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Human recombinant Laminin-511 and -521 stimulates neurite outgrowth and synaptogenesis of cerebellar progenitor cells

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1. Abstract

With increasing prevalence of neurodegenerative diseases the need for an effective treatment increases. To date pharmacological treatment is the golden standard. However, the medicines used have many adverse effects and, only work for a short time period. The best solution would be to cure the disease and thus stop the degradation of neurons and replace the already lost neurons by healthy ones. With the development of regenerative medicine, strategies to replace the lost neurons are emerging. One of the approaches includes the use of neuronal progenitor cells to differentiate and grow into functional neurons. To achieve this, a suitable *in vitro* environment is needed that includes an optimal use of culture medium and substrate. Laminin has been shown to be an effective substrate for neuronal cell culture purposes as it is part of the native extracellular matrix in the nervous system. However, laminin represents a family of 20 isoforms as known so far and there is still a lot unknown about the functions of each isoform on neuronal cultures. In this study the effects of eight available human recombinant laminin isoforms (i.e., -111, -121, -211, -221, -411, -421, -511 and -521) on cell attachment, differentiation, neurite outgrowth and connectivity of cerebellar progenitor cells have been assessed by means of immunofluorescence techniques. A general laminin substrate stemming from mice sarcoma (L2020) was used as a control. The results show that Laminin- 211, -221 and -411 do not possess any neurite outgrowth and differentiation abilities and thus were not included in the remaining experiments. Between the remaining substrates used no difference was found on cell attachment and neuronal differentiation. Laminin-511 and -521 increased neurite outgrowth the most and laminin-421 the least. Laminin-111 took third place. Laminin-111, -511, -521 and L2020 were included in the connectivity experiments. Laminin-521 stimulated synaptogenesis to the largest extend followed by laminin-511 and then -111. Overall, laminin-511 and -521 showed the best results in this study and performed better than a general laminin substrate that studies mostly use.

2. Introduction

With the aging of the western world the prevalence of neurodegenerative diseases such as Parkinson, Alzheimer, Ataxia and Huntington increases. Neurodegenerative diseases are characterized by the progressive atrophy of neuronal cells (Nopoulos 2016; Portugal et al. 2013). Neuronal atrophy takes place in the substantia nigra, hippocampus, cerebellum and striatum in the case of Parkinson, Alzheimer, Ataxia and Huntington respectively. The loss of neurons in these diseases cause chronic cognitive and motor disabilities in patients. Mature neurons lack the ability to self-regenerate making it impossible for the human body to replace the lost neurons on its own (Hsu et al. 2013). Neurodegenerative tissue shows a chronic immune activation and inflammation (Amor et al. 2010). Which eventually does more harm than good by causing neuronal damage and stimulates the reappearance of the original trigger and this way creates a vicious cycle (Amor et al. 2010). The loss of neurons leads to the following symptoms: hyper- and hypokinesia, tremors, dystonia, bradykinesia, eye movement abnormalities, clumsiness, sensory neuropathy, loss of executive functioning, dementia and loss of social etiquettes (Akbar and Ashizawa 2014; Nopoulos 2016; Portugal et al. 2013). To date there is no effective treatment to cure, improve the overall clinical output or stop the progression of neurodegenerative diseases. Pharmacological therapy is the golden standard to treat neurodegenerative diseases at the moment (Portugal et al. 2013). However, the medicines used have adverse effects on the well-being of patients, are costly and only show short-term clinical effects which decline over time (Portugal et al. 2013; Yu et al. 2014). As told above the hallmark of neurodegenerative diseases is the loss of neurons over time. So, what if these neurons can be replaced with new ones? In theory at least, the clinical condition of the patients should improve and the progression of the disease should decrease. The development of regenerative medicine gives the medical world the strategies it needs to test this theory. In fact it has already been tested and shown to improve neuronal regeneration and the clinical outcome of mice suffering from neuronal lesions or traumas (Frick et al. 2017; Hsu et al. 2013; Koh et al. 2008; Seo et al. 2013; Tate et al. 2009). However, there is still a long road ahead in the research and development of regenerative medicine before it can be clinical tested in patients.

In general, the quality of the end product of regenerative medicine, which is the restored, functional tissue, depends mainly on three factors, namely the type and quality of the cells, the medium and its soluble factors and the substrate or scaffold used. To improve the quality of the end product all three factors need to be optimized. In recent years, research has evidence that the properties of the extracellular environment influences cell survivability, proliferation, differentiation and morphology to a great extend (Powell and Kleinman 1997; Sur et al. 2014; Tavakol et al. 2015). The extracellular environment of neurons includes the extracellular matrix (ECM) which is build out of connective tissue such as collagen, laminin, fibronectin and perlecan (figure 1) (Arends and Lieleg 2016). Out of these ECM molecules especially laminin has shown to influence cell fate and behaviour to a large extend (Ichikawa-Tomikawa et al. 2012; Li et al. 2012). It is well-known that laminin is an important molecule during embryonic and post-natal brain development. However, it is not clear whether laminin is also present in the adult brain. If it is present than laminin will be an important molecule that supports neurons throughout the life of an organism. This will make laminin a promising substrate for neuronal cell cultures. At the first part of this research we found that laminin is present in the adult cerebella in mice. However, just being present during development and in adulthood does not make a molecule a good neuronal cell culture substrate. More knowledge about the molecule and the effect on neurons needs to be attained. The laminin family represents 20 isoforms known so far with specific chemistries and presumably functionalities. To date, most studies, successfully, used a

general laminin substrate derived from mouse sarcoma in neuronal cell culture studies. It is not known which isoforms these substrates contain and in which concentrations. This makes it hard to interpret research outcomes. At the moment eight pure human recombinant laminin isoforms are on the market (i.e. laminin-111, -121, -211, -221, -411, -421, -511 and -521). How these laminin isoforms affect cerebellar progenitor cells is not known. Therefore the focus of this paper will be on these laminin isoforms as a substrate for neuronal cell culture.

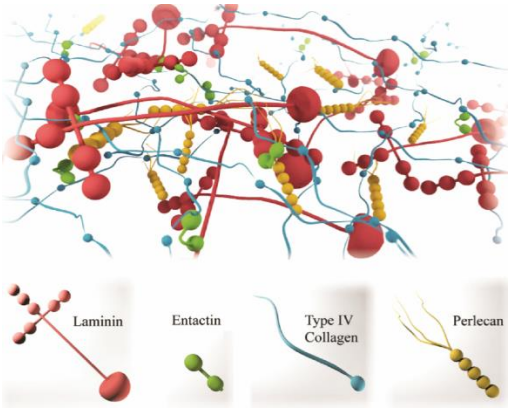

Figure 1: Schematic overview of the extracellular matrix, containing laminin, entactin, collagen and perlecan (Arends and Lieleg 2016)

Laminin is a common cruciform shaped glycoprotein (figure 2) (Colognato and Yurchenco 2000; Li et al. 2012; Porcionatto 2006). It exists out of three laminin subunits, namely an α, β and γ chain. This makes laminin a heterotrimeric molecule (figure 2) (Armony et al. 2016; Colognato and Yurchenco 2000; Fusaoka-Nishioka et al. 2011; Ichikawa-Tomikawa et al. 2012; Li et al. 2012; Porchionatto 2006; Powell and Kleinman 1997). Currently, there are five known α, four β and three γ laminin chains and together they make up the 20 known laminin isoforms (table 1) (Yao 2016). The laminin molecule contains several active sites through which it interacts with other molecules or cells (figure 2) (Luckenbill-Edds 1997; Moran et al. 2015; Powell and Kleinman 1997). The tail of the coiled coil structure of the laminin molecule contains the globular (LG) domains (Armony et al. 2016; Moran et al. 2015; Yao 2016). In general, the LG domains bind to integrins found on the cell surface and are associated with cell adhesion, neurite outgrowth, spreading and differentiation (Barczyk et al. 2009; Carafoli et al. 2009; Colognato and Yurchenco 2000 and Mecham 1991). At the tip of each side-chain the LN domains are found. These affect network formation with other ECM molecules (Moran et al. 2015 and Yao 2016).

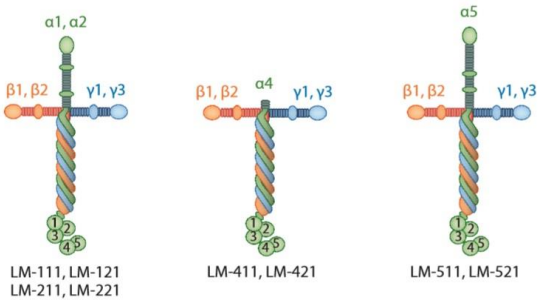

Figure 2: Structural model of laminin-111, -121, -211, -221, -411, -421, -511 and -521. The 1-5 circles at the bottom are the 5 LG-domains (Domogatskaya and collegues 2012)

Table 1: The known laminin isoforms. The left column represents the old name for the molecules and the right column the new abbreviations (Yao 2016).

Name	Abbreviation
Laminin-α1β1γ1	Laminin-111
Laminin-α2β1γ1	Laminin-211
Laminin-α1β2γ1	Laminin-121
Laminin-α2β2γ1	Laminin-221
Laminin-α3(A)β3γ2	Laminin-3(A)32
Laminin-α3Bβ3γ2	Laminin-3B32
Laminin-α3β1γ1	Laminin-311
Laminin-α3β2γ1	Laminin-321
Laminin-α4β1γ1	Laminin-411
Laminin-α4β2γ1	Laminin-421
Laminin-α5β1γ1	Laminin-511
Laminin-α5β2γ1	Laminin-521
Laminin-α2β1γ3	Laminin-213
Laminin-α3β2γ3	Laminin-323
Laminin-α4β2γ3	Laminin-423
Laminin-α5β2γ3	Laminin-523
Laminin- α2β1γ2	Laminin-212
Laminin- α2β2γ2	Laminin-222
Laminin- α3β3γ3	Laminin-333
Laminin- α5β2γ2	Laminin-522

In general, laminin affects cellular behaviour by binding to transmembrane receptors such as integrins and dystroclycans (Hall et al. 2003; Mills et al. 2006; Qu and Smith 2004 and Tavakol et al. 2016). Laminin-integrin binding affects cellular behaviour by recruiting cytoplasmic proteins which connect the cytoskeleton to integrins (Mills et al. 2006). An example of such a protein is integrin linked kinase (ILK) (Mills et al. 2006). In turn ILK triggers proliferation by activating glycogen synthase kinase 3β and protein kinase (Mills et al. 2006). Another example is the activation of ERK, JNK and PI3K/AKT signalling pathways upon laminin-integrin binding which in turn affects cell survival, proliferation and differentiation. Dystroclycans work in a similar manner as intergins. In general laminin binding to cell surface receptors alters cellular behaviour by activating signalling pathways that in turn elicit changes in gene expression or cytoskeleton arrangements.

In recent years, numerous studies have focused on laminin as neuronal substrate. The results of these studies show that laminin affects neuronal differentiation, migration, neurite outgrowth, adhesion, regrowth, survivability, dendritic sprouting and myelination of several cell lines, such as: cerebellar neurons, hippocampal cells, spinal cord neurons, the PC12 cell line originating from the adrenal medulla, skin derived precursor cells and neural stem cells (Czeisler et al. 2016; Freire et al. 2002; Frick et al. 2017; Fusaoka- Nishioka et al. 2011; Harris et al. 2016; Hsu et al. 2013; Huang et al. 2011; Koh et al. 2008; Lei et al. 2012; Martinez-Ramos et al. 2007; Porcionatto 2006;

Seo et al. 2013; Sur et al. 2014; Tate et al. 2009; Xing et al. 2010). However, most of the studies performed so far used laminin in general as a substrate for neuronal cell culture. This means that they used an unknown combination of laminin isoforms originating from mice sarcoma as substrate, such as laminin L2020 (Sigma-Aldrich) (Fong et al. 2015). If laminin in general was not used than laminin-111 was considered (Fusaoka-Nishioka et al. 2011; Harris et al. 2016; Kirkeby et al. 2016; Nael et al. 2009; Powell and Kleinmann 1997; Seil et al. 2014; Sur et al. 2014), with a few studies also found on laminin-211 on neuronal cell cultures (Powel et al. 1998; Seo et al. 2013). Comparative studies on the effects of various laminin isoforms are very rare and include only few different isoforms. Nevertheless, the results indicate differential effects of the investigated laminin isoforms. For example, Fusaoka-Nishioka and colleagues (2011) studied and compared the effects of laminin-111, -211, -411 and -511 on neurite outgrowth of hippocampal neurons. They found that laminin-511 increased neurite outgrowth the most and laminin-411 affected neurite outgrowth the least. However, from the 20 known isoforms only four were tested with regard to the effect on, neurite outgrowth only of a specific type of neurons, namely hippocampal neurons. Because this study was specifically focused on hippocampal neurons the results cannot be extrapolated to other type of neuronal cells, such as cerebellar progenitor cells. In this study eight different human recombinant laminin isoforms (i.e. laminin-111, -121, -211, -221, -411, -421, -511 and -521) will be tested as a substrate. This study will use cerebellar progenitor cells originating from mice. The ability of the laminin substrates to stimulate cell attachment, neuronal differentiation, neurite outgrowth and synaptogenesis will be assessed and compared with each other and laminin-L2020.

3. Materials/methods

3.1 Preliminary research

Before laminin was used as a substrate in this study the distribution of laminin in a postnatal and adult mice cerebellum was assessed via immunofluorescence techniques. This was done because laminin is known to be an important glycoprotein during the embryonic development of the nervous system, but a lot less is known about the presence and function of laminin during the postnatal and adult stages. If laminin is also present in post-natal and adult cerebella then laminin would be a glycoprotein that not only plays a role during early development, but is important throughout the entire lifespan of a vertebrate. During this experiment, several coupes belonging to two mice in each of the following categories: post-natal day 1 (P1), postnatal day 7 (P7), postnatal day (P21) and adult, were stained with calbindin-D 28k (Sigma), DAPI and either anti-laminin (Abcam, 11575) or anti-laminin (Sigma L9393). The results show that laminin is present in the pial surface, basement membrane of blood vessels, the molecular layer and the internal granule layer in all cerebella examined (figure 3). Figure 3 also suggests that laminin is present inside Purkinje neurons, but a free absorption specificity test shows this to be untruth. These results suggest that laminin is not only important during early development but also during post-natal development and in adulthood. This means that laminin as a substrate has the potential to positively influence neuronal development throughout different developmental stages. In the appendix this experiment is more thoroughly explained and discussed.

