulloblastoma EVs it is not possible to compare the effects of the medium EVs and the medulloblastoma EVs directly, something that would need to be rectified in the future.

4.2 Limitations

4.2.1 Using cell lines derived from a Group 3 medulloblastoma tumour

This study has several limitations. The cell lines used in this study originate from a Group 3 medulloblastoma tumour. As Group 3 tumours have the worst prognosis (Ramaswamy et al., 2016), it would be more clinically relevant to use a cell line derived from a subgroup with a better prognosis (such as Wnt or TP53-wildtype Shh) to investigate the long-term neurobiological effects of medulloblastoma on survivors. Consequently, more suitable cell lines such as MED5R (Wnt) and DAOY (Shh) (Ivanov et al., 2016) should be used in any future studies. This study used 2D cell culture rather than 3D for both the medulloblastomas cell lines and mouse primary cortical neurones. To create a more physiologically relevant model, 3D cell culture models could be used in the future. This is of particular importance for EV studies as several studies have shown that cell culture technique can significantly affect EV composition, yield and function (Jalilian et al., 2022; Kusuma et al., 2022; Thippabhotla et al., 2019). Regarding neuronal culture, even small 3D cultures produce complex electrical activity that is not observed in 2D monolayer culture (Ming et al., 2020).

4.2.2 Using a cross-species model of extracellular vesicle signalling

Although cross-species EV studies are common, it would be ideal to use human neurones (derived from induced pluripotent stem cells) rather than mouse neurones. Even though 79% of neural genes have a similar pattern of expression in humans and mice (Zeng et al., 2012), even small differences in amino acid sequence can change the function of the resulting protein in mice. For example, in Alzheimer's disease, the human amyloid precursor

protein (APP) forms aggregates but the same does not occur in the mouse brain due a difference of only 17 amino acids (Xu et al., 2015). These small differences are of particular importance when studying miRNAs (small non-coding RNAs that can regulate translation) as although many human miRNAs have mouse orthologues, not all do (although some have been shown to have a highly conserved sequence and function) (Haviv et al., 2018; Rahmanian et al., 2019). Consequently, it would be more biologically relevant to apply human medulloblastoma EVs to human cortical neurones. However, this is not to say that mouse primary cortical neurones are not a useful model. Cross-species findings are not unique to medulloblastoma. Like this study, Amit et al. used mouse primary neurones (dorsal root ganglion neurones rather than cortical) and EVs derived from human tumour cell lines. As this study also observed neuritogenesis this suggests that the cross-species effect of cancer EVs on neurones is not limited to medulloblastoma. Additionally, Zhou et al. observed consistent results across human primary neurones, mouse brain slices and neuroblastoma cells when applying human glioblastoma EVs, supporting the idea that mouse brains and neurones are still a useful model for EV work.

4.2.3 Adding a single addition of high concentration medulloblastoma extracellular vesicles to the mouse primary cortical neurones

Another limitation of this study is that the medulloblastoma-derived EVs were only added to the cortical neurones once. To make these experiments more physiologically relevant, it would be better to repeatedly dose the cells with a smaller concentration of EVs to mimic more closely what occurs in vivo. As EVs are endocytosed relatively quickly (one study observed uptake between 0 and 4 hours of incubation (Joshi et al., 2020)), it may be that after the initial dose the concentration of medulloblastoma-derived EVs in the cortical neurone cell culture medium decreases quickly. This could be investigated using fluorescently labelled medulloblastoma EVs in conjunction with nanoparticle tracking analysis to quantify EV concentration over time. This may help to explain why the increase in axon length was not seen after 24 hours. As the neurones grow and develop rapidly, the effect of the earlier EV

dose may be lost as the neurones 'recover' and return to the normal rate of growth. Another option would be to co-culture the medulloblastoma cells with the neurones so that they are continuously exposed to the EVs. However, the cell culture conditions would need to be suitable for both cell types and it would be difficult to isolate the effect of EVs from the effect of other secreted molecules (such as proteins).

By using EVs isolated from concentrated medulloblastoma conditioned medium, this study may have failed to reflect the concentration of medulloblastoma EVs that neurones would be exposed to in the brain. One study measured the concentration of EVs in the CSF from patients with brain tumours and with brain metastasis (K.-Y. Lee et al., 2020). They found that the average concentration of EVs in the CSF from patients with brain metastasis was 2.49×10^8 EVs per ml and approximately 3×10^8 EVs per ml for those with brain tumours. This study used a maximum concentration of 4×10^{10} EVs per ml which is over 100 times more concentrated than what is found clinically. As a result, any future work should either use a more physiologically relevant concentration of EVs or perform a dose response curve to investigate the effect of medulloblastoma-derived EVs on mouse primary cortical neurones at different concentrations.

4.3 Future directions

4.3.1 Extracellular vesicle characterisation

More work needs to be done to characterise the EVs released from the medulloblastoma cells as this was beyond the scope of the present study. This can be achieved in several ways. Firstly, mass spectrometry or western blot can be used to identify known protein markers of EVs such as CD63, CD81 and ALIX. Additionally, these techniques can be used to identify markers of common contaminants such as lipoproteins – lipid structures that closely resemble EVs (Simonsen, 2017; Zhang et al., 2020). Transmission electron microscopy or cryo-electron microscopy can be used to visualise the structure of EVs and confirm the presence of the phospholipid bilayer

membrane. This study used 'scatter mode' NTA to quantify the number of particles released and the size distribution. However, a 'fluorescent mode' can be used in which an antibody against a specific EV marker is conjugated to a fluorophore and used to visualise only the particles that are positive for that protein. This provides more accurate values as it does not measure contaminants within the sample. These methods of characterisation are all recommended by MISEV2018 and should be adhered to where possible (Théry et al., 2018).

