Substrate elasticity induces quiescence and promotes neurogenesis of primary neural stem cells – a biophysical *in vitro* model of the physiological cerebral milieu

Elasticity dependent modulation of primary neural stem cells in vitro

Stefan Blaschke[1,3], Sabine Ulrike Vay[1], Niklas Pallast[1], Monika Rabenstein[1], Jella-Andrea Abraham[2], Christina Linnartz[2], Marco Hoffmann[2], Nils Hersch[2], Rudolf Merkel[2], Bernd Hoffmann[2], Gereon Rudolf Fink[1,3], Maria Adele Rueger[1,3]

[1] Department of Neurology, University Hospital Cologne, Cologne, Germany

[2] Biomechanics Section, Institute of Complex Systems (ICS-7), Research Centre Juelich, Juelich, Germany

[3] Cognitive Neuroscience, Institute of Neuroscience and Medicine (INM-3), Research Centre Juelich, Juelich, Germany

Corresponding author:

Maria Adele Rueger, M.D.

Department of Neurology

University Hospital of Cologne

Kerpener Strasse 62

50924 Cologne, Germany

Phone: +49-221-478-87803

Fax: +49-221-478-89143

Email: maria.rueger@uk-koeln.de

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Abstract

In the brain, neural stem cells (NSC) are tightly regulated by external signals and biophysical cues mediated by the local microenvironment or “niche”. In particular, the influence of tissue elasticity, known to fundamentally affect the function of various cell types in the body, on NSC remains poorly understood. We, accordingly, aimed to characterize the effects of elastic substrates on crucial NSC functions.

Primary rat NSC were grown as monolayers on polydimethylsiloxane- (PDMS-) based gels. PDMS-coated cell culture plates, simulating the physiological microenvironment of the living brain, were generated in various degrees of elasticity, ranging from 1 - 50 kPa; additionally results were compared to regular glass plates as usually used in cell culture work.

Survival of NSC on the PDMS-based substrates was unimpaired. The proliferation rate on 1 kPa PDMS decreased by 45% compared to stiffer PMDS substrates of 50 kPa ($p<0.05$) while expression of cyclin-dependent kinase inhibitor 1B / p27Kip1 increased more than 2-fold ($p<0.01$), suggesting NSC quiescence. NSC

differentiation was accelerated on softer substrates and favored the generation of neurons (42% neurons on 1 kPa PDMS vs. 25% on 50 kPa PDMS; $p<0.05$). Neurons generated on 1 kPa PDMS showed 29% longer neurites compared to those on stiffer PDMS substrates ($p<0.05$), suggesting optimized neuronal maturation and an accelerated generation of neuronal networks.

Data show that primary NSC are significantly affected by the mechanical properties of their microenvironment. Culturing NSC on a substrate of brain-like elasticity keeps them in their physiological, quiescent state and increases their neurogenic potential.

Keywords: elasticity; mechanobiology; neurogenesis; primary neural stem cells;; polydimethylsiloxane; quiescence

Introduction

Neural stem cells (NSC) constitute the building blocks during development as well as during repair of the central nervous system (CNS; Gage, 2000). Although they remain in a functional state within their specific niches throughout the life span of all mammals (Alvarez-Buylla & Garcia-Verdugo, 2002; Bond, Ming, & Song, 2015), the regenerative capacity of the brain is limited if substantial damage occurs (Alvarez-Buylla, Seri, & Doetsch, 2002; Sohur, Emsley, Mitchell, & Macklis, 2006). Promising research focuses on enhancing this innate regenerative capacity of the brain by specifically targeting the endogenous NSC niche, e.g., by pharmacological means (Rabenstein & Rueger, 2018; Rueger & Schroeter, 2015; Vay et al., 2016). However, research on NSC grown in cell culture always faces the problem of artificiality when compared with the *in vivo* situation, since NSC functions crucially depend on the microenvironment in the stem cell niche (Doetsch, 2003; Regalado-Santiago, Juarez-Aguilar, Olivares-Hernandez, & Tamariz, 2016). For example, NSC change their differentiation fate when transplanted into a different region of the CNS (Sheen, Arnold, Wang, & Macklis, 1999), stressing the importance of the underlying host environment to alter cell fate. Likewise, the microenvironment of the stem cell niche affects proliferation, quiescence, and migration of NSC (Miller & Gauthier-Fisher, 2009; Regalado-Santiago et al., 2016). Signaling inside the niche is affected by many factors, for instance by cytokines or neurotrophins (Regalado-Santiago et al., 2016), cell-cell interactions (Stukel & Willits, 2016), as well as by mechanical properties of the tissue within the niche (Thompson & Chan, 2016).