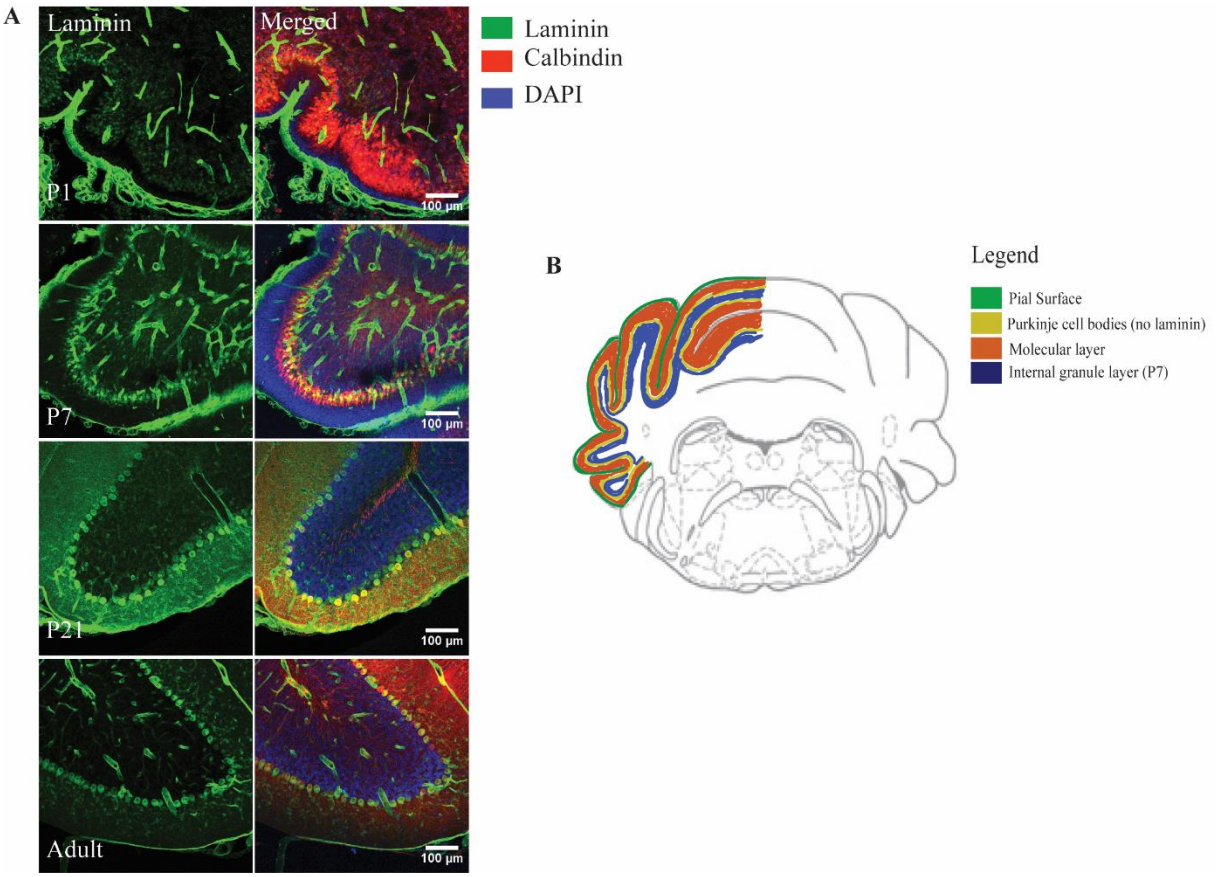

Figure 3: A shows microscopic images of stained cerebella belonging to the P1, P7, P21 and adult mice. Green represents laminin, red calbindin and blue DAPI. B is a schematic overview of the places where laminin was present in the cerebellum.

3.2 Cells used

Cerebellar progenitor cells were derived from mouse embryos and differentiated into neuro progenitor cells (NPCs) and frozen in liquid nitrogen according to the method described in (Higuera et al. 2017). Eleven separate frozen NPC vials were used in this study belonging to four different cell lines. Belonging to a different cell line means that the cells were extracted from different wild type mice. Two NPC vials belonging to two different cell lines were used during the screening of the following laminin isoforms (-111, -121, -211, -221, -411, -421, -511 and -521). Another two cell vials belonging to two different cell lines were used to further investigate the behavior of neurons on laminin-111, -121, -421, -511 and -521 after 5 days of culture. Four different cell vials belonging to four different cell lines were used to investigate cell attachment, differentiation and neurite outgrowth of laminin-111, -121, -421, -511 and -521 after 10 days of culture. Finally, two different cell vials belonging to two different cell lines were used to asses neuronal connectivity after 10 days of culture on laminin-111, -511 and -521. In all cases neurons were also cultured and examined when seeded on a generic laminin isoform (L2020, Sigma-Aldrich).

3.3 Experimental groups

In this study, 13 mm glass coverslips (Assitent, Germany) were coated with Poly-$_L$-Ornithine (PLO) (Sigma-Aldrich) and with one of the eight individual laminin isoforms (BioLamnina) or with laminin L2020 (Sigma-

Aldrich). The eight laminin isoforms coming from BioLamina are human recombinant laminin isoforms. Laminin L2020 is a laminin solution containing several unknown laminin isoforms derived from mice sarcoma. In the past, laminin L2020 has successfully been used as a substrate for neuronal cell culture studies, in this study it was used as a positive control. The study started with a screening of the eight different laminin isoforms used as substrate to culture neurons on for five days, these include the following isoforms: laminin-111, -121, -211, -221, -411, -421, -511 and -521. Figure 2 shows a schematic image of all included isoforms. There are some chemical differences between these eight isoforms which makes it likely that they will have differential effects on neuronal cell cultures. For example, the α4 chain had a shorter arm and has the lowest affinity for integrins compared to the other α chains included in this study (Domogatskaya et al. 2012). Laminins that contain the β2 chain have a higher affinity for integrins compared to a β1 chain (Domogatskaya et al. 2012). Furthermore, laminins which have an α1 or α5 chain can bind to intergins on both ends of the molecule which makes them efficient cell communicators (Domogatskaya et al. 2012). In the discussion section these differences will be further discussed. The PLO only condition is used during the screening to compare the results attained while using a laminin or non-laminin substrate.

In the screening, two coverslips were coated with each substrate (all eight isoforms, laminin L2020 and the PLO only condition). The screening was used to see which laminin isoforms would be used in future experiments. When the percentage of neurons compared to the total amount of attached cells and the mean total length of neurites cultures on the laminin isoforms closely resembled those of the PLO only condition they were excluded from the other experiments. This was the case for laminin-211, -221 and -411 (see result section). This means that laminin-111, -121, -421, -511 and 521, as well as laminin L2020 were used in the remainder of the experiments (table 2). Table 2 shows which isoform was used for which experiments.

To expand the neuronal differentiation, neurite outgrowth and cell attachment data after 5 days of cell culture another two cell vials were used to culture neurons on laminin-111, -121, -421, -511 and -521 as well as on laminin L2020 (table 2). Four cell vials were used to test for neurite outgrowth, cell attachment and neuronal differentiation after 10 days of culture (table 2) on coverslips coated with laminin-111, -121, -421,- 511, -521 and L2020. The three laminin isoforms that showed the highest neurite outgrowth were selected to test for neuronal connectivity after ten days of culture. These isoforms were laminin-111, -511 and -521 (table 2). Laminin L2020 was also included in this experiment because it is the control condition. Two cell vials were used in the connectivity experiment.

Table 2: This table shows which laminin isoforms were used for which experiments and how many coverslips were coated. Furthermore, it shows how many frozen cell vials were used.

Substrates	Screening (5 days)	Cell attachment, neuronal differentiation and neurite outgrowth (5 and 10 days)	Neuronal connectivity (10 days)
Laminin-111	2 coverslips, 2 cell vials	4 coverslips, 4 cell vials	3 coverslips, 2 cell vials
Laminin-121	2 coverslips, 2 cell vials	4 coverslips, 4 cell vials	-
Laminin-211	2 coverslips, 2 cell vials	-	-
Laminin-221	2 coverslips, 2 cell vials	-	-
Laminin-411	2 coverslips, 2 cell vials	-	-
Laminin-421	2 coverslips, 2 cell vials	4 coverslips, 4 cell vials	-
Laminin-511	2 coverslips, 2 cell vials	4 coverslips, 4 cell vials	3 coverslips, 2 cell vials
Laminin-521	2 coverslips, 2 cell vials	4 coverslips, 4 cell vials	3 coverslips, 2 cell vials
Laminin L2020	2 coverslips, 2 cell vials	4 coverslips, 4 cell vials	3 coverslips, 2 cell vials
PLO only	2 coverslips, 2 cell vials	-	-

3.4 Substrate preparation

First, the glass coverslips were sterilized for fifteen minutes using a 70% ethanol solution. Afterwards the coverslips were rinsed three times fifteen minutes using deionized water. After the sterilization process the coverslips were placed in individual wells of a 24-well plate and coated with a 0.01% PLO solution for 45 minutes on room temperature. Next, excess PLO was removed from the coverslips and the coverslips were rinsed three times using deionized water. The substrate preparation for the PLO only condition is now finished. To finish the laminin L2020 substrate, the coverslips were submerged in a 50 µg/ml laminin L2020 solution for two hours in a 37̊ degrees Celsius incubator. The coverslips belonging to the experimental groups were coated using 5 µg/ml of a certain laminin isoform for two hours in a 37̊ degrees Celsius incubator. The coating concentrations for the laminin isoforms was assessed during a preliminary experiment in which a dilution of 2.5 µg/ml, 5 µg/ml and 10 µg/ml was used to coat coverslips and culture cerebellar progenitor neurons on. BioLamina recommended a dilution of 5 µg/ml this was used as a point of reference. After 15 days of neuronal culture a dilution of 5 µg/ml showed the best results and thus was used in this experiment.

3.5 Cell culture methodology

3.5.1 NPC proliferation (preculture)

After reaching the NPC state according to the methodology described in (Higuera et al. 2017) the NPCs are frozen and stored in liquid nitrogen using Dimethyl Sulfoxide (DMSO; Sigma-Aldrich). Before the frozen NPCs can differentiate they need to proliferate into a sufficient amount of NPCs, which respond to an 80% confluency on a 10 cm^2 well. This is done by culturing the NPCs on a laminin L2020 substrate using a NPC medium that promotes proliferation but inhibits differentiation. Before the NPCs are collected from the storage, three 10 cm^2

wells of a 6-well plate (Greiner Bio-One) are coated using a 50 µg/ml laminin L2020 solution for 45 minutes on 37° degrees Celsius in an incubator for each batch of frozen NPCs. During this time, the NPC medium and deactivating serum were prepared. The deactivating serum needs to deactivate the DMSO that is frozen with the NPCs. The NPC medium consists out of DMEN/F12 (Gibco), N2 supplement (1%, Invitrogen), B27-RA supplement (2%, Invitrogen), Laminin L2020 (1 µg/ml, Sigma-Aldrich), penicillin/ streptomycin (1%, Sigma-Aldrich) and mouse BFGF (20ng/ml Invitrogen) and the deactivating serum contains: DMEN/F12 (Gibco) and 15% foetal bovine serum (FBS). The deactivating serum is made in a 15 ml Falcon tube. After the NPC medium and deactivating serum are finished they are kept in a 37° degrees Celsius warm water bath. After 35 minutes of coating the NPCs are taken from their storage and transferred in dry ice to the cell culture lab. Next, the cells are thawed in a 37° degrees Celsius warm water bath. When the NPC, DMSO solution is almost completely thawed and only a small floating ice crystal can be seen (which is the frozen DMSO) the cells are collected using a 5 ml pipet and transferred into the warm deactivating serum. Next, the deactivating serum containing the NPCs are placed in a 22° degrees Celsius centrifuge on 300 g's for 5 minutes. In the meantime the laminin coating is removed from the well plates and 1.9 ml NPC medium is added to each plate. After 5 minutes in the centrifuge the NPCs have sunken to the bottom of the Falcon tube. Now the deactivating serum is carefully removed from the tube using a suction system until only the NPCs with a little bit of serum is left at the bottom of the tube. Depending on the thickness of the NPC substrates, the NPCs are split in either two or three 10 cm^2 wells. This is done by adding 0.1 ml NPC medium to the NPC suspension for each well plate. After adding the NPC medium to the suspension a 200 µl pipet is used to gently pipet the suspension up and down 6 times to break the cell palate and mix the NPCs in the solution. In the final step 0.1 ml NPC medium with the NPCs is added to each well. Every second day the NPC medium is refreshed with new NPC medium until an 80% confluency is reached.

3.5.2 Neuronal differentiation and culture

When the NPC cultures are 80% confluent, usually after two to four days of culture, they are ready to be seeded on coated 13 mm glass coverslips and differentiate into healthy neurons (figure 4). Each 10 cm^2 NPC containing well is split into three 13 mm coated glass coverslips (figure 4). The medium used for neuronal differentiation and culture is the NS-21 medium. The NS-21 medium contains primary neural basal medium (Lonza), GS21 (2%, Global Stem), glutamax (1%, Gibco) and gentamycin (0.5 µg/ml, Gibco) and is pre-heated in a 37° degrees warm water bath before use. After preparing the NS-21 medium, the NPC medium is removed from the wells and are rinsed once using DPBS. Next, the NPCs are detached from the wells using 0.25%, 37° degrees Celsius, trypsin-EDTA for 5 minutes in a 37° degrees Celsius incubator. After detachment the NPCs are collected in pre-heated 37° degrees Celsius deactivating serum using a 5 ml pipet. Now, the deactivating serum containing the NPCs are placed in a 22 ° degrees Celsius 300 g's centrifuge for 5 minutes. In the meantime the already coated coverslips (as described in section 3.3) are submerged in 300 µl NS-21 medium. After 5 minutes in the incubator the deactivating serum is removed from the falcon tube until only the cells and a little bit of liquid is left. Now, 300 µl of NS-21 medium is added to the suspended cells and with a 200 µl pipet the cells are mixed in the NS-21 medium by pipetting up and down six times. Next, 100 µl of NS-21, NPC solution is added to each 13 mm coated coverslip and the well plates are stored in an incubator. After one hour the NS-21 medium is removed and 600 µl of new NS-21 medium is added. This is done to remove excess cells from the solution that did not attach to the coverslips. From this point onwards the cells are kept in the incubator and cultured for either 5 or 10 days (figure

4). Every second day the NS-21 medium is refreshed by new NS-21 medium. From the second day onwards, 0,001% cytosine β-D-arabinofuranoside (4 µM, Ara-C, Sigma-Aldrich) is added to the NS-21 medium (figure 4). Ara-C inhibits differentiation into glial cells. At day 5 or 10 days the coverslips are placed in a 4% paraformaldehyde (PFA) solution for 15 minutes and afterwards rinsed three times using 0.1 M PB (figure 4).

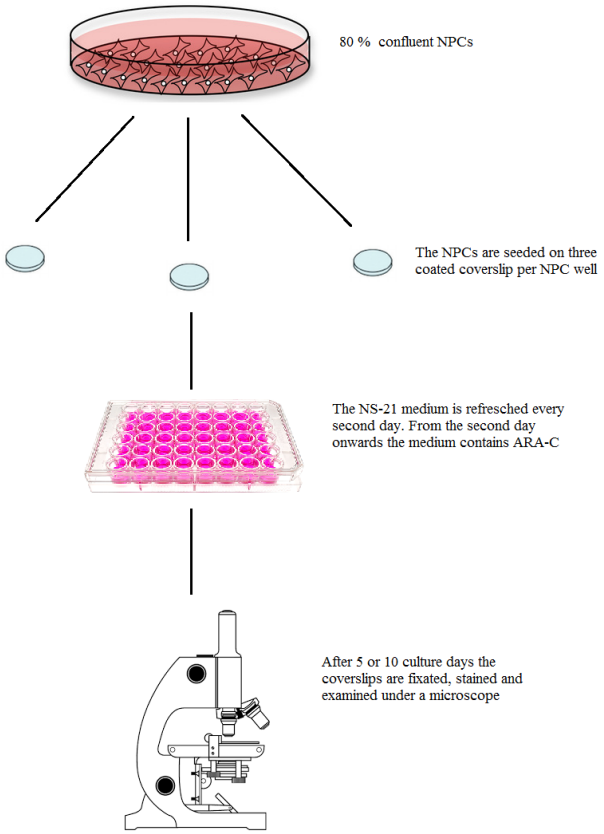

Figure 4: General procedure from 80% confluent NPCs until microscopic analysis.

