Synthetic Polymers Provide a Robust Substrate for Functional Neuron Culture

DOI: 10.1002/((please add manuscript number))
Article type: Communication

Synthetic Polymers Provide a Robust Substrate for Functional Neuron Culture

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Keywords: neuron culture, central nervous system regeneration, polymer microarrays, synthetic polymer substrate, progenitor cell maturation

Abstract

Substrates for neuron culture and implantation are required to be both biocompatible and display surface compositions that support cell attachment, growth, differentiation and neural activity. Laminin, a naturally occurring extracellular matrix protein is the most widely used substrate for neuron culture and fulfills some of these requirements, however, it is expensive, unstable (compared to synthetic materials) and prone to batch-to-batch variation. In this study, we used a high-throughput polymer screening approach to identify synthetic polymers that supported the *in vitro* culture of primary mouse cerebellar neurons. This allowed the

identification of materials that allowed primary cell attachment with high viability even under 'serum-free' conditions with materials that supported both primary cells and neural progenitor cells attachment with high levels of neuronal biomarker expression, while promoting progenitor cell maturation to neurons.

Damage to the adult central nervous system caused by physical injuries, inflammation or cancer cannot regenerate on its own.[1] As a consequence surgical treatments such as tissue transplantation and nerve grafting, have been used for reparation of damaged regions, but encounter limitations with regard to appropriate donor sites and shortages of material and are prone to infection.[2] Tissue engineering has become a promising alternative to conventional transplantation methods with a variety of scaffolds used as a support for neuron regeneration.[3] To minimise immunological issues, the use of a patient's own cells during tissue engineering is usually considered optimal. This requires in vitro expansion of cells, and using defined cell-culture substrates would aid the regulatory approval process.[4] Currently, the number of substrates, e.g. fibronectin, collagen, polylysine, that support *in vitro* culture and specifically neuronal expansion is limited; with laminin perhaps the most widely used surface coating for *in vitro* studies.[5]

In addition, it is often difficult to maintain primary neuron cultures under serum-free conditions,[6] especially on synthetic substrates (*e.g.* poly(L-lactic acid) or poly-Lysine).[7] Thus to conduct research on neural regeneration as well as to achieve clinical translation, factors that influence neuron growth need to be understood, ideally with neurons that are cultured in chemically defined media to minimise the influence of growth factors and/or extracellular matrix proteins – which again would cause problematic regulatory issues. Serum-free media such as Neurobasal medium (Gibco, Life Technologies) supplemented with B27 and L-glutamine has been found to be suitable, however, cells plated at low densities show significantly reduced viability.[8]

Hence, we set out to discover new synthetic substrates that support neuron cell culture looking at both primary mouse neurons[9] and mouse embryonic stem cell derived neural progenitor cells (NPCs).[10] Primary neurons are an archetypal source for cell replacement therapy in human neurodegenerative diseases, while the electrical responses of NPCs are well characterised in a range of neurobiologcal studies,[11] and as such are highly relevant for the evaluation of new biomaterials suitable for functional neuron culture.

To identify synthetic polymers for culturing functional neurons, polymer microarrays were applied, a high-throughput tool that allows the parallel screening of hundreds of polymers that in association with high-content microscopy allows the independent evaluation of the interactions of all polymer features with the cells of interest.[12] Contact[13] and inkjet printing[14] methods have both been used for the fabrication of polymer microarrays, with polymers identified for numerous biological applications including bacteria-repellent coatings for medical devices, controlled expansion of human embryonic stem cells, thermal harvesting of mesenchymal stem cells and activation of platelets.[13-14] In comparison to other synthetic substrates used for neuron culture, e.g. polylysine and polyornithine, polyacrylate based polymers permit tunable polymer properties and molecular weight, while providing the possibility of crosslinking of the linear polymers into 3D tissue engineering scaffolds.

In this study we targeted the identification of polymeric substrates that would support the ‘serum-free’ culture of neurons with the medium NS21 with no additional supplements with a polymer microarray screen conducted to identify those polymeric substrates that best supported neuron attachment, growth, differentiation and “communication”. The “lead” polymers were scaled-up and shown to support neuron growth with multiple biomarkers expressed at significantly higher levels than equivalent cells grown on laminin-coated substrates.

Dissociated primary cerebellar cells were isolated from E17.5 mice and used for microarray screening studies (**Figure 1**). The polymer microarray of 1536 features (that included 4 replicates for each polymer composition) was fabricated by contact printing of pre-synthesised polymers with cell attachment quantified by counting of stained nuclei.[13a] The chemical compositions of the 15 "hit" polymers that supported cell attachment with or without serum supplement are shown in ESI, **Figure S1**. It is worth noting that all of the polymers contained functional groups that would be positively charged under physiological conditions due to the presence of tertiary amines, pyridines and imidazole groups - that would interact with the surface of cells.[15]

Mitogen activated protein kinase (MAPKinase) is involved in cell growth, proliferation and signal transduction,[16] with some MAPKinases (*e.g.* p38 MAPKs) involved in the activation processes for neuron differentiation,[16a] and thus was used here as a marker during screening. Caspase-3 is a critical mediator of cellular apoptosis and was used to evaluate cell viability.[17] Detailed screening identified three "lead" polymers (PA186, PA414 and PA529) that were observed to bind cells (both with and without serum supplements, **Figure 1b**), while showing low Caspase-3 levels and expressing high levels of MAPKinase even without serum supplementation (see **Figure 1d** and **Table S1**). Other polymers such as PA299, while providing good levels of cellular attachment, showed low levels of MAPKinase expression. The three "lead" polymers (PA186, PA414, or PA529) have different chemical compositions but also some similarities: PA186 contains HEMA that will generate a layer of water of hydration on the surface, while 4-vinylpyridine will be protonated at physiological pH, thus promoting cell binding. The same is true of PA529 that contains DEAEMA – protonation will drive cell binding. PA414 contains MEMA, DEAEMA and MA. The surface of mammalian cells (unlike bacteria) contains both cationic and anionic groups and thus a zwitterion polymer would be expected to be both highly solvated as well as attractive to mammalian cells. Thus

the cell binding can be rationalised in all cases – but with subtle differences that explain cellular binding alterations/behaviour.