Elasticity, as a fundamental and ubiquitous mechanical cue, is mainly determined by the properties of the extracellular matrix (ECM) that differs notably in the brain as compared to other tissues (Barros, Franco, & Muller, 2011; Dityatev, Seidenbecher, & Schachner, 2010). In this regard, the brain is one of the softest human tissues, with

an estimated elastic module (Young's module) of 0.1 to 16 kPa (Tyler, 2012). In the CNS, integrins and other transmembrane proteins that bind to the ECM are the main determinants of cellular mechanosensing (Stukel & Willits, 2016). Interestingly, these molecules are not only known for the mechanical forces they transmit, but also for their various biological effects on cells in the brain (Klein et al., 2014; Seebeck et al., 2017; Williams, Furness, Walsh, & Doherty, 1994). Overall, elasticity constitutes an important mechanical factor influencing various cell types in the body, including embryonal stem cells (Chowdhury et al., 2010; Evans et al., 2009), mesenchymal stem cells (Engler, Sen, Sweeney, & Discher, 2006; Schellenberg et al., 2014), hematopoietic stem cells (Kumar et al., 2013), or cardiomyocytes (Hersch et al., 2013).

Polydimethylsiloxane- (PDMS-) based substrates offer the unique opportunity to investigate mechanical effects upon NSC due to their tunable elastic modulus and good biocompatibility (Schellenberg et al., 2014). Besides, the rather smooth surface topography in contrast to other commonly used substrates like polyacrylamide (PA), allows a specific investigation of elasticity while minimizing other mechanical influences (Palchesko, Zhang, Sun, & Feinberg, 2012). To characterize the biological effect of different grades of tissue elasticity, three compositions of PDMS-substrates were used, mainly reflecting physical properties of the brain (1 kPa), muscle (15 kPa; Engler et al., 2004) and bone (50 kPa; Engler et al., 2006), respectively. Under the hypothesis that substrate elasticity should affect key functions of NSC, and that culturing NSC on regular glass plates constitutes an artificial system, we here investigated survival, proliferation, quiescence, and differentiation of primary NSC grown on PDMS-based substrates in a physiological range of elasticity.

Methods

Preparation of elastomeric silicone rubber substrates

Cells were seeded on various substrates of PDMS, varying by its grade of elasticity by different conjugations of base (vinyl terminated PDMS) and cross-linker (methylhydrosiloxane-dimethylsiloxane copolymer), as described previously (Hersch et al., 2013). In brief, elastomeric substrates with elasticities of 50 kPa, 15 kPa, and 1 kPa were made of silicone rubber. For high-end microscopy, elastomers were spin-coated as 70 µm thin layers over 110 µm thin cover slides (Cover Slip, Ø 18 mm, #0, Menzel-Gläser, Braunschweig, Germany), and glued under a hole drilled into a petri dish. For all other experiments, elastomers were cross-linked as thick layer in cell culture dishes (Nunclon Δ Multidishes, 4-Well, flat bottom, Thermo Fisher Scientific, Massachusetts, USA). Elasticity of all cross-linked elastomeric mixtures was accurately calibrated as described earlier (Ulbricht et al., 2013).