3.6 Immunofluorescence

To study the neurons after 5 or 10 days of culture immunofluorescence was used (figure 4). The PFA treated coverslips are rinsed three times using PBS. Next, the coverslips were placed in a primary antibody solution overnight. The basis of the solution contains; 2% horse serum, 4% triton and PBS and either TUJ1 (1:2000 rabbit; Abcam) and synaptophysin (1:200 mouse; Sigma-Aldrich) or just TUJ1 (1:2000 rabbit; Abcam). The next day, the coverslips are rinsed three times using PBS. After rinsing the coverslips, they are placed in the secondary anti-body solution overnight. The secondary anti-body solution contains; 2% horse serum, 4% triton, PBS, Cy3 (1:200, Dako) and Cy5 (1:200, Dako). After the secondary anti-body treatment the coverslips are rinsed twice with PBS and once with PB (0.l M). Afterwards, the nuclei of the cells are stained using a 1:10.000 DAPI solution for 10 minutes. After rinsing the coverslips twice with PB (0.1 M), they are mounted on glass using mowiol and nail polish.

3.7 Imaging and quantification

A fluorescence microscope was used to examine all coverslips (figure 4). Using the fluorescence microscope six pictures were taken on six different locations from each coverslip on a ten times magnification. These six pictures per coverslip were later used to asses cell attachment and neuronal differentiation. To asses' synaptogenesis and neurite outgrowth four pictures were taken from each coverslip belonging to the 10 culture day conditions using a confocal microscope (LSM-700 Zeis) (figure 4). To asses neurite outgrowth a twenty times magnification lens was used for the 5 culture day conditions and a 10 times magnification lens for the 10 culture day conditions. In the case of connectivity a 40 time's magnification lens was used. From these four pictures 10 neurons were analyzed on their neurite outgrowth and 6 neurons on their connectivity.

3.7.1 Cell attachment and neuronal differentiation

In this paper cell attachment is defined as the total number of DAPI stained nuclei per square millimeter. This is calculated by counting all the DAPI stained cells in each picture belonging to a coverslip. The area of the picture is known. Next, an average over the total amount of DAPI stained nuclei of all six pictures is taken. Lastly, the average amount of nuclei per predefined area of a certain coverslip is transformed to the average amount of nuclei per square millimeter. This final number represent the cell attachment for a coverslip.

Neuronal differentiation is defined as the percentage of neurons to the total amount of attached cells. This is calculated by assessing the percentage of neurons in all of the six pictures belonging to a certain coverslip. Next, the average is taken over the six pictures. The final number represents the neuronal differentiation for a certain coverslip.

3.7.2 Neurite outgrowth

Neurite outgrowth is defined as the total neurite length of a neuron. Neurite outgrowth is measured using the Synapse Detection (Syn-D) Tool of Matlab (Schmitz et al. 2011). After loading a RGB image into the Syn-D tool the channels and resolution are specified. Afterwards, the tool detects somas by first reducing the noise of the image using a 2D adaptive Wiener filter (Schmitz et al. 2011). Next, thresholding is used to separate background from foreground (Schmitz et al. 2011). To detect soma's the program searches for places on the specified channel where neurites are connected by a disc like structure (Schmitz et al. 2011). If the tool could not find all the soma's or counted other structures as soma's they were manually selected or deleted respectively. Next, neurites are detected by following bright ridges surrounded by a dark background (Schmitz et al. 2011). The tool starts at the detected soma and traces the neurites using local criteria (Schmitz et al. 2011). The neurites were manually edited if needed. The paper of Schmitz and colleagues (2011) provides an extensive explanation and guide. Using this tool the total neurite length of 10 neurons per coverslip is calculated. This results in 40 neurons examined for each coverslips after both the 5 and 10 culture day conditions.

3.7.3 Connectivity

Connectivity is defined as the amount of synapses per µm neurite length found on a neuron. This is also measured using the Synapse Detection tool of Matlab (Schmits et al. 2011). As described above, the channels on a loaded picture are specified. This way the program knows which channel belongs to the synaptophysin. Synapses can be recognized as bright regions. To identify synapses the program first searches for possible synapses by thresholding the image (Schmitz et al. 2011). A possible synapse was identified as being one standard deviation above the mean intensity of the synapse channel (Schmitz et al. 2011). Synapses that are detected more than 1 µm away from a neurite are excluded (Schmitz et al. 2011). Furthermore, if the area belonging to a possible synapse is smaller than 0.35 μm^2 it is considered noise (Schmitz et al. 2011). In order to distinguish one synapse from the other the program looks for regions with unique local intensity maxima's in the channel (Schmitz et al. 2011). These regions are averaged to get a typical synapse kernel (Schmitz et al. 2011). Next, to separate individual synapses from each other the image is deconvolved with the obtained single synapse kernel (Schmitz et al. 2011). In the paper of Schmitz and colleagues (2011) and extensive explanation and guide is found. This tool calculates the amount of synapses found on neurons. Per coverslip 6 neurons are examined and their amount of synapses are calculated. Next, the amount of synapses found on a neuron is normalized to its neurite length. This is done because, not all neurons fitted completely on an image taken by the microscope. In total 18 neurons are examined per condition were examined.

3.8 Statistics

Statistics is used to look for differences on cell attachment, neuronal differentiation, neurite outgrowth and connectivity between culture conditions. Before testing for differences in the data, the assumptions for normality and equality of variance is tested via the Shapiro-Wilk and Levene's test respectively. If the assumption for normality is not met a logarithmic transformation is used. If the assumption for equality of variance is violated the Brown test in combination with Games-Howell is used. Otherwise just the normal ANOVA is used. A Factorial ANOVA was used to look at the differences on cell attachment, neuronal differentiation and neurite outgrowth between culture days of each group. In this study a significant difference is defined as having a p-value > 0.05.

4. Results

In this section the results regarding the screening test, cell attachment, neuronal differentiation, neurite outgrowth and connectivity will be presented.

4.1 Screening

The results of the screening is meant to determine which laminin isoforms would be further tested. This was done by comparing the results attained with a non-laminin substrate (PLO only condition). Laminin isoforms that would be included in further experiments need to perform better than the PLO only condition on neuronal differentiation and neurite outgrowth. Because the sample size for neuronal differentiation is just

two in this case a significant difference between the PLO condition and the laminin isoforms cannot be found. Therefore, whether the laminin isoforms differs from the PLO only condition will be judged visually based on the bar chart of figure 5 and table 3. In the case of neurite outgrowth, the sample size is big enough to determine whether the neurite length differs significantly between the PLO only condition and the laminin isoforms. If they do not differ significantly from the PLO only condition the laminin isoforms will be excluded. Figure 5A shows a confocal microscope picture of each condition included in the screening. Figure 5A shows that neuronal differentiation and neurite outgrowth of laminin-211, -221 and -411 are lower compared to the other isoforms. The same can be seen in table 3. This becomes even clearer in figure 5B and C which show a bar chart of the mean neuronal differentiation and outgrowth of every condition. Figure 5B and table 3 show that the neuronal differentiation of the PLO and laminin-211, -221 and -411 conditions are about equal. The same goes for neurite outgrowth (figure 5C).

A one-way ANOVA shows a significant effect between the different substrates used and neurite length F (9, 182) = 9.971, p <0.001. A Post hoc test shows no significant difference between the PLO only condition and laminin-211, -221 and -411, p = 1.000, p = 0.904 and p = 1.000 respectively. Combining these results with the fact that figure 5B shows no real difference in neuronal differentiation between the PLO only condition and laminin-211, -221 and -411 these three laminin isoforms are excluded from the remaining experiments.

*Table 3: This table shows the results of the screening tests. The table shows the mean neuronal differentiation and neurite length data ± the standard error. * shows the substrate that preformed the best and ** shows the substrates that did not differ from the PLO only condition*

Substrate	**Screening** **Neuronal differentiation**	**Screening** **Neurite length**
Laminin-111	*M = 29.5 ± 7.0% **	*M = 390.441 ± 45.232µm*
Laminin-121	*M = 16.6 ± 8.2%*	*M = 368.259 ± 37.630 µm*
Laminin-211	*M = 7.8 ± 1.9% ***	*M = 188.990 ± 16.126 µm ***
Laminin-221	*M = 11.5 ± 5.9% ***	*M = 256.031 ± 37.694 µm ***
Laminin-411	*M = 8.9 ± 4.4% ***	*M = 221.076 ± 35.554 µm ***
Laminin-421	*M = 22.9 ± 5.1%*	*M = 439.155 ± 45.454 µm*
Laminin-511	*M = 21.6 ± 6.3%*	*M = 412.105 ± 33.251 µm*
Laminin-521	*M = 23.2 ± 5.1%*	*M = 501.328 ± 64.593 µm **
L2020	*M = 23.8 ± 10.4%*	*M = 359.451 ± 56.696 µm*
PLO	*M = 9.9 ± 6.0%*	*M = 195.374 ± 19.380 µm*

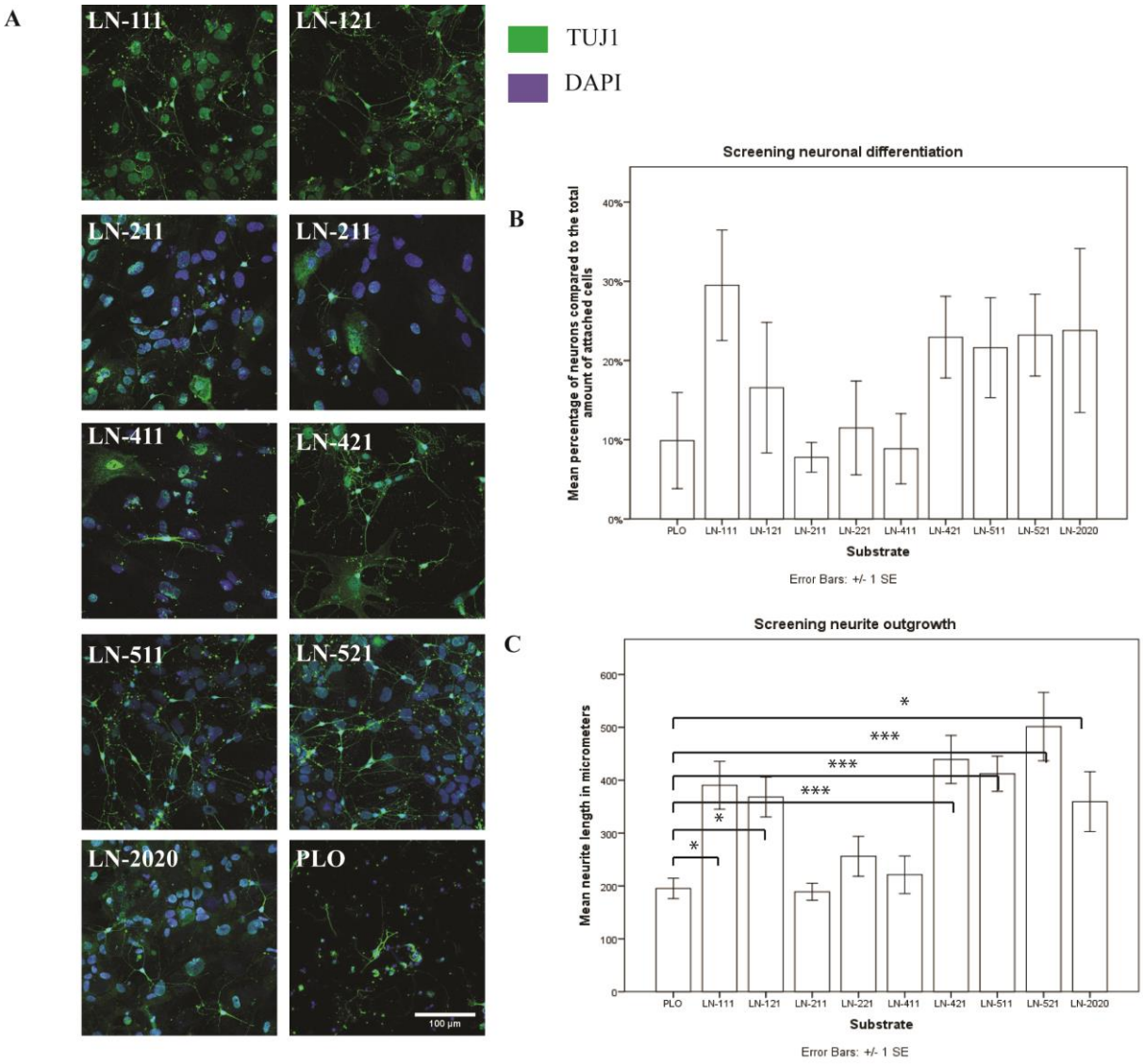

*Figure 5: As a whole figure 5 shows the results belonging to the screening tests. Figure 5A shows a confocal microscopic picture of each condition after 5 culture days. TUJ1 is shown in green and DAPI is shown with a blue color. Figure 5B shows the mean percentage of neuronal differentiation compared to the total amount of attached cells of each condition. Figure 5C shows the mean neurite length of each condition. * Means p < 0.05, ** means p < 0.01 and *** mean p < 0.001*

4.2 Cell attachment and neuronal differentiation

This section will present the results regarding the cell attachment and neuronal differentiation experiments after 5 and 10 culture days as well as the difference in cell attachment and neuronal differentiation between the different culture times.

Figure 6A shows microscopic images of all conditions after 5 and 10 culture days. The first row of figure 6A shows all conditions after 5 days and are taken with a 20x magnifying lens. The second row shows the same for 10 culture days and with a magnification of 10x. Figure 6B shows bar charts of cell attachment and neuronal differentiation after 5 and 10 culture days. The last column of figure 6B shows the cell attachment and neuronal differentiation of both culture times next to each other.