The three "lead" polymers were re-synthesised *en masse* (several grams) using free radical polymerization with characterisation data shown in **Table 1** and studies undertaken by coating of these polymers onto glass coverslips and evaluation with the culture of primary neuron cells. The polymer coatings with thickness of 193, 372 and 433 nm (for PA186, PA414 and PA 529, respectively) were confirmed using atomic force microscopy (AFM) (see ESI, **Figure S2**). The expression of MAPKinase by the cells growing on these three polymers under 'serum-free' conditions were similar to those observed for cells growing on laminin-coated coverslips with serum containing media (**Figure 2**).

As is usual in any high-thought assay initial validation was used to select "leads" that were subsequently evaluated in more detailed assays. Subsequent studies using NPCs (on the "lead" polymer PA186, PA414 and PA529) showed similar results to the primary neurons, with all three polymers supporting NPC growth (without serum) with comparable levels of MAPKinase expression to cells grown on laminin with serum supplement. One polymer PA186 consistently "allowed" cells to show higher levels of MAPKinase than the other two polymers and the "gold standard" laminin (see **Figure 3**).

Glial fibrillary acidic protein (GFAP) is an intermediate filament protein expressed by numerous cell types of the central nervous system including astrocytes. β-III-tubulin (TUJ1) is expressed almost exclusively by neurons. Therefore, comparison of expression of these two markers allowed quantification of the maturation of NPCs to neurons. Thus NPCs cultured on these three polymers were analysed for the expression of GFAP[18] and TUJ1[19] studied using immunohistochemistry, to determine the maturation progression of NPCs into neural cells.

As a proof of concept, NPC maturation was initially characterised on the current 'gold standard' substrate, laminin, over 15 days, quantifying total cell numbers and also the neuron *vs.* non-neuron populations (see ESI, **Figure S3**). The total number of cells/mm^2 (quantified

by nuclear staining) did not vary significantly during the 15-day culture period (**Figure S3b**), while in contrast, the percentage of both TUJ1-positive (neuron) and GFAP-positive (non-neuron) cells increased significantly between days 1 and 10 (**Figure S3c**). However, neither the percentage of TUJ1-positive nor the mean dendritic length varied significantly over day 10 to day 15. Therefore the incubation time of 10 days was selected for further studies. NPCs cultured for 10 days on PA186, PA414, PA529 and laminin coated coverslips (with no serum supplement) showed high cell numbers (except for PA414), while immunohistochemistry revealed that cells cultured on PA186 and PA414 had significantly higher TUJ1 expression than laminin, indicating the higher differentiation potential of NPCs to neurons (see **Figure 4**). It is worth noting that PA414 provided limited cell attachment (**Figure 1**) but promoted NPC maturation and expression of TUJ1.

The functionality of neurons was studied by comparing the firing frequencies of action potentials [11] of the cells cultured on the three 'hit' polymers (with no serum supplement) (see **Figure 5**).[9] Neurons on PA186 showed significantly higher firing frequency than cells grown on PA529 or laminin. Thus, this study has revealed a novel polymer, PA186, as an excellent substrate for neuronal culture (**Figure 5d**).

In summary, A library of 382 polymers was screened with primary cells dissociated from the cerebellum of mouse embryos, and a high-throughput screen identified three polymeric substrates that allowed attachment of these cells (with no/low caspase-3 expression), yet showing high levels of MAPKinase, even in the absence of serum supplementation. Scale-up studies conducted by culturing these primary cells on glass coverslips coated with the three polymers, PA186, PA414 and PA529, showed high cellular attachment and high levels of MAPKinase expression under 'serum-free' conditions, giving similar levels to cells grown on laminin with serum supplement. Thus these polymers have the potential to be the substrates for primary neuron culture. Culture of NPCs revealed that PA186 allowed higher MAPKinase and TUJ-1 expression and demonstrated higher intrinsic spike activity than the other 'hit'

polymers as well as the 'gold standard', laminin (which has hugely variable batch-to-batch limitations). This study shows that polymer PA186 has the potential to replace laminin as a substrate for the 'serum-free' culture of primary and progenitor cerebellar neurons.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements
We thank European Research Council (Advanced Grant ADREEM ERC-2013-340469), Netherlands Organization for Scientific Research (NWO-VENI-STW-13075), the Dutch Organization for Medical Sciences, Life Sciences and Social and Behavioral Sciences for funding and Dr Alison McDonald for assistance with AFM.

Y.Z., G.A.H. and S.V. contributed equally to this work.

Received: ((will be filled in by the editorial staff))
Revised: ((will be filled in by the editorial staff))
Published online: ((will be filled in by the editorial staff))

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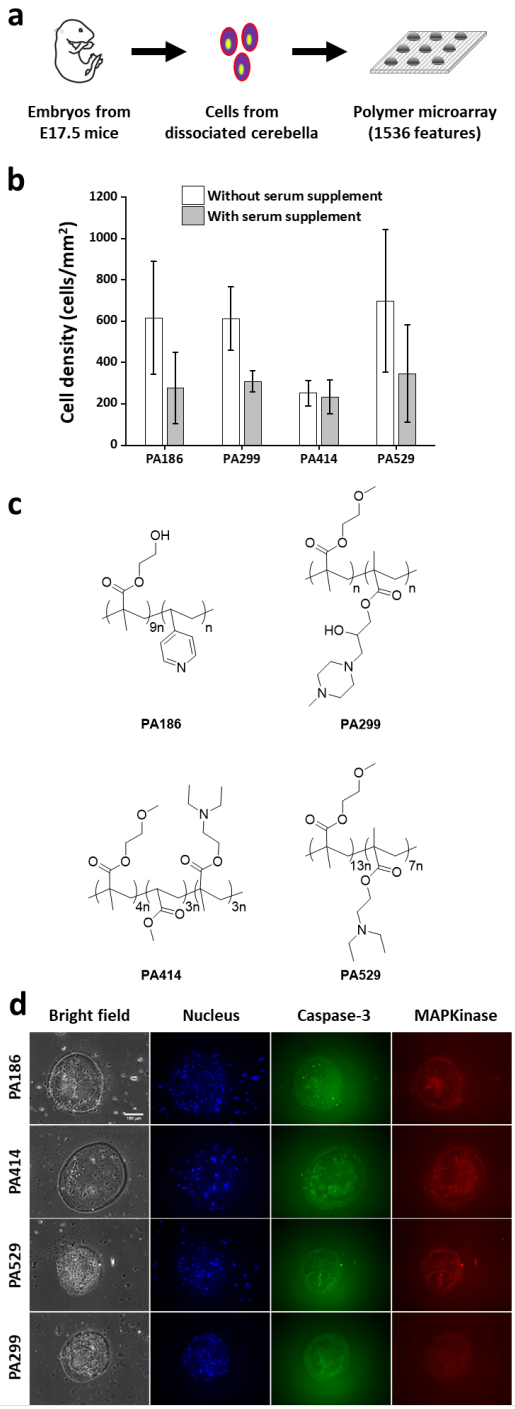