Culturing of primary NSC

All plates (glass and PDMS) were pre-coated with L-poly-ornithine (15%, Sigma Aldrich, St. Louis, USA), followed by bovine fibronectin (2.5 mmol/l, R&D Systems, Minneapolis, Canada). Primary monolayer NSC cultures were established from fetal cortical stem cells derived from Wistar rats of embryonic day 13.5 as described previously (Rueger et al., 2010). In brief, embryos were decapitated and meningeal layers removed. Additionally, the hippocampal formation was cut away. The residual cortical tissue was mechanically dissected and cells were first expanded on regular glass plates as serum-free monolayer cultures in DMEM/F12 medium (Life Technologies, Darmstadt, Germany) plus 1% N2 supplement (Gibco, Karlsruhe, Germany), 1% penicillin/streptomycin, 0.6mM L-glutamine and 1% sodium pyruvate; human recombinant basic Fibroblast Growth Factor (FGF-2; 10 ng/ml, Invitrogen,

Karlsruhe, Germany) was included as a mitogen throughout the experiments unless stated otherwise. If not specified otherwise, NSC were seeded at 10,000 cells / cm^2. After the expansion phase on glass plates, NSC from the second to fourth passage were re-plated on dishes coated with PDMS-substrates at various elasticity, prepared as described above, while 3.5 cm glass plates served as control. To assure stemness of cultured cells, stainings for anti-sex determining region Y box 2 (Sox2, mouse mAb, dilution 1:100, Cat# MAB2018, R&D Systems, Minneapolis, USA) as a marker of undifferentiated neural stem cells was performed before induction of differentiation.

Cell death assays

For assessment of cell viability, NSC were stained with propidium iodide to label dead cells (LIVE/DEAD Cell-Mediated Cytotoxicity Kit, Life technologies, Darmstadt, Germany) as described by the manufacturer, and counterstained with Hoechst 33342 (20 µg/ml, Biochemica, Billingham, United Kingdom) to visualize all cells regardless of viability. Ten images per condition were taken using a Keyence BZ-9000 inverted fluorescence microscope (Keyence Osaka, Japan). Labeled cells were counted manually, and cell death was assessed by the ratio of propidium iodide-positive dead cells to total cell number.

As a second assay of cell death, release of lactate dehydrogenase (LDH) from dying NSC was assessed using a colorimetric assay (Pierce LDH assay kit, Thermo Scientific, Waltham, USA) 24 hours after plating of NSC. The experiment was performed according to the manufacturer´s protocol. Color intensity was measured at a wavelength of 490 nm (FLUOstar Omega, BMG LABTECH, Ortenberg, Germany), being proportional to LDH activity. Color intensities were normalized to a positive control of lysed cells. Blank culturing medium (DMEM/F12) served as negative control.

Assessment of cell proliferation

To determine the ratio of proliferating cells, 10 µM bromodeoxyuridine (BrdU; Fluka, Munich, Germany) was added to cultures for 6 hours, before cells were fixed with 4% PFA. Cells were stained with monoclonal antibody (mAb) against BrdU to identify proliferating cells (mouse, clone BU-33, dilution 1:200, Sigma Aldrich, St. Louis, USA). For antigen-retrieval before staining, sections were incubated in 2N HCl for 30 minutes. For visualization, fluorescent-labeled anti-mouse or anti-rabbit immunoglobulin (IgG) were used as the second antibody (dilution 1:200; Invitrogen, Karlsruhe, Germany); all cells were additionally counterstained with Hoechst 33342. To calculate the ratio of proliferating cells, BrdU-positive cells were divided by the total cell number in each sample, and mean values were established among equally treated cells.