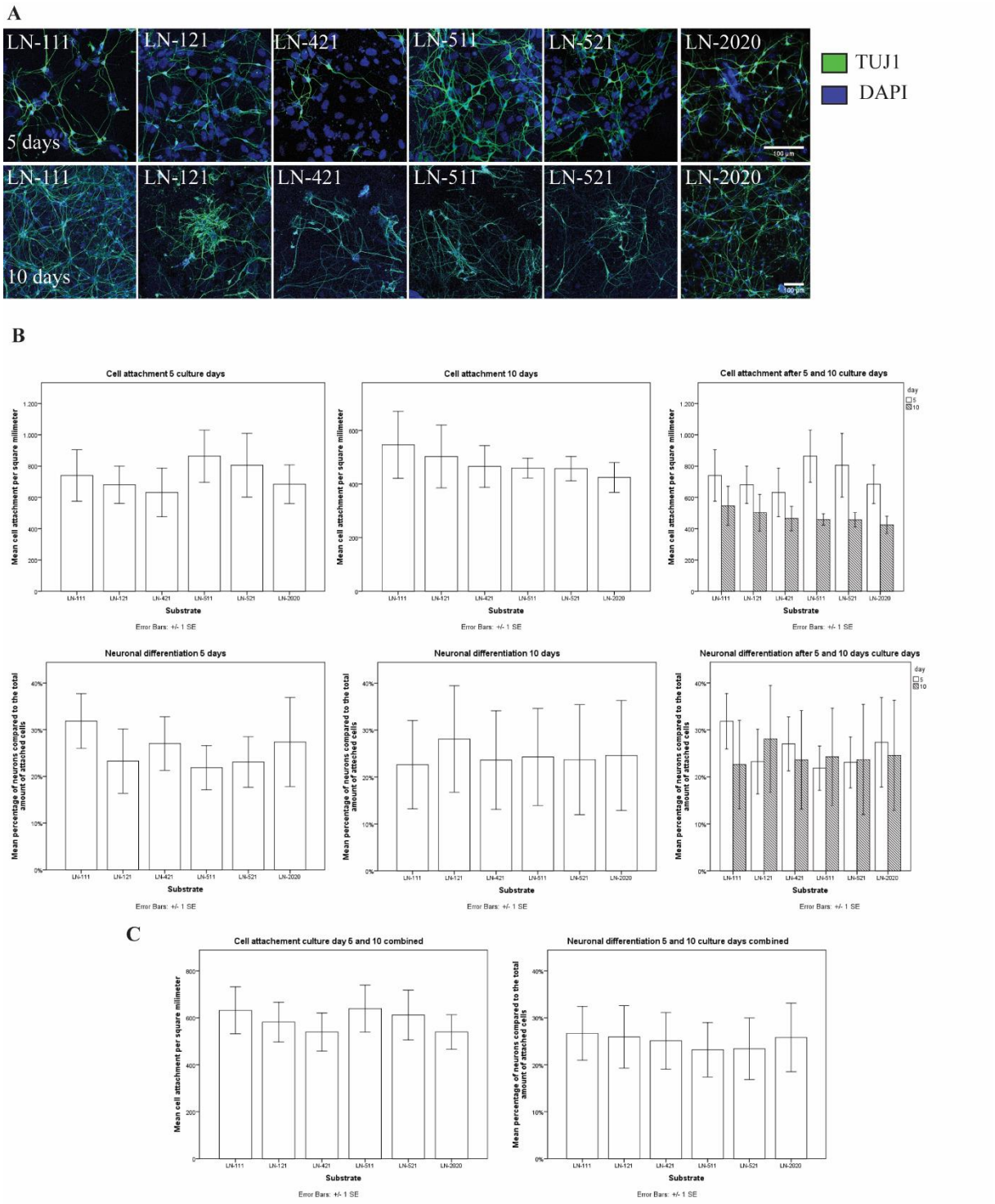

Figure 6: A shows microscopic images of all conditions after 5 and 10 days of culture. The green color represents TUJ1 and DAPI is depicted in blue. Figure 6B shows bar charts of cell attachment and neuronal differentiation after both 5 and 10 days of neuronal culture. The last column of figure 6B shows the cell attachment and neuronal differentiation after 5 and 10 days in one chart. Figure 6C shows the results for cell attachment and neuronal differentiation when the data of 5 and 10 cultures days is combined

From the first two columns of figure 6B and table 4 it can be seen that cell attachment and neuronal differentiation do not really differ between different conditions after both 5 and 10 days of culture. After 5 days the total amount of cells attached to the substrates was the largest for laminin-511 closely followed by laminin-521 and the smallest for laminin-421 (figure 6B and table 4). After 10 days laminin-111 was the most

populated substrate and laminin L2020 the least (figure 6B and table 4). After 5 culture days, the substrate used does not influence both neuronal differentiation and cell attachment, F (5, 18) = 0.324, p = 0.892 and F (5, 18) = 0.302, p = 0.905 respectively. The same is true after 10 culture days, F (5, 24) = 0.030, p = 0.999 for neuronal differentiation and F (5, 24) = 0.259, p = 0.931 for cell attachment. Furthermore, the substrate used does not affect cell attachment and neuronal differentiation when the data belonging to culture day 5 and 10 is combined, F (5,48) = 0.234, p = 0.946 and F (5,48) = 0.05, p = 0.998 respectively.

The last column of figure 6B compares cell attachment and neuronal differentiation between culture times. Close inspection does not show a difference in neuronal differentiations between 5 and 10 days of culture. However, it looks like there might be a difference in the number of attached cells after 5 and 10 days of culture. Statistical analyzes (Factorial ANOVA) indeed shows no significant difference for neuronal differentiation between 5 and 10 days of culture, F (1, 42) = 21.184, p = 0.821. There is a significant difference to be found in the amount of attached cells between 5 and 10 days of culture, F (1, 42) = 14.174, p = 0.001.

*Table 4: shows the results belonging to the cell attachment and neuronal differentiation experiments. The results are shown with their corresponding standard error. Everything depicted with a * had the biggest effect on either cell attachment or differentiation and everything depicted with ** the lowest*

Substrate	Attachment (5 days)	Attachment (10 days)	Attachment (combined)	Differentiation (5 days)	Differentiation (10 days)	Differentiation (combined)
LN-111	*M = 739.897 ± 164.711*	*M = 546.146 ± 124.794 **	*M = 632.258 ± 100.031*	*M = 31.8 ± 5.9% **	*M = 22.6 ± 9.4% ***	*M = 26.7 ± 5.7% **
LN-121	*M = 680.946 ± 119.456*	*M = 502.530 ± 117.086*	*M = 581.826 ± 84.669*	*M = 23.3 ± 6.9%*	*M = 28.0 ± 11.4% **	*M = 25.9 ± 6.7%*
LN-421	*M = 631.858 ± 155.598 **	*M = 465.325 ± 77.950*	*M = 539.340 ± 81.111 **	*M = 27.0 ± 5.8%*	*M = 23.6 ± 10.5%*	*M = 25.1 ± 6.0%*
LN-511	*M = 863.961 ± 167.292*	*M = 459.428 ± 36.859*	*M = 639.220 ± 100.462 **	*M = 21.9 ± 4.7% **	*M = 24.3 ± 10.3%*	*M = 23.2 ± 5.8%*
LN-521	*M = 805.938 ± 203.935 **	*M = 457.188 ± 45.448*	*M = 612.188 ± 106.110*	*M = 23.1 ± 5.4%*	*M = 23.7 ± 11.7%*	*M = 23.4 ± 6.6% **
L2020	*M = 683.988 ± 123.303*	*M = 424.332 ± 55.619 **	*M = 539.735 ± 73.987*	*M = 27.4 ± 9.5%*	*M = 24.6 ± 11.7%*	*M = 25.8 ± 7.3%*

4.3 Neurite outgrowth

The first part of figure 7 (A) shows a microscopic image of laminin-111 after 5 days of culture and an example of neurite length measurement using the Syn-D program. Table 5 shows the results of the neurite outgrowth experiment. Upon taking a look at these charts and table 5, small differences can be noted between the conditions after 5 and 10 days of culture. As can be seen laminin-521 affects neurite length to the biggest extend followed by laminin-511 after 5 days of culture (figure 7B and table 5). The neurite lengths of the neurons cultured on the other substrates are approximately equal after 5 culture days (figure 7B and table 5). After 10 days of culture laminin-511 affects neurite outgrowth the most with laminin-521 being a close second (figure 7B and table 5). Laminin-421 affects neurite outgrowth the least after 10 culture days (figure 7B and table 5). The first bar chart of figure

7C and the last column of table 5 show the results on neurite length when the data of both culture days is combined. With the data combined laminin-511 and -521 still affects neurite outgrowth to the largest extend and laminin-421 the least. The last figure of 7C shows the difference in neurite length along every condition after 5 and 10 days of culture. Except for laminin-421 all neurites seem to increase between 5 and 10 days of neuronal culture. Neurite length increases the most in the case of laminin-511 and -521.

*Table 5: This table shows the results regarding the neurite outgrowth experiment. The results are shown ± their standard error. * shows the substrate with the biggest effect on neurite length and ** shows the substrate with the smallest effect on neurite length*

Substrate	Neurite length (5 days)	Neurite length (10 days)	Neurite length (combined)
Laminin-111	*M = 363.224 ± ** 25.355 µm*	*M = 737.350 ± 68.562 µm*	*M = 525.888 ± 39.756 µm*
Laminin-121	*M = 364.355 ± 21.450 µm*	*M = 607.612 ± 69.373 µm*	*M = 466.593 ± 34.610 µm*
Laminin-421	*M = 364.480 ± 27.453 µm*	*M = 461.933 ± ** 46.255 µm*	*M = 406.851 ± ** 25.867 µm*
Laminin-511	*M = 422.996 ± 20.161 µm*	*M = 1024.120 ± * 102.629 µm*	*M = 680.620 ± 57.541 µm*
Laminin-521	*M = 514.024 ± * 38.312 µm*	*M = 1013.802 ± 108.931 µm*	*M = 728.214 ± * 59.143 µm*
L2020	*M = 390.966 ± 36.728 µm*	*M = 726.271 ± 67.681 µm*	*M = 536.750 ± 41.010 µm*

Statistical analysis shows a significant effect of the substrate used and the length of neurites after 5 culture days, F (5, 231) = 4.048, p = 0.002. A post hoc analysis shows that laminin-521 significantly differs from laminin-111, -121, -421 and 2020, p = 0.012, 0.022, 0.07 and 0.020 respectively. After 10 days of culture the substrate used also significantly influences the length of neurites, F (5, 173) = 10.545, $p < 0.001$. A post hoc test shows that the ability of laminin-121 to stimulate neurite outgrowth significantly differs from that of laminin-511 and -521, p = 0.001 and 0.002 respectively. Furthermore, laminin-421 is significantly less capable of stimulating neurite outgrowth compared to laminin-111, -511, -521 and L2020, with $p < 0.001$ for laminin-511 and -521, 0.007 for laminin-111 and 0.012 for laminin-L2020. When the data belonging to 5 and 10 culture days is combined the effect persists, F (5, 410) = 9.109, $p < 0.001$. A post hoc analysis shows that the neurite length between laminin-511 and 521 significantly differs from laminin-121 and 421, p = 0.005 and < 0.001 between laminin-511 and -121 and -421 respectively. While $p < 0.001$ and < 0.001 between laminin-521 and laminin-121 and -421 respectively. Furthermore, laminin-521 significantly differs from laminin-111, p = 0.013, and laminin L2020, p = 0.014. Lastly, a Factorial ANOVA shows a significant difference between the neurite lengths of the different culture times, F (1, 394) = 125.854, $p < 0.001$.

Because laminin-111, -511 and -521 show the largest neurites after 10 days of culture, they are used in the last experiment on connectivity.

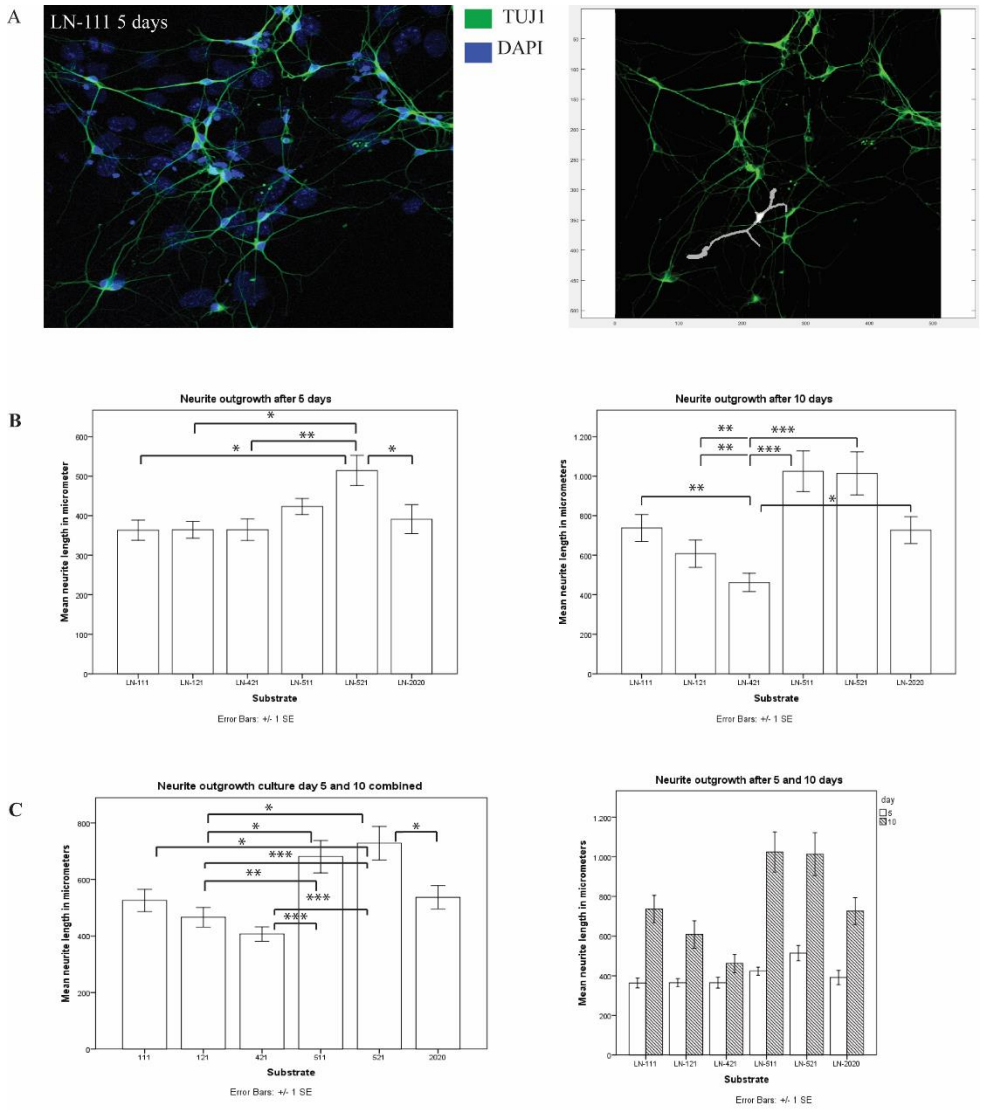

*Figure 7: This figure shows the neurite outgrowth results. Figure 7A shows a microscopic image of laminin-111 after 5 days of culture. The green color shows TUJ1 and DAPI is shown in blue. The second image of 7A shows an example of neurite measurement whilst using Syn-D. Figure 7B shows bar charts of the neurite length after 5 and 10 days. Figure 7C shows the results when the data of 5 and 10 days of culture are combined. The last image compares the neurite lengths of every condition between 5 and 10 days of culture. * means a p value < 0.05, ** a p value < 0.01 and *** a p value < 0.001*

4.4 Connectivity

The last experiment looked at the ability of laminin-111, -511, -521 and -L2020 to generate synapses. Connectivity is defined as the amount of synapses found on a neuron. The top part of figure 8A shows an image taken with a confocal microscope of laminin-511 stained with synaptophysin, TUJ1 and DAPI after 10 days of cultures. The green staining represents TUJ1, the red staining synaptophysin (which is a marker for synapses) and DAPI is depicted with blue. The bottom part of figure 8A shows the result of using the Syn-D tool. The gray dots are synapses and the synapse channel is depicted in red. The results that came out after using Syn-D is shown in the bar chart of figure 8B and in table 6. The amount of synapses found per µm neurite length in neurons cultured on laminin-111, -511 and -521 is relatively equal to each other, as can be seen in figure 8B and table 6. However, the mean amount of synapses found per µm neurite per neuron is the largest when cultured on laminin-521 (figure 8B). Laminin L2020 showed to stimulate synaptogenesis the

least. However, a statistical analyses by means of the Brown-Forsythe ANOVA test does not show an effect between the substrates used and the amount of synapses found, F (3,46.346) = 0.162, p = 0.162.