Figure 1. (a) Dissociated primary cerebellar cells from mouse embryos were cultured (24 h) on polymer microarrays. (b) Three "lead" polymers (PA186, PA414 and PA529) along with PA299 (as a cell binding control) supported robust primary cerebellar cell attachment (both with and without serum supplement) with cell densities determined by counting DAPI-stained nuclei (n = 8); (c) Chemical structures of the four polymers PA186, PA414, PE529 and PA299; (d) Examples of polymers showing cell attachment and expression of Caspase-3 and MAPKinase (see quantification of MAPKinase expressions in ESI, Table S1). Nucleus (blue, $\lambda_{Ex/Em}$ = 364/454 nm), Caspase-3 (green, $\lambda_{Ex/Em}$ = 490/520 nm), MAPKinase (red, $\lambda_{Ex/Em}$ = 548/562 nm). Scale bar: 50 µm.

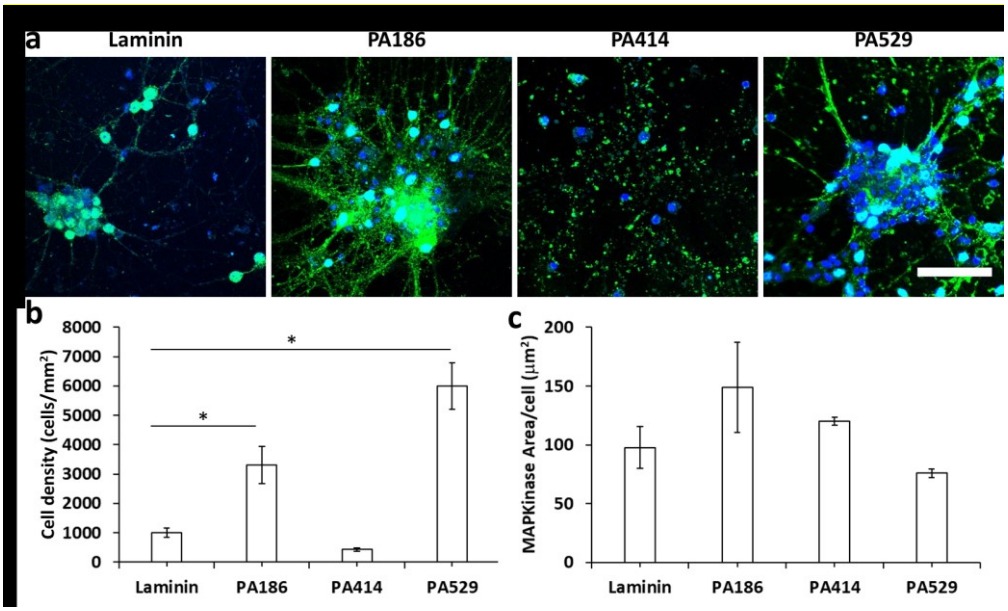

Figure 2. (a) Dissociated cerebellar primary cells as cultured (for 10 d) on 13 mm diameter coverslips coated with Laminin, PA186, PA414 and PA529, showing MAPKinase expression (green) and cell number (DAPI, blue). Scale bar: 50 µm. (b) Densities of cells attached to different substrates and (c) their MAPKinase expression level.

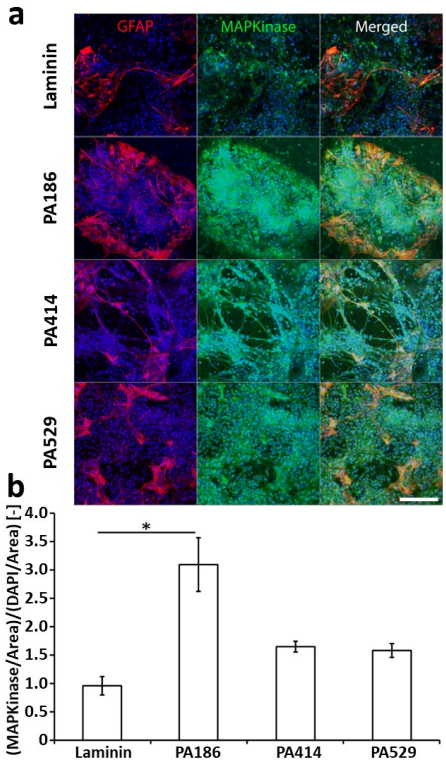

Figure 3. NPCs cultured (10 d) on glass coverslips coated with Laminin, PA186, PA414, or PA529 with all cells stained with DAPI. (a) NPCs expressing glial fibrillary acidic protein (GFAP) (red) and MAPKinase (green); (b) MAPKinase expression by the NPCs. Data are represented as the mean and standard error of mean (n = 10 images from 5 experimental runs from 5 cell batches), which were assessed *via* one-way ANOVA followed by Tukey's with statistical significance set at $P \leq 0.01$. Scale bar: 100 µm.