Assessment of cell differentiation

To analyze NSC differentiation, the mitogen FGF2 was withdrawn after plating, and NSC were fixed with 4% PFA after 3, 7, or 14 days of differentiation. Cells differentiating for 14 days were switched to Neurobasal medium (Life Technologies, Darmstadt, Germany) supplemented with GlutaMAX (200 mM; Life Technologies, Darmstadt, Germany), penicillin/streptomycin (50.000U; Life Technologies, Darmstadt, Germany), L-glutamine, sodium selenite, B27 (10 µg/ml; Life Technologies, Darmstadt, Germany), NT3 (50 ng/ml; R&D Systems, Minneapolis, USA), and BDNF (25 ng/ml, R&D Systems, Minneapolis, USA) after the first week of differentiation, as described earlier (Androutsellis-Theotokis et al., 2008). After fixation, different cell types were immunocytochemically stained with primary antibodies against young neurons (neuron specific beta-III tubulin monoclonal

antibody anti-TuJ1; dilution 1:100, R&D Systems, Minneapolis, USA), astrocytes (rabbit anti-glial fibrillary acidic protein GFAP, clone GA5; dilution 1:1000, Millipore, Billerica, USA), oligodendrocytes (mouse anti-2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase); clone 11-5B, dilution 1:500, Millipore, Billerica, USA), or undifferentiated NSC (mouse anti-sex determining region Y box 2 (SOX2); mAb, dilution 1:100, Cat# MAB2018, R&D Systems, Minneapolis, USA). In selected cultures, double-stainings with anti-TuJ1 plus anti-GFAP were performed. For visualization, fluorescein-labeled anti- mouse or anti-rabbit IgG were used (dilution 1:200, Invitrogen, Karlsruhe, Germany). All cells were additionally counterstained with Hoechst 33342. Ten images per condition were taken and cells counted manually.

Assessment of neurite outgrowth

For examination of neurite outgrowth, cells were seeded at a low density of 5.000 cells / cm^2 to facilitate the assessment of single cells. Differentiation was induced by mitogen withdrawal and cells were fixed after 14 days as described above. After immunofluorescent staining against TuJ1 labeling young neurons, ten images were taken per condition. The pixels of the raw data were normalized to a value range between [0,1]. Subsequently, the contrast of the neuronal structures was enhanced by using histogram equalization. Based on the resulting image, the local mean intensity around the neighborhood of the pixel was used as the threshold for image binarization. To reduce the amount of image artifacts, all connected components that had fewer dimensions than P = 2000 pixels were removed. Another morphological filter decreased the diameter of the neuronal structure by removing all pixels on the boundaries of the object. An analysis skeleton algorithm was applied, as previously proposed (Strzodka & Telea, 2004). The total amount of endpoints for each neuron

was identified by finding the k-nearest neighbor (X_k). By summarizing all existing paths, we defined the overall extension of the neuron, and stored the path with the most non-zero pixels between the endpoint (X_i) and its related junction (Y_i). The whole algorithm was implemented in MATLAB 2017b (The MathWorks Inc., Massachusetts, USA), to ensure that each image was treated identically in this automated analysis. In order to validate the described processing pipeline, neurons from a representative field of view of each image were measured manually using ImageJ (NIH, Bethesda, USA) and the NeuronJ plugin (Meijering et al., 2004).

Real-time quantitative PCR (qPCR)

To investigate cell quiescence, gene expression of p21 and p27 was examined. Twenty-four hours after plating NSC on PDMS or glass, respectively, mRNA was harvested from three wells of the same condition, and qPCR was performed. After RNA isolation via the RNeasy Mini Kit (Qiagen, Hilden, Germany), total RNA concentration and purity were evaluated photometrically. The Quantitect reverse transcription kit (Qiagen Hilden, Germany) was used to convert total RNA to c-DNA by reverse transcription. The primers were obtained from Biolegio (Nijmegen, The Netherlands). The sequences of the primers were: A) p21-forward: GAGTTAAGGGGAATTGGAGG, B) p21-reverse: AAGTCAAAGTTCCACCGTT, C) p27-forward: ACTCACTCGCGGCTC, and D) p27-reverse: CGTTAGACACTCTCACGTTT. The qPCR reaction was carried out using 10 ng total RNA in a 20µl reaction using Quantitect Reagents (Qiagen, Hilden, Germany) in accordance with the manufacturer's recommendations. Sample amplification and quantification was performed using the CFX Connect™ Real-Time PCR Detection System (Bio Rad, Hercules, California) with the following thermal cycler conditions: activation: 95°C, 10 min; cycling: 45 cycles, step1: 95°C, 15 s, step 2: 55°C, 15 s,

step 3: 72°C, 40 s. Each sample was normalized to GAPDH as reference gene, and individual mRNA levels were normalized to endogenous Glycerinaldehyd-3-phosphat-Dehydrogenase (GAPDH) expression (ΔCq); normalized values were then expressed as $2^{-\Delta\Delta Cq}$ relative to glass condition. Mean values ± SEM were calculated for the different treatment conditions.