*Table 6: This table shows the results regarding the neuronal connectivity experiment. The results is shown together with its standard error. * shows the substrate with the biggest effect on connectivity and ** the substrate with the smallest effect on connectivity*

Substrate	Mean amount of synapses per µm neurite length
Laminin-111	*M = 0.187 ± 0.025*
Laminin-511	*M = 0.218 ± 0.020*
Laminin-521	*M = 0.234 ± 0.030 **
L2020	*M = 0.151 ± 0.026 ***

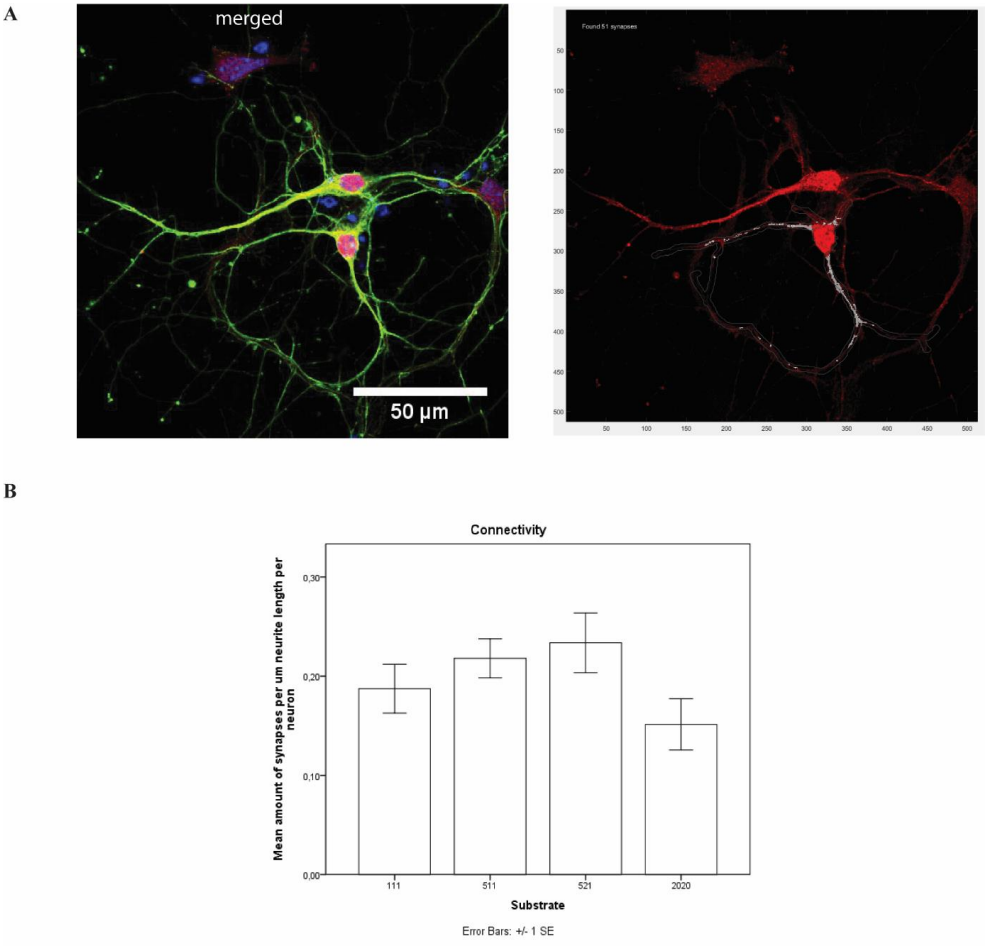

Figure 8: This figure shows the connectivity results. Part A shows microscopic images taken from the laminin-511. In green the TUJ1 marker is shown, red shows synaptophysin and blue DAPI. The right part of 8A shows the results after a neuron is processed using Syn-D. Part B shows a bar chart of the mean amount of synapses found per neuron for each substrate

5. Discussion

The focus of this paper has been to study the effects of laminin-111, -121, -211, -221, -411, -421, -511 and -521 on cell attachment, neuronal differentiation, neurite outgrowth and connectivity of cerebellar progenitor cells. This was done after 5 and 10 days of culture.

5.1 Screening

The first experiment conducted was a screening test in which the neuronal differentiation and neurite outgrowth of laminin-111, -121, -211, 221, -411,- 421, -511, -521 and laminin L2020 were compared with a non-laminin coated condition, namely the PLO only condition. The bar chart of figure 6B did show a lower neuronal differentiation of laminin-211, -221 and -411 in comparison with the other laminin isoforms and laminin L2020. Furthermore, the same bar chart showed that the same three laminin isoforms affected neuronal differentiation approximately to the same extend as a non-laminin coated coverslip (PLO only condition). Therefore, it is likely that laminin-211, -221 and -411 do not further stimulate neuronal differentiation. The study done by Ge and colleagues (2015) showed that PLO possesses the ability to stimulate neuronal differentiation of neuronal progenitor cells. The results of this study show that when a laminin-211, -221, and -411 coating is added atop PLO it does not further enhance neuronal differentiation. This would mean that laminin-211, -221 and -411 do not stimulate neuronal differentiation themselves, but that all neuronal differentiation observed comes from the PLO coating. However, it could be that because the sample size of the screening was small, no significant effect could be found. So, these statements and results need to be interpreted with caution. A bigger sample size is to strengthen statements made above.

During the screening the ability of every substrate to enhance neurite outgrowth was also examined. A study done by Flanagan and colleagues (2006) showed that a general laminin substrate stimulated neurite outgrowth to a significantly larger extend than a PLO substrate. In this study the same effect was found. Laminin L2020 significantly differed from the PLO only condition. The same was true for laminin-111, -121, -421, - 511 and -521. However, laminin-211, -221 and -411 did not stimulate neurite outgrowth more than the PLO only condition. Meaning that laminin-211, -221 and -411 do not have the ability to stimulate neurite outgrowth more than a non-laminin containing substrate. Because, laminin-211, -221 and -411 showed no real difference in neuronal differentiation and neurite outgrowth compared to a non-laminin containing substrate they were excluded from the remainder of this study.

The screening results showed that laminin-211, -221 and -411 did not stimulate neuronal differentiation and neurite outgrowth more than a non-laminin containing substrate. In this study only two laminins containing the α2 chain were included and both were poor neuronal differentiators and neurite outgrowth stimulators. Table 1 shows that beside laminin-211 and -221, laminin-212 also contains an α2 chain. If laminin-212 also does not promote neuronal differentiation and neurite outgrowth, than this would mean that the α2 chain does not possess any neuronal differentiation and neurite outgrowth properties. The difference between laminin-211 and -212 is a γ2 chain. Gene ID: 3918 NCBI (2017) shows that chemically the γ2 chain does not differ much from the γ1 chain except being shorter and missing some domains. Furthermore, it is primarily expressed in the skin, lung and kidney (NCBI 2017). The primary role of the laminin γ2 chain is to act as an epithelium attachment molecule and mutations in the γ2 chain causes junctional epidermolysis bullosa (NCBI 2017). Given this information it is unlikely that laminin-212 will have neuronal differentiation and neurite outgrowth capabilities. Meaning that the α2 chain does not have any inert neuronal differentiation or neurite outgrowth abilities. Kirkeby and colleagues (2017) looked at the adherent differentiation of ventral mesencephalon progenitor cells on 7 different laminin isoforms (laminin-111, -211, -221, -411, -421, -511 and -521). They found that four of the seven laminin isoforms (laminin-111, -421, -511 and -521) supported differentiation of ventral mesencephalon progenitor cells. This

would mean that laminin-211, -221 and -411 did not. These results support the results found in this study. Furthermore, this would also mean that there is a reasonable possibility that the results found in this study does not only translate to cerebellar progenitor cells. However, Kirkeby and colleagues (2017) only make this statement, they do not mention or show any numbers. Seo and colleagues (2012) used the LN2-P3 motif of laminin-211 to coat nerve grafts and compared its nerve regenerative capabilities with nerve grafts coated with scrambled peptides. They found that the LN2-P3 coated nerve crafts performed better (Seo et al. 2012). The regenerating nerve was longer at certain time points compared to the control (Seo et al. 2012). This could be because of LN2-P3 capability to enhance cell attachment and cell spreading providing an environment better suited for nerve regeneration compared to the control (Seo, et al. 2012). However, Seo and colleagues did not research the neuronal differentiation and neurite outgrowth capabilities of LN2-P3 compared to the controls. Furthermore, a study conducted by Powell and colleagues (1998) found that laminin-111 and -211 promoted neurite outgrowth equally in granule cells after 2 to 5 days of culture. In this study laminin-111 promotes neurite outgrowth to a larger extend. The difference between both studies is that they used purified granule cells and this study used cerebellar progenitor cells. Fusaoka-Nishioka and colleagues (2011) compared the dendrite length and axon length of 18 days old embryonic hippocampal neurons after 3 days of culture on laminin-111, -211, -411 and -511. They found that laminin-211 significantly increased dendritic length and axon length compared to coverslips coated with poly-l-lysine, while this was not the case in this study (Fusaoka-Nishioka et al. 2011). Furthermore, while the dendrite length of laminin-411 did not differ from the poly-l-lysine condition, the axon length did in the study of Fusaoka-Nisioka and colleagues (2011). In this study axon length and dendrite length were put together, this could result in the overall neurite length being not significantly different from a non-laminin condition in the case of laminin-411. However, this analysis was not included in this study. To date there is no study that compared the neurite outgrowth capabilities of poly-l-lysine with PLO. It could be that PLO enhances neurite outgrowth to a larger extend than poly-l-lysine. Another possibility is that laminin-211 in combination with PLO diminishes the neurite outgrowth capabilities of the substrate. Furthermore, they found no difference in axon and dendrite length between laminin-111, -211 and -411, while laminin-111 promotes neurite outgrowth more strongly in this study (Fusaoka-Nishioka et al. 2011). However, in the paper of Fusaoka-Nishioka and colleagues (2011) it is not mentioned how dendrite and axon length was measured. Because their method is unknown, it is hard to compare their results to the ones of this study. Furthermore, they examined neurite and axon length after three days of culture (Fusaoka-Nishioka et al. 2011). It could be that laminin-111 needs more time to stimulate neurite outgrowth. Figure 7B shows a large increase of neurite length after 10 days of culture for laminin-111 compared to 5 days. While such increase is not seen in laminin-121 and -421. This would support the statement that 3 days of culture is too short to see the effect that laminin-111 has on neurite outgrowth. Furthermore, Fusaoka-Nishioka and colleagues (2011) used hippocampal neurons while cerebellar progenitor cells were used in this study. Laminin-111 could have more affinity for cerebellar progenitor cells compared to hippocampal neurons. Plantman and colleagues (2008) assessed the ability of laminin-111, -211, -411 and -511 on neurite outgrowth of dorsal root ganglion neurons. They found that laminin-111 and -511 stimulated neurite outgrowth to a larger extend than laminin-211 and -411 (Plantman et al. 2008). This is in agreement with the results of this study. In conclusion cerebellar progenitor neurons did not show more neuronal differentiation and neurite outgrowth after 5 culture days while seeded on laminin-211, -221 or -411 in comparison with the PLO substrate. However, on laminin-111, -121, -421, -511 and -521 they did. The study by Kirkeby and colleagues (2017) supports the results attained in this study regarding neuronal

differentiation. However, because the body of literature is still small on this subject more studies are needed to strengthen this conclusion. There is no conclusive support to be found in the literature regarding the neurite outgrowth results of the screening test. Because of a lack of studies focusing on this subject and different methodologies used, it is hard to compare these results with the current literature. More research is needed to strengthen our current knowledge regarding this subject.

5.2 Cell attachment and neuronal differentiation

After the screening test, two 5 day long cultures and four 10 day long cultures of cerebellar progenitor cells on laminin-111, -121, - 421, -511, -521 and L2020 were done. So, after those cultures there was a total of four 5 day long and four 10 day long cultures for laminin-111,-121, -421, -511, -521 and L2020. After 5 or 10 days the cells were fixated, stained and tested on cell attachment, neuronal differentiation and neurite outgrowth. The results showed that after 5 or 10 days of culture there is no significant difference in cell attachment or neuronal differentiation between cells cultures on all substrates belonging to the same culture times. It is important to note that even after four cultures done on both culture times the sample size is still small, namely four (each substrate was used four times). With such a sample size it is generally hard to find a significant effect. However, if we take a look at figure 6B and compare the size of the bars for cell attachment and neuronal differentiation for both culture times we see that the bars belonging to every substrate belonging to the same culture time hardly differ from each other. This means that even if the sample size increases enough to spot a significant effect it would be a small effect. There are some but not a lot of studies that compared different laminin isoforms to each other. Powell and colleagues (1998) compared the ability to promote neurite outgrowth of laminin-111 to that of laminin-211, but they did not look at neuronal differentiation or cell attachment. The same goes for the study done by Fusaoka-Nishioka and colleagues (2011). The study done by Kirkeby and colleagues (2017) did look at the adherent differentiation of ventral mesencephalon progenitor cells of laminin-111, -421, -511 and -521. They mention that these four laminin isoforms efficiently supported neuronal differentiation of ventral mesencephalon progenitors. But, they do not mention if any differences between the ability to promote neuronal differentiation exists between these four laminin isoforms. To date there are no studies to be found that compared the ability of different laminin isoforms on cell attachment or neuronal differentiation. Furthermore, the ability of laminin-111, -121, -421, -511 and -521 to promote cell attachment and neuronal differentiation did not differ from laminin L2020.

The ability of laminin-111, -121, -421, -511, -521 and L2020 to promote cell attachment and neuronal differentiation between 5 and 10 days of culture was also assed. A statistical analysis did not find any difference in the ability of the different substrates to promote neuronal differentiation between 5 or 10 culture days. Again the sample size is really small but, looking at the bar chart of figure 6B it is likely that the effect would be small on neuronal differentiation if the sample size is increased sufficiently. However, a significant difference is found in the amount of attached cells between the different culture times. Because, the percentage of neurons relative to the total amount of attached cells remains equal between 5 and 10 days, it is likely that in the 5 days between the two culture times some cells die and are removed during the refreshing of the culture medium resulting in less attached cells. It is also possible that the substrates used degrades over time. This results in less substrate being present after 10 days to which cells can adhere and therefore the degraded substrate components and cells that were attached to the degraded places are removed during the refreshing of the culture medium. Because the

percentage of neurons compared to the total amount of attached cells should be roughly the same everywhere on the coverslip this would result in a decrease in total cell attachment but not in neuronal differentiation, because of how it is defined in this paper. Future studies are needed to test whether the substrates used degrades over time.