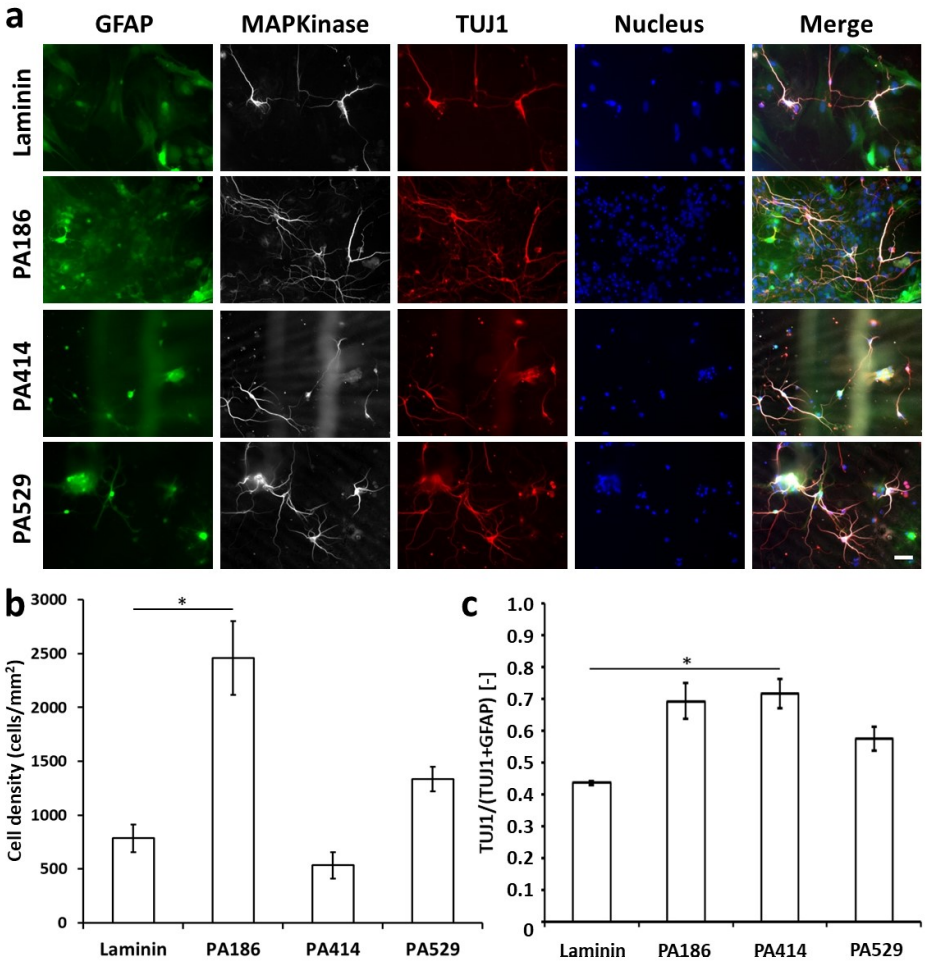

Figure 4. (a) GFAP-positive cells expressed GFAP (green), MAPKinase (white) and TUJ1 (red) with nucleus shown in blue. (b) Density of NPCs attached to substrate. (c) The ratio of TUJ1-positive cells to GFAP-positive cells grown on laminin, PA186, PA414 or PA529 coated coverslips. Data are shown as the mean and standard error of mean (n = 10 images from 3 experimental runs consisting of 3 cell batches), which were statistically evaluated *via* one-way ANOVA followed by Tukey's with statistical significance set at $P \leq 0.01$. Scale bar: 50 µm.

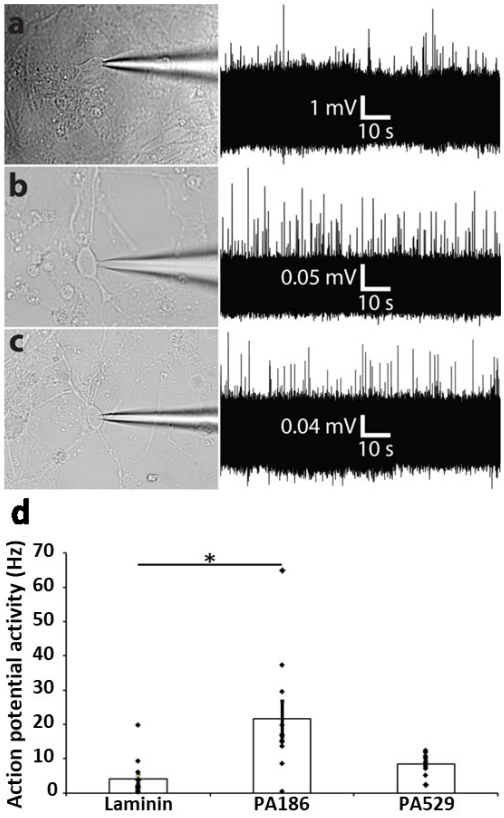

Figure 5. Spiking activity of neurons recorded in cell-attached configuration (n = 15 cells/substrate) on: (a) Laminin; (b) PA186; and (c) PA529; (d) Average intrinsic spike activity of neurons on the substrates. Data are shown as the mean and standard error of mean (n = 15 cells from 5 experimental runs consisting of 5 cell batches), which were statistically compared *via* one-way ANOVA followed by Tukey's with statistical significance set at $P \leq 0.01$. (Neurons cultured on PA414 did not survive the preparation necessary for electrophysiological recordings.)

Table 1. Characterisation of the "hit" polymers with the details of the specific functional groups of the polymers. M_n, M_w and dispersity (Đ) were quantified by GPC (DMF) with calibration using poly(methyl methacrylate) as standards.

Polymer	Yield (%)	M_n (kDa)	M_w (kDa)	Đ	Functional groups	
PA186	89	41	58	1.4	Hydroxy	Pyridine
PA414	95	50	72	1.4	Tertiary amine	-
PA529	95	183	565	3.0	Tertiary amine	-

Synthetic polymeric substrates for *in vitro* neuron culture were identified via a high throughput approach. Polymeric substrates were shown to be superior to the current gold standard laminin showing high levels of neuron and progenitor cell attachment, with the substrate significantly promoting progenitor cell maturation with remarkably high expression of neuron related biomarkers in the absence of serum.

Keyword neuron culture, central nervous system regeneration, polymer microarrays, synthetic polymer substrate, progenitor cell maturation

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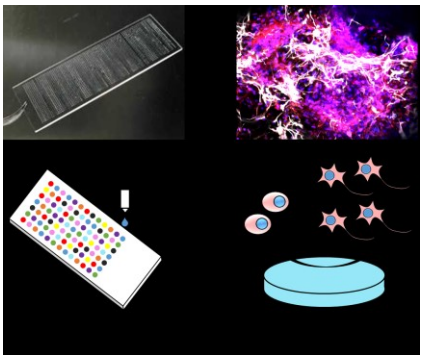