Statistical analysis

Descriptive statistics and Student's T-Test were performed in Microsoft Excel 2010 (Microsoft). For statistical evaluation of more than two groups, one-way analysis of variance (ANOVA) was performed using SPSS Statistics (Version 24, IBM, Armonk, USA). ANOVA was followed by pairwise comparisons using Tukey-honest significant difference or, in case of heteroscedasticity, Game's Howell test. For nonparametric data, the Kruskal-Wallis ANOVA test (KWT) was performed. Statistical significance was set at the <5% level ($p < 0.05$).

Results

Soft substrates do not affect NSC viability but reduce their proliferation rate

We first investigated the viability of primary fetal rat NSC grown for 24 hours as an undifferentiated monolayer on soft PDMS substrates (50, 15, or 1 kPa), additionally investigating regular glass dishes (>1 GPa) to allow evaluation of these results in the context of regular cell culture work. In the presence of the mitogen FGF-2, all NSC expressed SOX2 as a marker for undifferentiated cells irrespective of the substrate, suggesting a homogenous NSC culture (fig. 1A' & A''). The Live/Dead assay – staining dead cells with propidium iodide to establish a ratio of cell death – revealed no adverse effect on NSC viability (**fig. 1B**). Likewise, an independent assay to detect cell death via LDH production corroborated these findings (**fig. 1C**), suggesting that substrate elasticity as low as 1kPa, mimicking the softness of the brain, did not impair NSC viability. Strikingly, however, we observed lower numbers of NSC when cultivated on soft substrates (**fig. 1B' & B''**). Since reduced cell viability had been ruled out as a reason, we exposed NSC to BrdU in order to assess their proliferative activity immunocytochemically. Indeed, NSC proliferation significantly decreased by 45% when cells were grown on 1 kPa elasticity compared to stiffer PDMS substrates of 50 kPa ($p < 0.05$, **fig. 2A**). To ensure that differences in cell number were not (additionally) mediated by an insufficient cell adherence to the very soft PDMS substrates, higher concentrations of fibronectin for plate coating were assessed in parallel to ensure NSC adherence, demonstrating that effects were independent of the concentration of fibronectin used (**fig. 2A**).

Soft substrates promote NSC quiescence by upregulation of $p27^{Kip1}$

To further explore the reason for reduced NSC proliferation on softer substrates, we assessed their expression of the cyclin dependent kinase inhibitor (CDKI) 1b ($p27^{Kip1}$)

as a central player of the molecular program to keep NSC out of the cell cycle, and thus in a quiescent state. The relative mRNA levels of $p27^{Kip1}$ as measured by qPCR after 24 hours in culture increased with the elasticity of the substrate they were cultivated on, with a 2-fold difference when cells were grown on 1 kPa elasticity compared to stiffer PDMS-substrates of 50 kPa ($p < 0.001$, **fig. 2B**). Relative expression of another gene implied in quiescence, CDKI 1a/$p21^{cip1}$, was not altered by substrate elasticity (**fig. 2C**), suggesting that $p27^{Kip1}$ was sufficient to maintain NSC quiescence. Thus, the data suggest that the softness of the brain keeps NSC in a quiescent state, i.e., a state that is physiological to the healthy brain.