5.3 Neurite outgrowth

The results shows that the substrate used affects mean neurite length per neuron after 5 days of culture. Figure 7B shows that laminin-521 stimulated neurite outgrowth to the largest extend after 5 culture days compared to the other substrates. A post hoc test reveals that the ability to promote neurite outgrowth of laminin-521 significantly differs from laminin-111, -121, -421 and L2020. The ability to promote neurite outgrowth of all other laminin isoforms and laminin L2020 do not significantly differ from each other. Five days later the story is a bit different. Again the substrates used significantly affect neurite outgrowth, but now a post hoc test reveals more individual differences between substrates. Laminin-511 and -521 promotes neurite outgrowth more than laminin-121 and -421. Laminin-421 potentiates neurite outgrowth the least and its capability to do so differs significantly from all substrates used except for laminin-121. Except for laminin-421 the ability to promote neurite outgrowth does not differ from a general laminin substrate (L2020). When the ability to promote neurite outgrowth for each substrate is compared between 5 and 10 days of culture a significant difference is found. Total neurite length is significantly larger after 10 days of culture compared to 5 days. Figure 7B shows a bar chart in which mean total neurite length per neuron on a substrate is compared between both culture days. From this it can be seen that the neurite length of neurons cultures on all substrates except for laminin-421 largely increased between 5 and 10 days of culture. This would mean that the neurite outgrowth capacity of laminin-421 is really limited compared to the other substrates. Furthermore, the results show that more time is needed than 5 days of culture to really assess the potential of each substrate to stimulate neurite outgrowth. In the literature there are two studies that compared the ability to promote neurite outgrowth of some of the laminin isoforms included in this experiment to each other, which is the study of Fusaoka-Nishioka and colleagues (2011) and Plantman and colleagues (2008). Fusaoka-Nishioka and colleagues (2011) looked at dendrite length and axon length instead of total neurite length. Furthermore, it is not clear how they measured both dendrite and axon length. Another difference to this study is that they used hippocampal neurons while cerebellar progenitor cells were used in this study. Fusaoka-Nishioka and colleagues (2011) used laminin-111, -211, -411 and -511 in their study. The results of Fusaoka-Nishioka and colleagues (2011) show that the hippocampal neurons on laminin-511 showed the biggest dendrite and axon length after three days of culture. This is in agreement with this study, were laminin-511 also showed to have a bigger effect on neurite outgrowth compared to laminin-111. However, the difference was not significant in this study. Laminin-211 and -411 were excluded for future testing in this study and will not be further discussed here. Because, Fusaoka-Nishioka and colleagues (2011) used cells that lost their potency and only cultured them for three days, it is hard to compare our results with theirs. Plantman and colleagues (2008) assessed the ability of laminin-111, -211, -411 and -511 on neurite outgrowth of dorsal root ganglion neurons. As stated above they found that laminin-111 and -511 stimulated neurite outgrowth to a larger extend than laminin-211 and -411 (Plantman et al. 2008). Furthermore, laminin-511 stimulated neurite outgrowth to a larger extend than laminin-111 (Plantman et al. 2008). This is in agreement with the results of this study.

5.4 Connectivity

In the last experiment included in this study the ability to generate synapses of laminin-111, -511, -521 and L2020 were assessed and compared. To date this is the first experiment to do so. Figure 8B shows a bar chart of the mean amount of synapses found per neuron on each substrate. As can be seen from this bar chart laminin-521 seems to potentiate synaptogenesis to the largest extend. While laminin L2020 performed the poorest. Laminin-511 was a close second. However, a statistical analysis does not show a significant effect of the substrate used on the mean total amount of synapses per µm neurite length. The sample size of this experiment was just 18 per substrate. It is likely that an effect is present if the sample size is increased.

5.5 Explaining the differences

At the present, the literature that describes mechanical and chemical differences of all laminin isoforms and their affinity for cell receptors is still too limited to explain why some isoforms affect cerebellar progenitors in culture differently than others. However, some chemical differences between the laminin isoforms included in this study will be discussed and linked to their performance. Laminin-411 and -421 both performed poorly on neurite outgrowth compared to laminin-111, -121, -511 and -521. It is known that the α4 chain is much shorter compared to the α1 and α5 chain (Domogatskaya et al. 2012). Furthermore, the α4 chain has a low affinity for integrins and α-dystroglycans, which are both important cellular receptor through which laminin can communicate with cells (Domogatskaya et al. 2012). Plantman and colleagues (2008) showed that laminin-411 uses the α6β1 integrin for neurite outgrowth. Given the similarities between laminin-411 and -421 it is likely that laminin-421 also uses integrin α6β1 for neurite outgrowth. However, Nishiuchi and colleagues (2006) show that laminin-411 and -421 have a poor affinity for integrin α6β1. So, even though laminin-411 and likely laminin-421 use integrin α6β1 for neurite outgrowth their affinity for the integrin is poor (Nishiuchi et al. 2006; Plantman et al.2008). This will result in laminin-411 and -421 having poor neurite outgrowth abilities. This is also reflected in the results of this study. The same argument can be made for laminin-211 and -221. Plantman and colleagues (2006) showed that laminin-211 uses the α3β1 integrin for neurite outgrowth. Nishiuchi and colleagues (2008) show that laminin-211 and -221 have a poor affinity for integrin α3β1. Again this will result into poor neurite outgrowth capabilities for laminin-211 and -221. Still, laminin-421 performed better than laminin-411 this could be accounted to the fact that the laminin-421 molecule has the β2 chain (Domogatskaya et al. 2012). It has been shown that the β2 chain has a higher affinity for α3β1 and α7Xβ1 integrins compared to the other β chains this could potentially explain why laminin-421 performed better than laminin-411 (Domogatskaya et al. 2012). Especially because it is known that the binding between laminin-111 and α3β1 promotes neurite outgrowth, this could also be the case for laminin-421 (Domogatskaya et al. 2012). Furthermore, it could also explain why laminin-521 is a better promoter of neurite outgrowth than laminin-511 after 5 days of culture. However, it is important to note that Plantman and colleagues (2008) found that laminin-111 uses the α3β1 and α7Xβ1 integrins to stimulate neurite outgrowth while laminin-511 uses α6β1. Nishiuchi and colleagues (2006) found that laminin-111, -511 and -521 have a high affinity for the α6β1 integrin. However, Plantman and colleagues (2008) showed that laminin-111 does not use integrin α6β1 for neurite outgrowth while laminin-511 does. Because, laminin-511 and -521 are closely related to each other it is likely that laminin-521 also uses integrin α6β1 for neurite outgrowth. It could be that laminin-521 also uses α3β1 and α7Xβ1 integrins because of the affinity that the β2 chain has for both integrins, however this is not known yet.

This can be confirmed by studies that block integrin α3β1 and α7Xβ1 on laminin-521 cultures. Furthermore, in the case of laminin-421 and -521 their effect on neurite outgrowth decreased after longer culturing time. Neurite length of the neurons cultures on laminin-421 did not really increase after 10 days of culture compared to 5 and laminin-521 shared its top spot after 10 days of culture with laminin-511. Meaning that the β2 integrin could only be playing a role in neurite outgrowth at the early stages of development. The reason that laminin-111, -511 and -521 performed better than laminin-411 and -421 can also be contributed to the fact that the α1 and α5 chain can bind to integrins on both ends of the chain while other α chains cannot (Domogatskaya et al. 2012). This could also be the reason that they performed better than laminin-211 and -221. Lastly, laminin-511 and -521 stimulated neurite outgrowth to a larger extend than laminin-111. This could be attributed to the fact the laminin-511 and -521 use integrin α1β1 to modulate neurite outgrowth (Katagiri et al. 2012).

Lastly, an attempt is made to explain the differences in the abilities of laminin-111, -511 and -521 to generate synapses. Nikonenko and colleagues (2003) showed that integrin β1 plays a role during synaptogenesis. All laminin isoforms tested in the connectivity experiments are known to have a high affinity for β1 integrins (α1β1, α3β1, α6β1 and α7Xβ1). Of the three laminin isoforms tested laminin-521 likely has the highest affinity for these β1 integrins. It is known that the affinity for α1β1, α3β1 and α6β1 is the highest for laminin-511 and -521 (Domogatskaya et al. 2012; Nishiuchi et al. 2006; Katagiri et al. 2012). Domogatskaya and colleagues (2012) showed that the laminin α5 chain can bind to the αVβ3 integrin. Milner and Campbell (2002) showed that the β3 integrin plays a role during the maturation of synapses. Furthermore, because of the laminin β2 chain, laminin-521 has a higher affinity for α3β1 and α7Xβ1 integrins compared to laminin-511 (Domogatskaya et al. 2012). Considering these facts, laminin-521 should stimulate synaptogenesis to the largest extend and laminin-111 the least. The connectivity experiment conducted in this study confirms this statement. Future research is needed to investigate these proposed explanations and turn them into scientifically supported statements. This can be done by studies in which certain integrins on the neurons in culture get blocked by antagonist and compare these results with controls in which no integrins are blocked.

5.6 Recommendations

In this study, no differences on neuronal differentiation and cell attachment were found between laminin-111, -121, -421, -511, -521 and L2020. However, during the neurite outgrowth and connectivity experiments, although not always significant, differences were noted. Laminin-511 and -521 seem to perform better than all other substrates used. To date, most studies use a general laminin substrate stemming from mice sarcoma. This study shows that human recombinant laminin-511 and -521 perform better on neurite outgrowth and connectivity of NPCs than the general laminin substrate L2020. Knowing this, it is better to use the purified human recombinant laminin-511 and -521 isoforms for neuronal cell culture studies than laminin L2020. Using the purified laminin-511 and -521 isoforms, researchers know exactly what their substrate looks like and what they can expect. While with using a general laminin substrate they do not. Because it is unknown which laminin isoforms are contained in a general laminin substrate and in which concentrations they are present. Furthermore, this can differ from batch to batch. Knowing the substrate used makes it easier to interpret results after a cell culture study. Moreover, using human recombinant isoforms is recommended over laminin stemming from mice because, these experiments are

done so that human diseases can be treated. Focusing on human laminin substrates and learning how to use them instead of animal substrates will save a lot of time in the end.

The literature regarding the effects of different laminin isoforms on neuronal cell cultures is still very limited and more research is needed to broaden our understanding on this subject. More knowledge is needed to be able to create an optimal laminin substrate that can generate a large and healthy neuronal network for future clinical applications. Furthermore, in this study only eight of the currently 20 known isoform were tested. If more laminin isoforms become available their effects on neuronal cell cultures should also be assessed. Lastly, this study used mice cerebellar progenitor cells. To translate these results to human neurons, studies are needed that use human cells.

6. Conclusion

In conclusion, laminin-211, -221 and 411 do not possess neuronal differentiation and neurite outgrowth capabilities. The ability of laminin-111, -121, -421, -511, -521 and L2020 to promote cell attachment and neuronal differentiation is equal after both 5 and 10 days of culture. After 5 days of culture laminin-521 promotes neurite outgrowth significantly better than laminin-111, -121, -421 and L2020. Five days later laminin-521 shares the top spot with laminin-511 in their ability to promote neurite outgrowth. Laminin-511 and -521 promotes neurite outgrowth to a significantly larger extend than laminin-111, -121 and -421. The ability of laminin-421 to promote neurite outgrowth is the smallest and is significantly different from laminin-111, -511, -521 and laminin L2020. Furthermore, laminin-421 did not stimulate neurite outgrowth more after 10 days of culture compared to 5 days. No statistical difference could be found between the ability of laminin-111, -511, -521 and L2020 in generating synapses. However, neurons cultured on laminin-521 followed by laminin-511 showed the most synapses while laminin L2020 the least. Overall, human recombinant laminin-511 and -521 seem to promote neurite outgrowth and synaptogenesis better than laminin-111, -121, -421 and L2020. In the future laminin-511 and -521 should be used as a substrate for cerebellar progenitor neurons and possibly for other type of neurons.

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8. Appendix

In this sections some additional data and experiments conducted in this study are shown and briefly discussed. This includes the preliminary research on laminin in post-natal and adult mice cerebella, data regarding the percentage of astrocytes compared to neurons on each coverslip and the percentage of bi-polar neurons compared to multipolar neurons on each coverslip. The reason that the cell culture data is not included in the main part of this paper is that the data shows inconsistencies or other forms of distrust.

8.1 Laminin in mice cerebella

In this section the methods/materials and results will be addressed.

8.1.1 Materials/methods

8.1.1.1 Perfusion. Embedding and sectioning of the cerebella

For each experimental condition (P1, P7, P21 and adult mice) two mice cerebella were used and thus in total eight mice were perfused using the method described in this section. The fixative used during the perfusion of the mice is a 4% paraformaldehyde (PFA) solution. Depending on the age of the mice the speed of the perfusion pump was adjusted to prevent rupture of blood vessels. After a successful perfusion the cerebellum is taken out of the skull and post fixed in a 4% PFA solution for two hours on a shaker in room temperature. Next, the cerebella are rinsed once using a 0.1 molar PB solution containing 10% sucrose for the adult cerebella and a 0.1 molar PB containing 30% sucrose for the postnatal cerebella. Afterwards, they are kept in the same solutions overnight on a shaker in the four degrees Celsius fridge. For the embedding a 12% gelatine and 10% sucrose solution is used for the cerebella belonging to adult mice and a 12% gelatine and 30% sucrose solution for the cerebella belonging to the postnatal mice. Before embedding the cerebellum in gelatine, the cerebella were kept in the embedding solution for one hour in a 55 degrees Celsius oven. After an hour in the embedding solution the cerebella are taken out of the oven and are embedded using the same solution. After embedding the cerebella are placed into a four degrees Celsius fridge for about 15 minutes. After 15 minutes the cerebella are taken out of the fridge. The gelatine solution is sufficiently solid by this time. Next, the hardened embedding solution containing the cerebella are cut into square blocks. To discriminate the left and right side of the cerebellum a cut is made in the corner belonging to the top right side of the cerebellum. Next, the blocks belonging to the postnatal mice are placed in a jar containing a 10 % fixative, 30% sucrose solution for two hours on a shaker in a four degrees Celsius fridge to make sure that the gelatine is firmly attached to the cerebellum. The blocks belonging to the adult mice receive the same treatment but are kept in the fixative for three hours instead of two. Lastly, after two/three hours the blocks are rinsed once using a 30% sucrose solution in 0.1 M PB and kept in the same solution on a shaker in the four degrees Celsius fridge overnight. The next day the cerebella were sectioned along their coronal axis using a freezing cryotome that uses carbon dioxide gas. The sections were 40 µm thick in the case of the adult cerebella and 50 µm thick in the case of the postnatal cerebella. The sections were collected in jars containing a 0.1 M PB solution. Depending on the age of the mice to which the coupes belonged, they were collected either in 3, 4 or 8 jars, for the P1, P7 and

P21 and adult mice respectively. Of every experimental condition (P1, P7, P21 and adult) one jar was used as a negative control for the immunofluorescence staining.