Supporting Information

Synthetic Polymers Provide a Robust Substrate for Functional Neuron Culture

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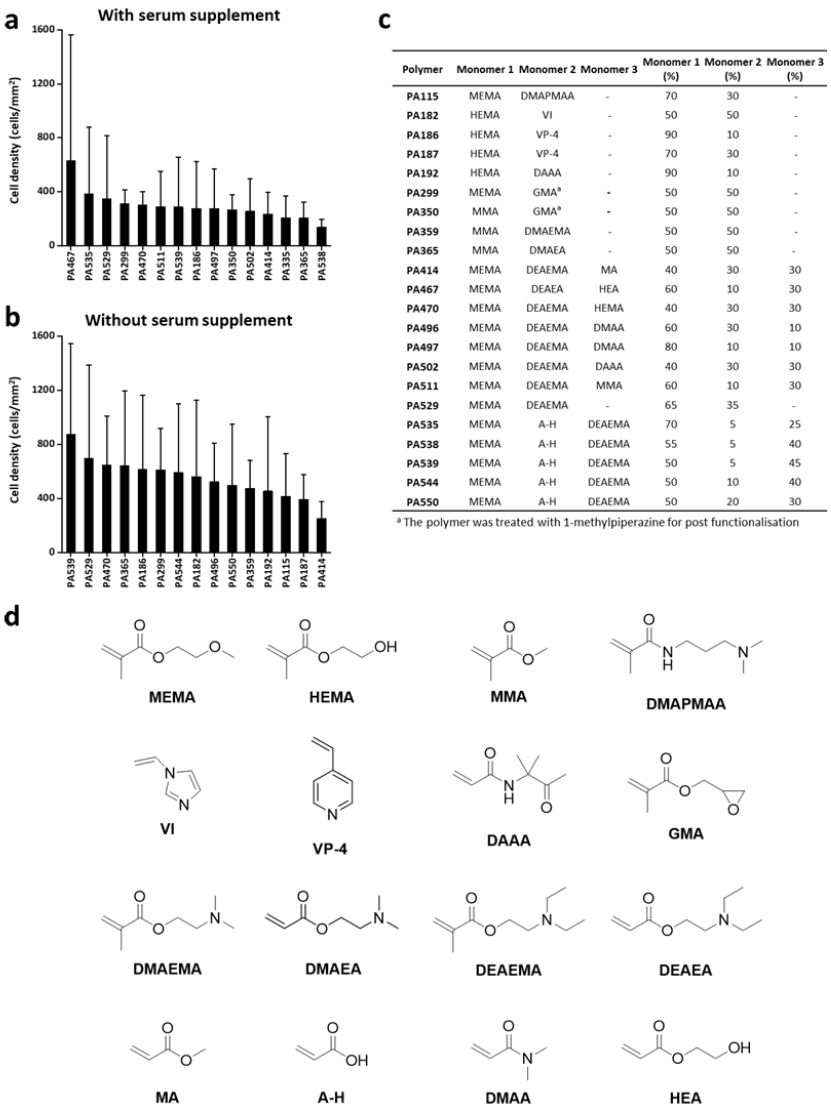
S1. Supporting Tables and Figures

Table S1. Quantification of MAPKinase expressions of primary neurons cultured on the "lead" polymers PA186, PA414 and PA529, with PA299 as a negative control (features without polymer coating were used as a blank to give background fluorescence levels). Fluorescence intensities were quantified using ImageJ.

Polymers	Fluorescence intensity (A.U.)	Fluorescence intensity difference from blank (A.U.)	Standard deviation
Background	251.8	-	0.47
PA186	283.7	31.9	6.42
PA299	261.9	10.1	3.10
PA414	279.4	27.6	1.59
PA529	302.9	51.0	8.17



Polymer	Monomer 1	Monomer 2	Monomer 3	Monomer 1 (%)	Monomer 2 (%)	Monomer 3 (%)
PA115	MEMA	DMAPMAA	-	70	30	-
PA182	HEMA	VI	-	50	50	-
PA186	HEMA	VP-4	-	90	10	-
PA187	HEMA	VP-4	-	70	30	-
PA192	HEMA	DAAA	-	90	10	-
PA299	MEMA	GMA[a]	-	50	50	-
PA350	MMA	GMA[a]	-	50	50	-
PA359	MMA	DMAEMA	-	50	50	-
PA365	MMA	DMAEA	-	50	50	-
PA414	MEMA	DEAEMA	MA	40	30	30
PA467	MEMA	DEAEA	HEA	60	10	30
PA470	MEMA	DEAEMA	HEMA	40	30	30
PA496	MEMA	DEAEMA	DMAA	60	30	10
PA497	MEMA	DEAEMA	DMAA	80	10	10
PA502	MEMA	DEAEMA	DAAA	40	30	30
PA511	MEMA	DEAEMA	MMA	60	10	30
PA529	MEMA	DEAEMA	-	65	35	-
PA535	MEMA	A-H	DEAEMA	70	5	25
PA538	MEMA	A-H	DEAEMA	55	5	40
PA539	MEMA	A-H	DEAEMA	50	5	45
PA544	MEMA	A-H	DEAEMA	50	10	40
PA550	MEMA	A-H	DEAEMA	50	20	30

[a] The polymer was treated with 1-methylpiperazine for post functionalisation

Figure S1. (a) and (b) The top 15 polymers that supported dissociated embryonic cerebellar neurons with and without serum supplement (n = 8). Cell density was determined by counting stained nucleus over the area of polymer spots. (c) Chemical compositions of the top polymers with monomer structures shown in (d).

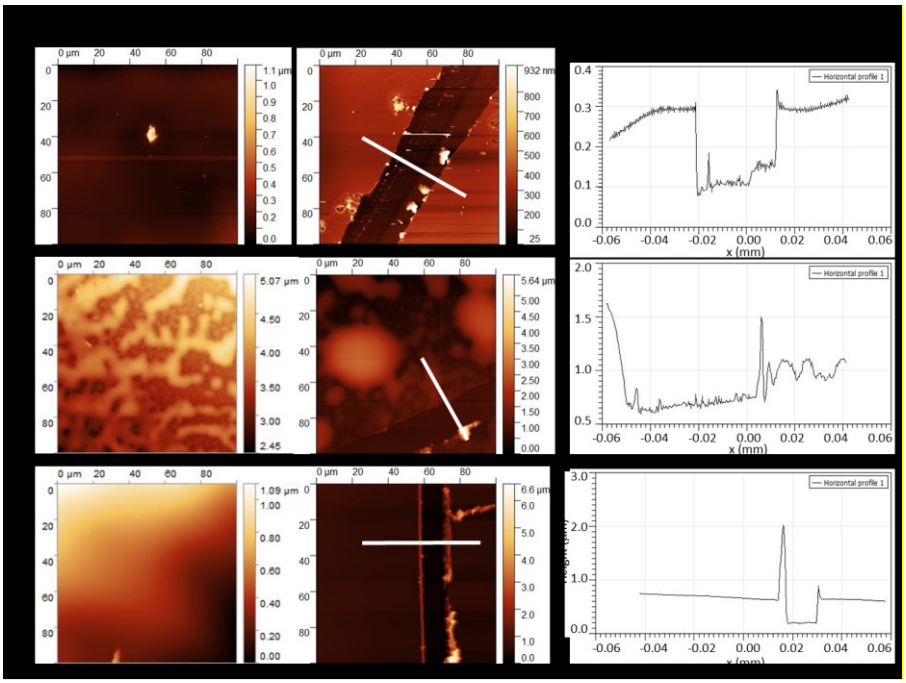


Figure S2. AFM analysis of "lead" polymer PA186, PA414 and PA529 coated coverslips showing the successful polymer coatings on coverslips with a "scratch" experiment quantifying the thicknesses of the coatings: 193 ± 12, 372 ± 27 and 433 ± 35 nm for PA186, PA414 and PA529, respectively (n = 5).