4.3.2 Integrating more controls into the experimental design

Although this study did perform controls to see whether EVs or other bovine-derived contaminants from the cell culture medium could affect neuronal development, these experiments were performed independent of the medulloblastoma-derived EV treatments. As a result, it is not possible to directly compare the effect of contaminants within the cell culture medium and the medulloblastoma EVs which should be rectified in any future studies. It will also be necessary to perform medium controls at all time points studied (not just at 24 hours) but this was beyond the scope of this study due to time constraints. Few studies include medium controls but it is recommended by MISEV2018 to use non-conditioned medium as a negative control (Théry et al., 2018). They also recommend comparing the functional effects of the total conditioned cell culture medium, the conditioned cell culture medium with the EVs removed and the isolated EV fractions which could be incorporated into future work.

Additionally, as noted by the MISEV2018 guidelines (Théry et al., 2018), it is difficult to attribute distinct functions to different cell types without testing a range of cell types and cell lines (such as cancer-derived EVs promoting neuritogenesis or other changes in neurone growth and development). Consequently, it would be useful to include EVs from another cell type to act as a control. For example, one study investigating the effect of non-small cell lung cancer-derived EVs on non-tumourigenic cells used EVs from lung epithelial cell lines as a control (Hasan et al., 2022). However, there are

limited cell types which would produce EVs that are physiologically relevant to cortical neurones due to their relatively inaccessible location within the CNS. Also, there is no guarantee that the EVs from the chosen control cell line are 'inert' and have no effect on the growth of mouse primary cortical neurones in vitro.

4.3.3 Functional studies, improved analysis of neuritogenesis and investigating underlying mechanisms

This study only considered the morphological changes of the mouse primary cortical neurones, but it would be interesting to investigate whether medulloblastoma EVs also elicit functional changes. Calcium imaging can be used to record the levels of calcium within a cell as a measure of electrical activity (Clarkson et al., 2017). Alternatively, a multiple electrode array or patch clamp could be used to record the electrical activity of the cells directly (Hales et al., 2010; Noguchi et al., 2021). It would also be important to investigate whether the mouse primary cortical neurones, which have been exposed to medulloblastoma EVs, behave in a similar way to neuronal models of ADHD and ASD. For example, induced pluripotent stem cell derived neurones from ASD patients show calcium signalling hyperactivity when cultured in vitro (Avazzadeh et al., 2019).

This lab has experience of culturing mouse primary cortical neurones in microfluidic chambers which enable the separation of the cell body and axon (Lucci et al., 2020). These could be used to add EVs to the axons or the cell bodies of the neurones and identify whether any morphological changes are mediated by uptake at the axon or at the cell body.

Although analysis of neuritogenesis was able to capture early changes in the process of polarisation, more work is needed to fully comprehend the differences that were observed. For example, it would be beneficial to count the number of immature neurites protruding from each cell, measure the length of each individual neurite, identify the proportion of cells that are at each distinct stage of polarisation and identify any multipolar neurones. To

do this, an antibody to visualise JIP-1 could be used, a protein which localises in the neurite that will become the developing axon (Dajas-Bailador et al., 2008). This would enable us to identify whether medulloblastoma-derived EVs result in multipolar neurones and investigate the changes in neuritogenesis more thoroughly.

These findings suggest that medulloblastoma-derived EVs can alter neuronal development but the mechanism behind this has not yet been investigated. EVs are known to contain a range of proteins, lipids and nucleic acids – all of which could contribute to the changes in neuronal growth. One idea would be to look at whether these EVs contain miRNAs known to be involved in regulating cell growth and development such as miR-21, miR-199a-3p and miR-26a (Jiang et al., 2015; Kar et al., 2021; Lucci et al., 2020). This would complement yet unpublished work produced by the Dajas-Bailador lab which suggests that miRNAs within EVs can regulate axon development in mouse primary cortical neurones. Also, other medulloblastoma cell lines could be used to see if there are any consistent differences between primary and recurrent tumours, different disease subgroups and cell lines with different known mutations.

4.4 Final conclusions

Cancer research has long focused on cancer treatment and prevention, for obvious reasons, but more work needs to be done to understand the long-term biological effects on survivors who experienced cancer at a young age. This study has identified that EVs derived from both primary and recurrent medulloblastoma tumour cells are able to increase neuritogenesis in mouse primary cortical neurones after 24 hours. EVs derived from the recurrent cell line also increased axon length in the mouse primary cortical neurones after 24 hours. It has long been known that PBTS are more likely to develop symptoms of neurodevelopmental disorders but the underlying biological mechanisms of this are poorly understood. If EVs released from medulloblastoma cells can permanently alter the neuronal structure of the cortex, although this is yet to be seen, it may be necessary to prevent EV

release from the tumour to reduce the risk of the emergence of neurodevelopmental disorders and cognitive impairments later in life. Overall, this study has uncovered an unknown ability of medulloblastoma EVs to alter cortical neurone development and is as a steppingstone towards a better understanding of the long-term neurobiological effects of paediatric medulloblastoma on survivors.

5. References

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