8.1.1.2 Immunofluorescence staining of the coupes.

Of every mice one jar is used for immunofluorescence staining with anti-laminin (Abcam 11575), calbindin-D 28k (sigma) and DAPI. Furthermore, one jar is used as a control. The controls do not contain the laminin primary anti-body, but do contain calbindin-D 28k, DAPI and both secondary anti-bodies. To test whether the basement membranes of blood vessels contain laminin one jar belonging to a P7 mouse was stained for laminin and CD-31. CD-31 is a marker for angiogenesis. The general immunofluorescence method is the same for all experimental conditions, controls and the double staining for laminin and CD-31. First, all the jars are rinsed three times ten minutes with PBS before putting the jars in a block solution for one hour under the same conditions. The block solution used contains 10% normal horse serum, 0.5% triton and PBS. After being in the block solution for an hour the coupes are put into the primary anti-body solution for 72. The primary anti-body solution contains: 2% normal horse serum, 0.4% triton, the primary anti-bodies and PBS. The ratio's in which the primary antibodies used were added are: 1.10.000, 1:7000 and 1:200 for anti-laminin, calbinding-D 28k and CD-31 respectively. After 72 hours in the primary anti-body solution all the coupes were rinsed three times ten minutes in PBS before being placed into the secondary anti-body solution. The secondary anti-body solution contains 2% normal horse serum, 0.4% triton, secondary anti-bodies and PBS. The secondary anti-bodies used are: Cy3 donkey-anti-mouse and Alexa 488 donkey-anti-rabbit. Both secondary anti-bodies were added with a ratio of 1:200. The coupes were kept in this solution for 1.5 hours in room temperature on a shaker while being covered from light. Next, the coupes were rinsed two times ten minutes in PBS and afterward in 0.1 M PB for five minutes. After five minutes in PB the coupes are placed on a shaker under the same conditions in a 1:10000 DAPI solution with 0.1 M PB for 10 minutes. Before mounting the coupes they are rinsed twice for five minutes in 0.1 M PB. The coupes are mounted using a 1:5 chroomoliun solution. After the coupes and the mount glasses dried, they were covered using glass and mowiol. Note that every step from the secondary anti-body solution onward was performed in the dark as much as possible.

8.1.1.3 Laminin specificity tests

A big concern after the immunofluorescence staining explained above is the question regarding the specificity of the laminin anti-body used. It could be that the laminin anti-body used also reacts to molecules other than laminin. To exclude the possibility of non-specific binding we used two tests. The first test is rather simple and straightforward and includes using a different general laminin anti-body and compare the immunofluorescence results attained with the results from the Abcam laminin anti-body. The second laminin anti-body used is the L9393 anti-laminin from the firma Sigma. Another firma was chosen to maximize the difference between both laminin anti-bodies. To test the specificity using this different laminin anti-body one jar of the adult mice was stained exactly the same way as described above, but instead of adding anti-laminin (Abcam 11575), anti-laminin (Sigma L9393) was added in a ratio of 1:1000.

The second test to lower the possibility of non-specific binding influencing our results an immunofluorescence method called free absorption was used. This method includes the use of the target molecule to absorb the anti-bodies contained in the primary anti-body solution before staining the coupes. This was done by coating glass coverslips with laminin L2020 (Sigma) in a 24 well-plate in the same way as described in section 3.3. The primary anti-body solution containing anti-laminin was added to the wells containing the coverslip. Afterwards the resulting primary anti-body solution is transferred to the coupes and the normal staining process as described above was followed. Only now anti-laminin should be absent from the solution. Because we now have two different laminin anti-bodies this test was performed twice, one time for each anti-body.

8.1.1.4 Qualification of laminin in the cerebella

To qualify laminin in the cerebellum all coupes were inspected using a fluorescence microscope first. This was done to get a general idea of the locations containing laminin and to select coupes to further analyse using the confocal microscope. Using the confocal microscope sufficient data was collected by means of images. For both microscopes lasers 405, 488 and 505 and 10x, 20x, 40x, 63x and 100x magnification were used.

8.1.2 Results

First the results regarding the specificity tests and negative controls are shown. Next, the results regarding the spatial temporal distribution of laminin will be shown.

8.1.2.1 Specificity and control

Figure 9 shows the immunofluorescence results attained using both laminin anti-bodies. Both non-related laminin anti-bodies show the same specificity. Because both anti-bodies are manufactured by different companies, this result weakens the possibility of non-specific binding. However, it could be that both anti-bodies suffer from the same non-specific binding. The results attained from the free absorption study is needed to draw definite conclusion.

Figure 10 shows the results from the free absorption technique using both laminin anti-bodies. The left panel of figure 10 shows the results of the free absorption technique when the Abcam anti-body was used and the right side when the Sigma anti-body was used. When the free absorption technique is used, as is described in the method section, the primary anti-body solution does not show a big reaction to the pial surface and basement membrane of blood vessels anymore. However, the reaction of both anti-bodies to the inside of Purkinje cells does not diminish at al after applying the free absorption technique. Meaning that the chance of non-specific binding occurring inside Purkinje cells to both of these laminin anti-bodies is very high.

Figure 11 shows that when the same exposure time is used for Alexa 488 (970 ms), which reacts with the laminin anti body, between both the experimental conditions and the controls, that the controls do not show any reaction to the laser. Which means that the results attained should not be affected by artefacts and auto-fluorescence. Furthermore, the same figure shows that the light intensity caused by the reaction to the laser (Alexa

488) decreases along experimental conditions starting from the P1 condition. Meaning that the amount of laminin decreases throughout postnatal development into adulthood.

In the following subsections the spatial-temporal distribution of laminin in mouse cerebella will be discussed.

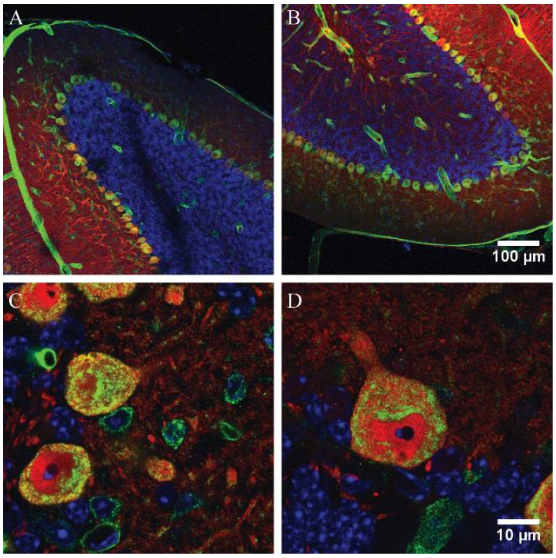

Figure 9: This confocal image shows the immunofluorescence results attained using both the Sigma and Abcam laminin anti-body. Picture A and C show the results attained using the Sigma laminin anti-body while B and D show the results attained using the Abcam anti-body. The big cells in pictures C and D are Purkinje cells.

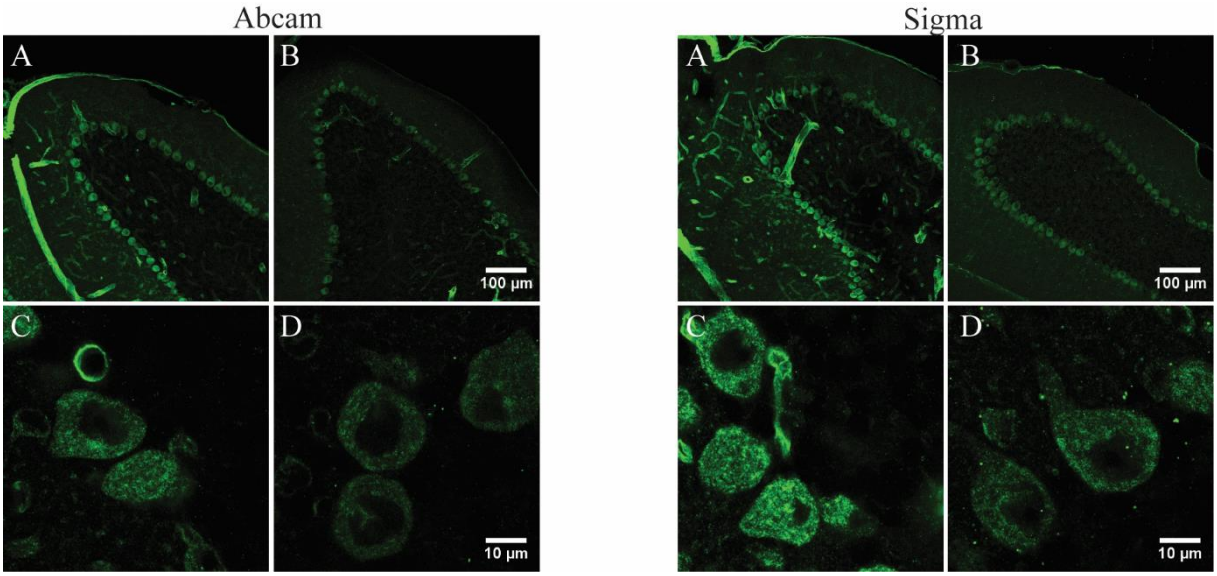

Figure 10: Free absorption images acquired using the confocal microscope. The left panel of image represent the free absorption test done on the Abcam laminin anti-body and the right on while using the laminin anti-body from Sigma. Everything coloured green represent the reaction to the laminin anti-body. Images A and C are the result attained using the normal staining procedure and image B and D represent the results attained using the free absorption technique.

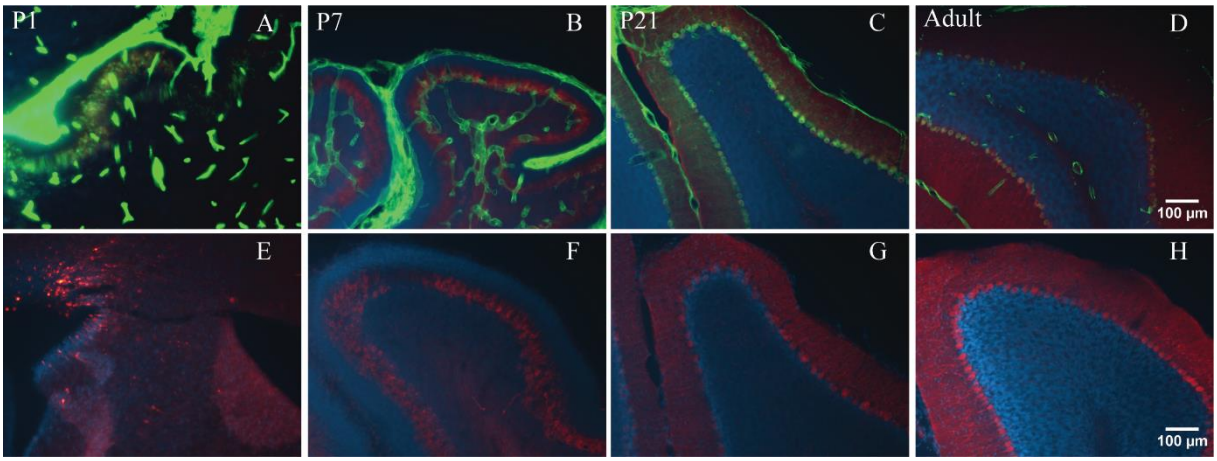

Figure 11: All these images were made while using the same exposure time for Alexa 488 (which reacts to the laminin anti-body). A-D are sections from the experimental conditions of respectively P1, P7, P21 and adult cerebella. While E-H are sections of the control conditions of respectively P1, P7, P21 and adult cerebella. The green colour shows laminin, red calbindin and blue DAPI. The laminin anti-body used was the Abcam one.

8.1.2.2 Spatial-temporal laminin distribution

Figure 12 shows the distribution of laminin in P1, P7, P21 and adult mouse cerebella respectively. As can be seen from figure 12 laminin is found in/around vessel like structures in all experimental groups. These vessel like structures highly resemble blood vessels. Figure 13 confirms this hypothesis. Figure 13 shows that the vessel-like structures react both the laminin anti-body and CD-31 (which is a marker for angiogenesis). This not only means that these structures are indeed blood vessels, but also that laminin is present in the basement membrane of these vessels. Besides the basement membranes of blood vessels figure 12 shows that laminin is found in the pial surface and in the molecular cell layer across all groups. Moreover, as can be seen in figure 11 the area containing laminin seems to decrease over time. Laminin is strongly present in P1 cerebella and while its presence decreases from P7 onwards. The lowest area of laminin is found in the adult cerebella. Below a closer look at the immunofluorescence results will be made.

At postnatal day one the cerebellum is still very immature. The cerebellum will undergo change during the postnatal development. Figure 12 shows that in P1 the lobules are still very small and hence hard to distinguish from each other. Furthermore, the molecular layer is thin as can been seen from the small and still immature Purkinje cells. At this stage the internal granule layer is not present yet and even the external granule layer is hard to find. Laminin can mostly be found in the pial surface and the basement membrane of blood vessels. Moreover, figure 12 shows that laminin is also present in and around the molecular layer. Figure 12 also shows a reaction to the laminin anti-body inside the Purkinje cell soma, this is a trend that will continue along the other conditions. However, this reaction is not due to laminin being present inside the cell soma but due to non-specific binding as can be seen in figure 10.

At post-natal day seven the shape of the molecular layer starts to look more mature and clear lobules are starting to from (figure 12). Still, the molecular layer is very thin in comparison to a fully developed molecular layer (figure 12). At this stage it is easier to distinguish individual Purkinje cells and the internal granule layer starts to form (figure 12). However, the external granule layer is still clearly present (figure 12). Again laminin is found in the pial surface and basement membrane of blood vessels (figure 12). Moreover, there seems to be

laminin inside the internal granule layer (figure 12). There is more laminin in the internal granule layer than in the external.

P21 cerebella look a lot like adult cerebella as can be seen in figure 11, a clear molecular layer, lobules and internal granule layer have been formed (figure 12). Furthermore, the external granule layer does not exist anymore. However, as can be seen in figure 12 and 11 laminin seems to be more concentrated in the molecular layer of the P21 cerebella in comparison to the adult one. This is also the case with the amount of laminin found in the internal granule layer (figure 12). Furthermore, laminin is found in the pial surface and the basement membrane of blood vessels (figure 12).

In the last row of figure 12 we see a characteristic cerebellar lobule belonging to an adult mice. A dense packed molecular layer and internal granule layer can be seen (figure 12). Laminin is found in the pial surface, the molecular layer and basement membrane of blood vessels (figure 12).

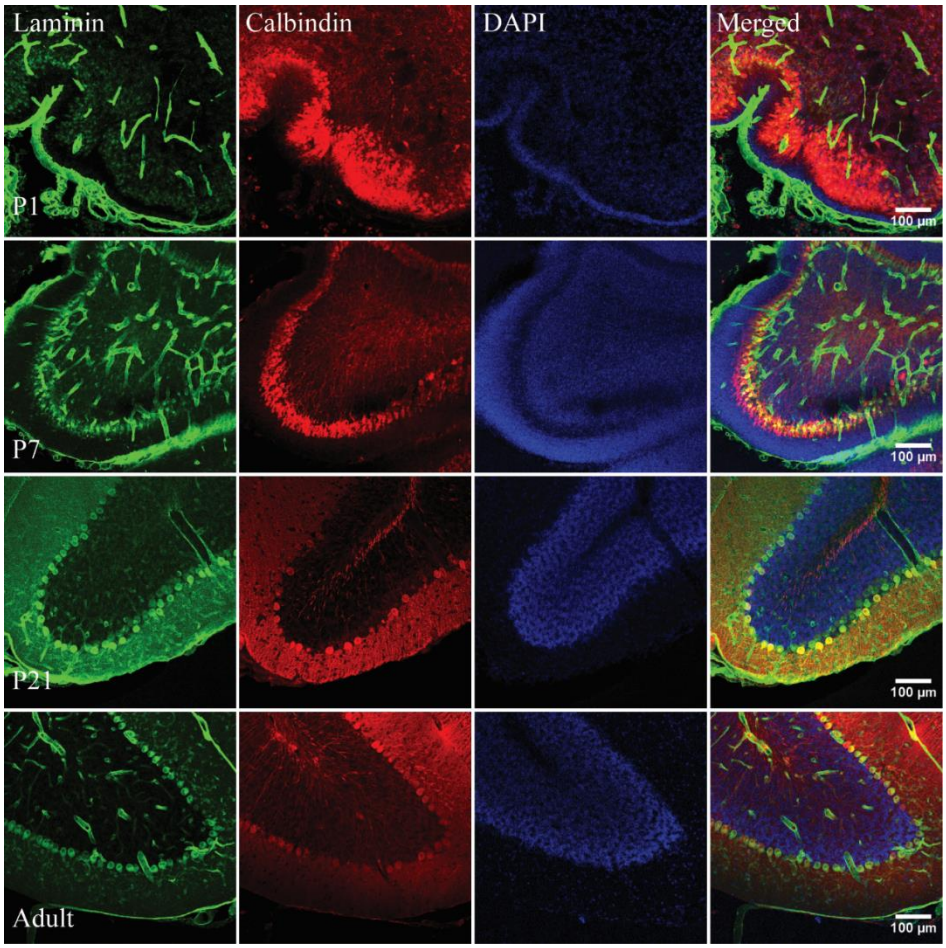

Figure 12: Each row shows a different experimental condition. Starting from the top this image shows sections of the P1, P7, P21 and adult cerebella. Each column shows the reaction to different anti-body staining's. Starting from the left this image shows staining for calbindin, laminin (Abcam), DAPI and a merged image.