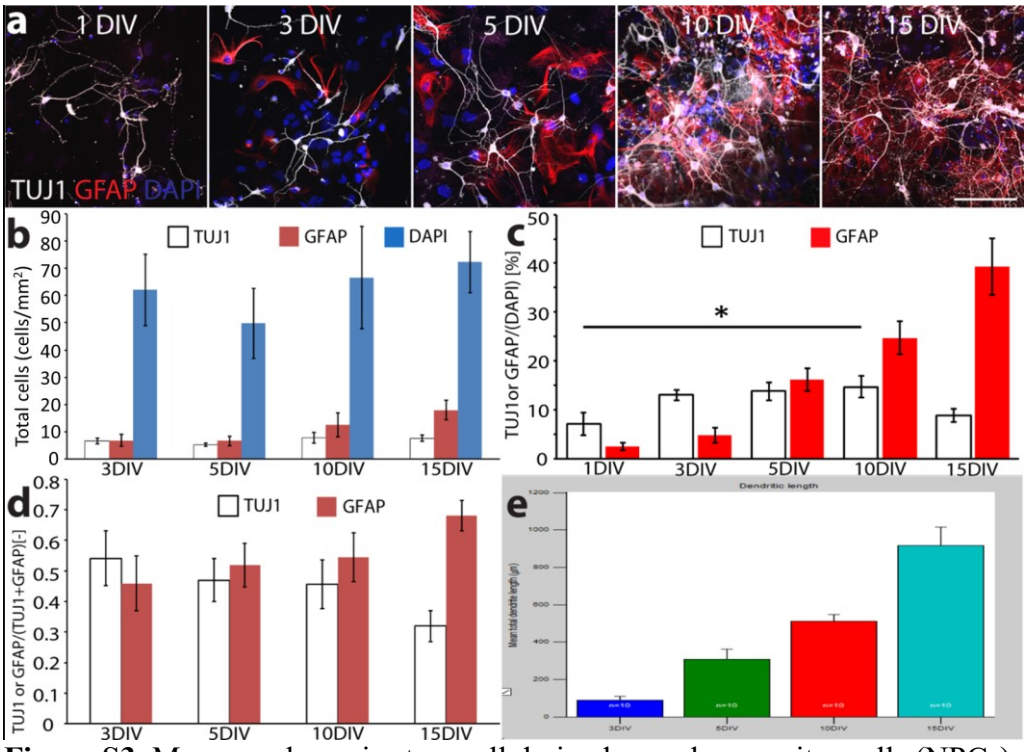


Figure S3. Mouse embryonic stem cell derived neural progenitor cells (NPCs) cultured on Laminin were analysed for up to 15 days *in vitro* (DIV) with respect to cell numbers, the changes in neuron vs. non-neuron cell populations, and the mean dendritic length. (a) NPCs matured in NS21 medium displayed β-III-tubulin (TUJ1), glial fibrillary acidic protein (GFAP) and DAPI during 15 DIV. (b) Cell densities (cells/mm^2) of TUJ1-positive cells and GFAP-positive cells were calculated from the total number of cells (DAPI). (c) Percentage of TUJ1-positive and GFAP-positive cells were estimated from the total number of cells (DAPI). (d) The ratio of TUJ1-positive cells to GFAP-positive cells (TUJ1/TUJ1+GFAP) and vice

versa were quantified. (e) Mean dendritic length of neurons was quantified via sholl analysis. The dendritic length of neurons was traced with semi-automated neurite tracing adapted MATLAB-code Syn-D[20] and were processed further with MATLAB-2010a (Mathworks, USA). Data are shown as the mean and SEM (n = 10 images from 5 experimental runs consisting of 5 cell batches), which were statistically evaluated via one-way ANOVA followed by Tukey's with statistical significance set at $P \leq 0.01$. Scale bar: 100 µm.

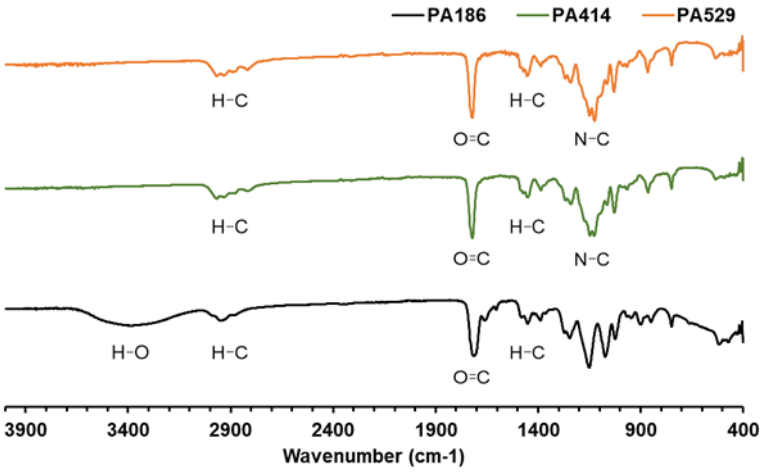


Figure S4. FTIR spectra of "lead" polymers PA186, PA414 and PA529.

S2. Materials and Methods

S2.1. General information

All chemicals and solvents were purchased from Alfa Aesar, Fisher Scientific or Sigma Aldrich and used as received. Glass coverslips (13 mm) were purchased from Assistent, Germany. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AVA500 spectrometer (500 and 125 MHz, respectively) at 298 K in deuterated solvents. Chemical shifts for proton and carbon spectra are reported on the δ scale in ppm. Polymers were analysed by gel permeation chromatography (GPC) using two PLgel MIXED-C columns (200-2,000,000 g mol^{-1}, 5 µm) using DMF with 0.1% w/v LiBr at 60 °C at 1 mL min^{-1} as an eluent. Molecular weights obtained were relative to narrow dispersity poly(methyl methacrylate) standards. The IR spectra were recorded on an IRAffinity-1S compact Fourier transform infrared spectrophotometer (SHIMADZU). Each spectrum from 4000 to 400 cm^{-1} was averaged over 32 scans at a resolution of 2 cm^{-1}.

S2.2. Animals

All experimental procedures involving animals were approved by an independent animal ethical committee (DEC consult, Soest, The Netherlands) and/or by the national authority (*Centrale Commissie Dierproeven*, The Hague, The Netherlands) and performed in accordance to Dutch legislation and institutional guidelines (Erasmus MC, Rotterdam, and Netherlands Institute for Neuroscience, Amsterdam).

S2.3. Dissociation of cerebellar neuron cells

Mouse embryos were removed by caesarean section from deeply anesthetised pregnant female mice at E16-18. After rapid decapitation, the cerebella were dissected and dissociated as previously described.[1] A suspension of the dissociated neurons from 16 mouse embryos (E17.5) from 3 pregnant females was prepared in NS21 medium.

S2.4. Polymer microarray fabrication and screening using dissociated embryonic cerebellar neurons

Polymer microarrays were fabricated by contact printing pre-formed polymers as described previously.[2] Briefly, solutions of pre-synthesised polymers (1% in *N*-methyl-2-pyrrolidinone) were contact printed (Qarray mini, Genetix) onto an agarose coated 25 x 75 mm glass slide with 1536 features of 382 polymers with 2 negative controls (NMP treated agarose) in quadruplicate. The slides were dried overnight under vacuum at 40 °C and sterilised using ultraviolet light on both sides (20 min each side) prior to use.

Primary neurons were seeded onto the microarrays at a density of 1×10^7 cells/mL and incubated for 24 h in NS21 medium on four identical polymer microarrays under standard cell culture conditions.[1] The NS21 medium of two of the microarrays was supplemented with 10% FBS, while two of the arrays contained no FBS. After 24 h, the medium was removed and polymer arrays were washed with PBS (x1), fixed with paraformaldehyde (4%), stained with DAPI and immunohistochemistry performed for expression of MAPKinase and Caspase-

3 followed by high-content imaging of each feature using a Nikon Eclipse 50i microscope with the Pathfinder software (IMSTAR™, France). Quantification of was carried out by counting number of DAPI-stained nuclei on polymer spots.

S2.5. Cell culture of neural progenitor cells

NPCS were cultured as previously described.[3] Thus NPCs from two C57BL/6 mice and one actin-GFP mouse[4] were cultured in NPC medium containing DMEM/F12 (Gibco), N2 supplement (1%, Invitrogen), B27-RA supplement (1:50, Invitrogen), laminin (1 µg/ml, Sigma-Aldrich), mouse bFGF (20 ng/ml, Invitrogen), penicillin/streptomycin (1%, Sigma-Aldrich). 6-well plate (10 cm^2 per well, Greiner Bio-One), coated with laminin (50 ug/ml, 30 min incubation at 37℃) were used for the experiments. NPCs were seeded (3×10^5) immediately after removing excess of laminin and adherent cultures were refreshed every other day with NPC medium (2 mL) and passaged (1:4) every 4 days for up to 6 passages. NPCs were either used for experiments after passaging or preserved for later use after washing with PBS, detaching with 0.25% trypsin-EDTA (Sigma-Aldrich) for 5 min at 37℃, resuspension in DMEM/F12 with 10% FBS (Lonza), centrifugation (300 g, 5 min), followed by resuspension of FBS containing DMSO (10%) in cryotubes and freezing in liquid nitrogen.

S2.6. Synthesis and characterisation of 'hit' polymers

PA 186

2–Hydroxyethyl methacrylate (6.84 mL, 56.4 mmol) and 4-vinyl pyridine (0.676 mL, 5.64 mmol) were filtered through basic alumina and added to a reaction vessel. Azobisisobutyronitrile (AIBN) (10.1 mg, 0.62 mmol) in DMF (12 mL) was then added to the mixture. The solution was purged with N_2 for 30 min and stirred at 60 °C for 24 h. The solution was then poured to a mixture of hexane/diethyl ether (4:1, 500 mL) and the precipitant was collected by centrifugation and was re-dissolved in minimum amount of DCM

and precipitated from hexane (500 mL). The purified polymer was dried *in vacuo* at 40 °C in quantitative yield. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 7.49, 7.07, 4.88, 4.11, 2.86, 2.63, 2.03, 1.91, 1.28; ^{13}C NMR (125 MHz, Chloroform-*d*) δ 182.70, 157.05, 140.27, 130.34, 96.15, 92.94, 63.53, 58.39, 54.30, 37.97, 21.23; GPC: M_n 41 kDa; M_w 58 kDa; Đ 1.4.

PA 414

Ethylene glycol methyl ether methacrylate (2.85 mL, 24.8 mmol), methyl acrylate (1.68 mL, 18.6 mmol) and 2-(diethylamino)ethyl methacrylate (3.74 mL, 18.6 mmol) were filtered through basic alumina and added to a reaction vessel. AIBN (10.1 mg, 0.62 mmol) in toluene (12 mL) was then added to the mixture. The solution was purged with N_2 for 30 min and stirred at 60 °C for 24 h. The solution was then poured into hexane (500 mL) and the precipitant was collected by centrifugation which was re-dissolved in a minimum amount of DCM and precipitated from hexane (500 mL). The purified polymer was recovered in quantitative yield after drying *in vacuo* at 40 °C . ^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.23 – 3.95, 3.61, 2.72, 2.66 – 2.54, 2.11 – 1.75, 1.62, 1.28, 1.07, 0.93; ^{13}C NMR (125 MHz, Chloroform-*d*) δ 177.47, 70.04, 69.90, 63.89, 58.87, 50.51, 47.64, 45.11, 44.76, 13.78, 12.13; GPC: M_n 50 kDa; M_w 72 kDa; Đ 1.4.