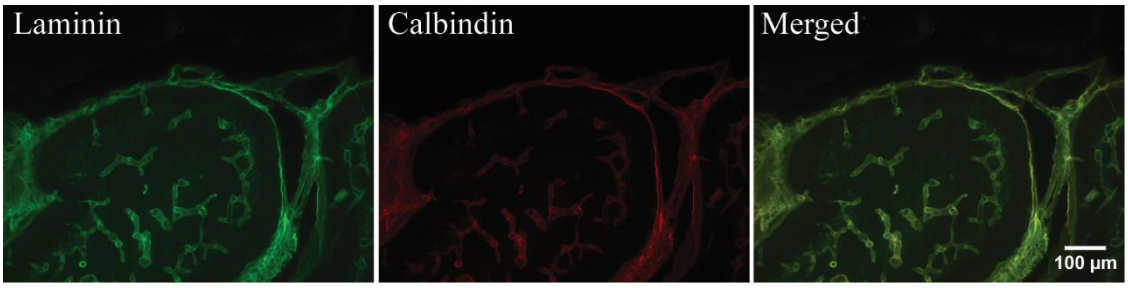

Figure 13: This image shows the separate and combined staining of CD-31, laminin (Abcam) and DAPI respectively

8.1.3 Conclusion

Laminin is found in the pial surface, molecular layer and basement membrane of blood vessels in P1, P7, P21 and adult mice cerebella. Furthermore, laminin is present in the internal granule layer of P7 mice cerebella.

8.2 Additional cell culture experiments

8.2.1 Astrocytes

The percentage of astrocytes compared to the total amount of neurons per coverslip is assessed by staining coverslip of each of the eight laminin isoforms with GFAP (1:15000, mouse, Sigma). Next, six pictures of each coverslip are taken with a confocal microscope. On each picture the amount of astrocytes and neurons are counted and the percentage of astrocytes compared to the amount of neurons is calculated. Lastly, the mean percentage of astrocytes per coverslip is assessed by taking the mean of the six pictures. Two vials were used in this experiment and two coverslips of each laminin substrate was used.

Figure 14A shows confocal images of substrates stained with GFAP, TUJ1 and DAPI. It was hard to find six places on a coverslip that contained astrocytes. This means that during the process of making images, specific areas of the coverslip needed to be found that contained astrocytes. So, the images could not be taken in random places. This makes the data gathering part of this experiment already biased. Figure 14B shows bar charts of the mean percentage of astrocytes compared to neurons for each substrate. As can be seen in the first bar chart the percentage of astrocytes on laminin-221 and -411 is the largest. The percentage of astrocytes compared to neurons is the least on laminin-421 and -511. However, the standard error of the data is really large in laminin-221 and -411 making the data untrustworthy. Furthermore, the second bar chart shows the mean percentage of astrocytes compared to neurons per cell vial used. The trend shown by both bars representing different vials differ greatly. Because, of the aforementioned reasons this data was not included into the main paper. The reason for the inconsistency could stem from the fact that random images of the coverslip could not be taken but, an extensive search was needed to find astrocytes on the coverslip. The lack of astrocytes could be due to the adding of ARA-C to the culture medium, which is known to halt glial cell formation.

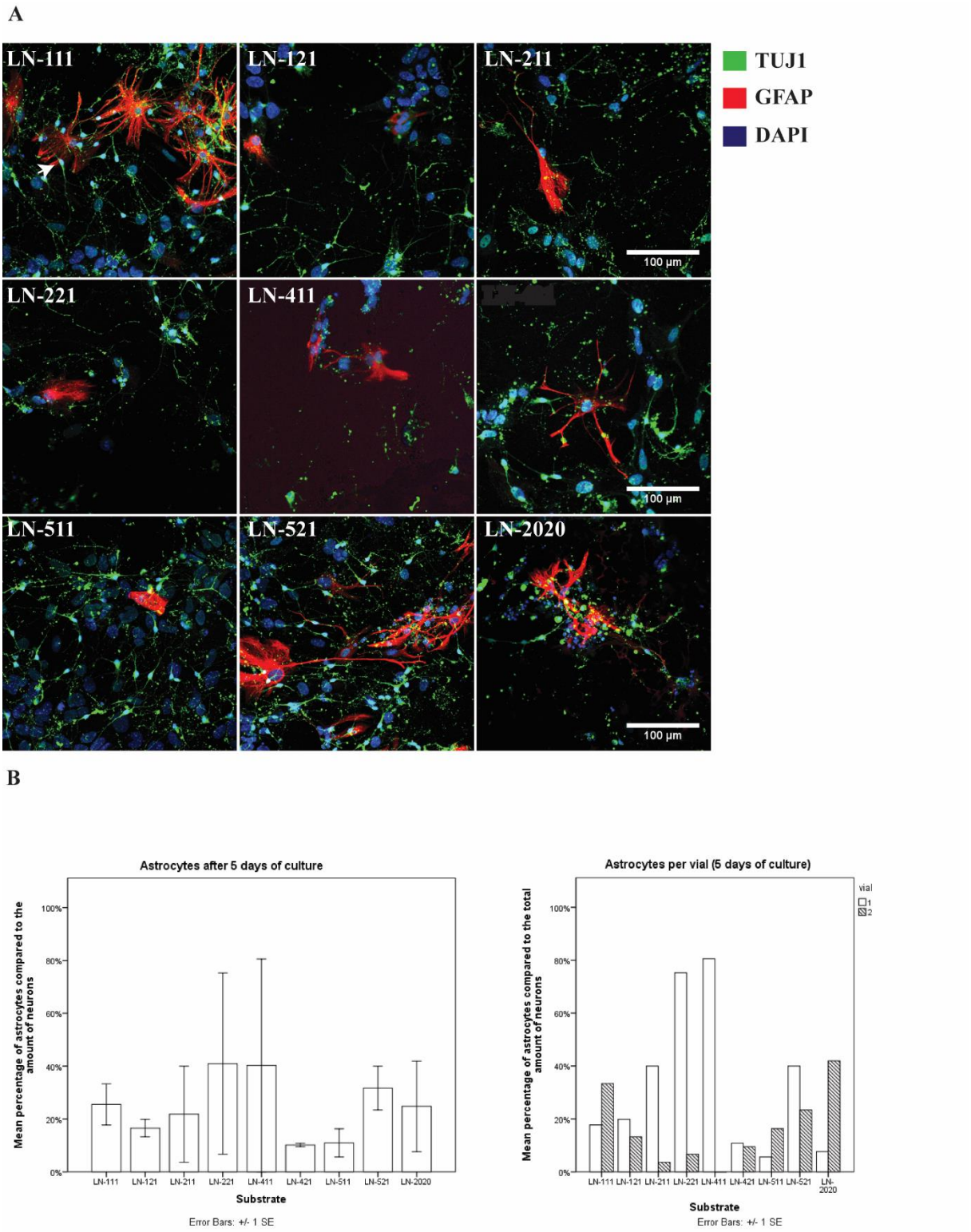

Figure 14: A shows confocal images of astrocyte and neuronal staining for all of the eight included isoforms and laminin L2020. Part B shows two bar charts. The first one displays the mean percentage of astrocytes compared to the total amount of neurons between different substrates. The second one compared the astrocyte data between the different vials used

8.2.2 Morphology

In this study morphology was defined as the polarity of neurons (uni-polar, bi-polar or multi-polar neurons). After studying the neuronal cultures on the different coverslips no uni-polar neurons were detected. The bulk of the neurons developed into multi-polar neurons. Bi-polar neurons were also detected but to a smaller amount. Therefore, morphology was calculated as the percentage of bi-polar neurons compared to the total amount of neurons. This gives both an overview of the ability of substrates to culture bi-polar neurons as well as multi-polar neurons. Bi-polar neurons were defined as having not more than two extensions originating from the cell soma, while multi-polar neurons have three or more neurites extending from their soma. The first image of figure 14A shows a bi-polar neuron via an arrow. The morphology was assessed both after 5 days and 10 days of culture by taking 6 pictures of each coverslip and calculating the percentage of bi-polar neurons compared to the total amount of neurons for each picture. Next, the average percentage of bi-polar neurons compared to the total amount of neurons for each coverslip was calculated.

Figure 15 shows the bar charts belonging to the morphology data. With 15A showing the morphology data belonging to the substrates after 5 days of culture, 15B after 10 days of culture and 15C compared the data between culture days. Figure 15A does not show large differences between the morphology data and the substrates used. A statistical analyses by means of an ANOVA confirms this, F (8, 21) = 2.199, p = 0.071. Figure 15B also does not show large difference between morphology data. However, this time a significant difference is found, F (5, 24) = 6.788, $p < 0.001$. A post hoc analyses shows that the mean percentage of bi-polar neurons compared to the total amount of neurons significantly differs between laminin-111 and laminin-511, -521 and L2020, p = 0.024, 0.003 and 0.007 respectively. Figure 15C shows that the percentage of bi-polar neurons is higher after 10 days compared to 5 days of culture than 5 days. A factorial ANOVA does indeed show a significant effect of the amount of culture days on the percentage of bi-polar neurons compared to the total amount of neurons, F (1, 42) = 26.573, $p < 0.001$.

The reason why this data is not in the main paper is because it is hard to distinguish between bi-polar and multipolar neurons on images of low magnification. However, a high magnificent will not visualize a lot of neurons. If six pictures are taken randomly the changes are high that there are no bi-polar neurons present on the picture. If it is not taken randomly than a bias will be created because of the specific search of bi-polar neurons. So, in short because it is likely that neurons were counted as bi-polar or multi-polar even though they are not it is hard to make definite statements. This could be solved by letting multiple people investigate the pictures and determine the percentage of bi-polar neurons compared to the total amount of neurons. In the end an average over the data could be made.

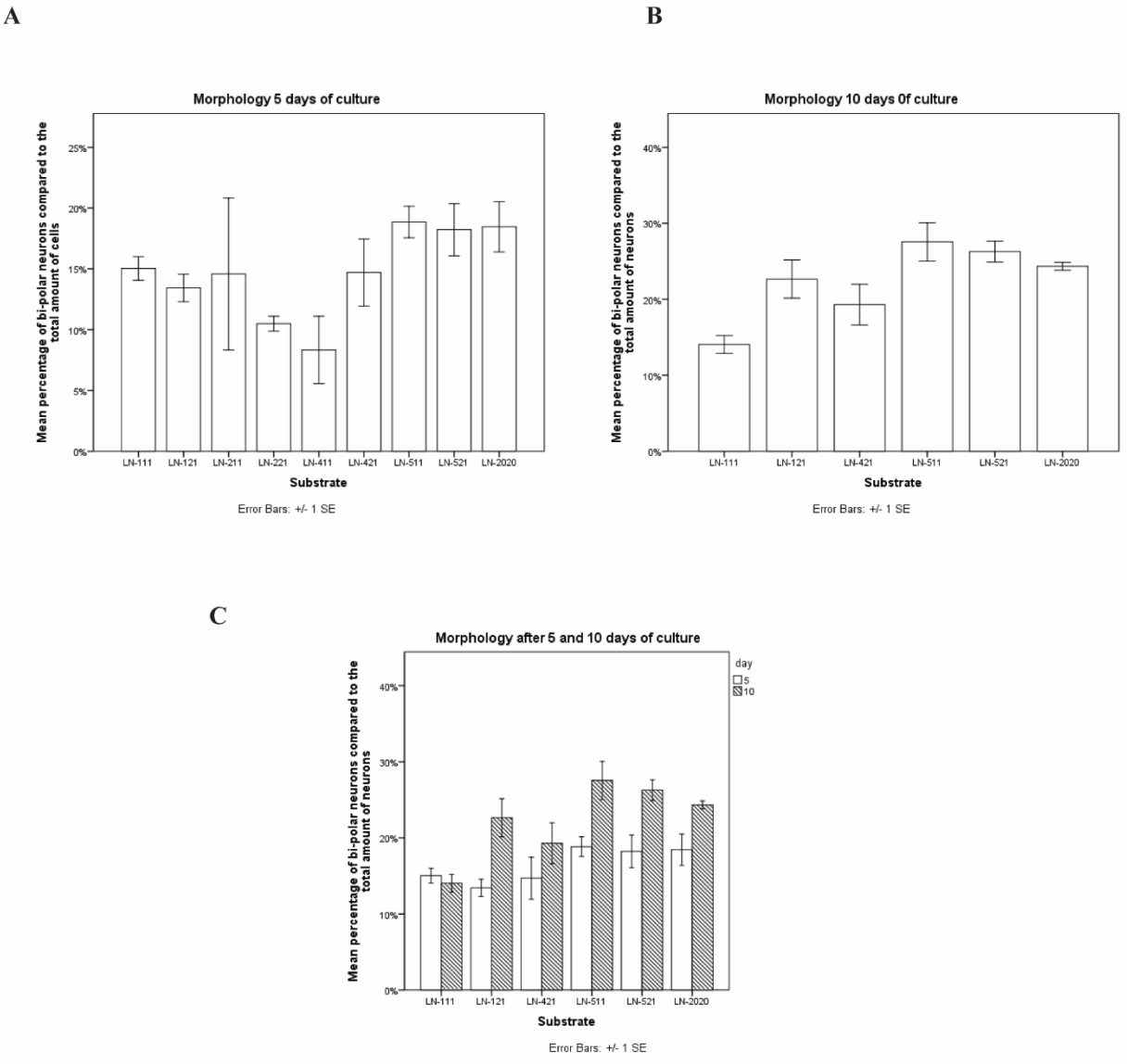

Figure 15: This image shows three bar charts containing information regarding the morphology experiments. Part A shows the mean percentage of bi-polar neurons compared to the total amount of neurons after 5 days of culture. Part B shows the mean percentage of bi-polar neurons compared to the total amount of neurons after 10 days of culture. Lastly, part C compares the morphology data between culture days.