PA 529

Ethylene glycol methyl ether methacrylate (5.85 mL, 40.3 mmol) and 2-(diethylamino)ethyl methacrylate (4.36 mL, 21.7 mmol) were filtered through basic alumina and added to a reaction vessel. AIBN (10.1 mg, 0.62 mmol) in toluene (12 mL) was then added to the mixture. The solution was purged with N_2 for 30 min and stirred at 60 °C for 24 h. The solution was then poured into hexane (500 mL) and the precipitant was collected by centrifuge and was re-dissolved in minimum amount of DCM and precipitated from hexane (500 mL). The purified polymer was recovered in quantitative yield after drying *in vacuo* at

40 °C. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 4.20 – 4.08, 4.02, 3.60, 3.46 – 3.34, 2.72, 2.65 – 2.57, 2.05 – 1.71, 1.11 – 0.99, 0.94; ^{13}C NMR (125 MHz, Chloroform-*d*) δ 178.10 – 176.32, 70.03, 69.89, 63.84, 63.32, 58.87, 54.16, 50.51, 47.64, 45.13, 44.79; GPC: M_n 183 kDa; M_w 565 kDa; Đ 3.0.

S2.7. Preparation of coverslips coated with 'hit' polymers or laminin

Polymer coated glass coverslips (13 mm) were prepared as follow. The coverslips were incubated in sodium hydroxide (10% aq with gentle shaking) for 5 h, washed with water (x5), dried in an oven at 110 °C and spin-coated with a 5% polymer solution (tetrahydrofuran for PA414 and PA529 or methanol for PA186) and dried at room temperature for 3 days. Laminin coated coverslips (13 mm) were prepared as follow. The coverslips were incubated in a polyornithine aqueous solution (0.5 mg/ml) overnight, washed with water (x5), allowed to dry at room temperature and stored at room temperature. Prior to use, the coverslips were incubated in an aqueous laminin sloution (50 µg/ml) at 37 °C for 30 min. The coverslips were removed, dried at room temperature and used directly for cell culture.

S2.8. Immunohistochemistry

Neurons on coverslips were fixed (4% PFA in water for 15 min at 4 °C), permeabilized with Triton X-100 (0.1% in PBS for 10 min), blocked with BSA (0.8% in PBS for 30 min) (washed twice with PBS after each step), and incubated overnight with primary antibodies and then for 2h with fluorescently labelled secondary antibodies at 4 °C (see below for antibodies and dilutions). The following antibodies were used at the described dilutions: GFAP (Mouse 1:15000, Sigma-Aldrich, Rabbit 1:8000, Dako), MAPkinase (Sheep 1:200, R&D Systems), β-III-tubulin (Mouse 1:2000, Chemicon), Caspase-3 (Mouse 1:7000 Swant, Rabbit 1:7000 Swant). Cy3 (mouse/rabbit/goat 1:200, Dako), and Cy5 (mouse/rabbit/sheep 1:200, Dako) were used as secondary antibodies. DAPI (Molecular Probes) was used as a

nuclear stain. Images were obtained on an upright LSM 700 confocal microscope (Zeiss), assessed with FIJI[5] and assembled by Adobe illustrator CS6.

S2.9. MAPKinase expression of primary cells on polymer-coated coverslips

Isolated dissociated cerebellar cells were directly suspended in NS21 medium and 300 µl of this medium providing a cell density of 1×10^6 cells/mL were seeded on polymer/laminin coated coverslips in a 24-well plate. The non-adherent cells were removed after 1 hour and NS21 medium (600 µl) was added to the culture (0 d), with half-volume refreshments (300 µl) of medium supplemented with cytosine β-D-arabinofuranoside (4 µM, Ara-C, Sigma-Aldrich) performed every two days over the 10 days of culture. The level of expression of MAPKinase was quantified by immunohistochemistry.

S2.10. MAPKinase expression of NPCs on polymer-coated coverslips

NPCs were cultured on PA186, PA414 and PA529 coated glass coverslips with no serum supplement, as well as laminin coated glass coverslips with 10% FBS supplement, and the expression of MAPKinase and GFAP was studied. The coverslips were imaged by confocal microscopy (LSM 700, Zeiss) and upon quantification of the normalised area expressing MAPKinase (MAPKinase area/total area over DAPI area/total area).

S2.11. Differentiation of NPCs to neurons

NPCs were suspended (10^6 cells/mL) in NS21 medium containing primary neural basal medium (PNBM, Lonza), GS21 (2%, GlobalStem), glutamax (1%, Gibco) and gentamycin (0.5 µg/ml). A 300 µl drop of NPCs suspension was seeded on 13 mm coverslips coated with laminin or the polymers, PA186, PA414, and PA529. The polymer-coated coverslips were sterilised using ultraviolet light on both sides (20 min each side). Subsequently, coverslips were washed with water (x1), and NS21 medium (x1) before cell seeding. The volume of non-

attached cells was removed after 1 hour and NS21 medium (600 µl) was added to the culture (0 d) and refreshed (600 µl) with NS21 medium supplemented (without serum) with cytosine β-D-arabinofuranoside (4 µM) every 2 days during the 10 days culture. The total cell number was quantified by measuring the area of stained nucleus and the ratio of neuron *vs.* non-neuron populations was revealed by measuring the areas of the stained glial fibrillary acidic protein (GFAP) and β-III-tubulin (TUJ1) using immunochemistry as described above.

S2.12. Action potential frequency of neurons cultured on polymer-coated coverslips

Cells cultured on coverslips (in 24-well plate with 300 µL NS21 medium) were transferred to aCSF containing (in mM): NaCl, (124); KCl, (2.5); Na_2HPO_4 (1.3); $MgSO_4$ (2); $CaCl_2$ (2); $NaHCO_3$ (26); and D-Glucose (20) (osmolarity: 295 Osm/L) for electrophysiology recordings. Since NS21 medium has a lower osmolarity than the aCSF (255 Osm/L), and in order to prevent an osmotic shock, the neurons were gradually brought to equilibrium prior to electrophysiological recordings. 200 µL of the abovementioned aCSF were added to the 1 ml NS21 medium containing the neurons, followed by removing of the added volume of the mixed solution after 3 minutes (repeated for approximately 20 min). Electrodes with resistances of 4 to 8 MΩ were pulled from thick-walled borosilicate glass capillaries and filled with the above extracellular bath solution. Data were acquired using an Axopatch 700B amplifier (Molecular Devices) and a pClamp 10 software (Molecular Devices). Offline analysis was performed with Clampfit 10.5 (Molecular Devices). All recordings were performed between 13 and 18 DIV, at room temperature.[3] Action potential activity measurements were obtained in cell-attached configuration.

S2.13. Statistical Analysis

The mean and deviation of measurements were evaluated in Matlab (Mathworks) using one-way ANOVAs followed by Tukey's post-hoc testing with statistical significance defined at p

< 0.01, unless stated otherwise. All data are reported as mean ± standard error of measurements (SEM).

S3. References

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