Differentiation of NSC is accelerated on soft substrates

Upon mitogen withdrawal, NSC start differentiating towards a terminal cell fate, a process that can be quantified by the loss of SOX2-immunoreactivity, a marker for undifferentiated NSC. In the presence of the mitogen FGF2, the majority of NSC expressed SOX2, regardless of the elasticity of the substrate (67% on glass, 65% on PDMS substrates of 50 kPa vs. 72% on PDMS of 1 kPa, mimicking the brain, n.s.; **figs. 3A and C**). Already three days after mitogen withdrawal, more cells on soft substrates had lost SOX2-immunoreactivity (56% on glass vs. 42% on PDMS of 15 kPa, $p < 0.05$, **fig. 3C**). The effect was most pronounced seven days after mitogen withdrawal (**fig. 3B**), with a reduction of undifferentiated cells to 19% on the 1 kPa substrate, mimicking the softness of the brain, compared to stiffer PDMS-substrates of 50 kPa, on which almost 30% of NSC still expressed SOX2 ($p < 0.05$, **fig. 3C**), or glass, with 40% of Sox2 positive cells ($p < 0.01$, **fig. 3C**). Additionally, NSC grown on 15 kPa substrates, resembling the elasticity of, e.g., muscle tissue, lost stemness faster than those grown on glass plates (28% vs. 40% on day 7 of differentiation, $p < 0.01$; **fig. 3C**).

On soft substrates, the fate of NSC differentiation shifts towards neurogenesis

In addition to the speed of NSC differentiation, we investigated the fate of NSC differentiating on substrates of various elasticities. On a hard glass surface as the standard culture condition, 82% of NSC differentiated into astrocytes expressing GFAP (**figs. 4A and C**). When cultivated on PDMS of 50 kPa similar to, e.g., the elasticity of bone, less astrocytes (62%) and more young neurons expressing TuJ1 (25%) were generated (**figs. 4A', B and C**). Most pronounced, this effect was observed on the soft PDMS substrates of 1 kPa, mimicking the elasticity of the brain (**fig. 4A''**), on which 42% of NSC had differentiated into neurons after 7 days, compared with 17% on the >1 GPa glass plates ($p < 0.01$, **fig. 4B**), still comprising 66% more neurons compared to stiffer PDMS-substrates of 50 kPa ($p < 0.05$, **fig. 4B**). This promotion of neurogenesis by PDMS of 1 kPa remained stable for the entire observation period of 14 days (**fig. 4B'**). In turn, the generation of astrocytes from NSC was diminished to 50% on PDMS of 1 kPa compared to 62% on stiffer PDMS-substrates of 50 kPa ($p < 0.05$; **fig. 4C**) or even 82% on >1 GPa glass plates ($p < 0.001$; **fig. 4C**).

Soft elasticities promote neurite outgrowth

To further investigate the effects of substrate elasticity on the maturation of neurons differentiating from NSC, we assessed neurite outgrowth after 14 days of differentiation. As a morphological observation, neurites appeared considerably longer when neurons matured on soft PDMS of 1 kPa elasticity compared to glass surfaces (**fig. 5A**). In order to quantify this observation, the longest neurite from each neuron was measured, revealing an increase of 29% between 1 kPa PDMS (184 µm) compared to 50 kPa PDMS (143 µm, $p < 0.01$; **fig. 5B**). As an additional measure of

neuronal maturation, especially with regard to the generation of neuronal networks, we quantified the overall (sum) length of all neurite processes developing from NSC-derived neurons. Likewise, this sum length was increased from 662 µm on stiffer PDMS substrates of 50 kPa to 855 µm on 1 kPa PDMS, $p < 0.05$; **fig. 5C**), representing an almost 30% increase in the sum length of all neurites. Automatic quantification of neurite length was validated by manual measurements, showing good reliability of the automated method with mean differences <6% comparing the data from both methods (n.s.). As a further morphological observation, neural networks built between maturing neurons appeared more clustered on soft 1 kPa PDMS when compared to glass, where neurons seemed to be more scattered (**fig. 5D**), again suggesting that not only single neurons matured faster but also neuronal networks developed better on soft substrates mimicking the brain.

Discussion

Mechanical cues such as pressure, stretch, surface topology, or tissue stiffness, are increasingly recognized as important modulators of cell behavior during embryogenesis (Keller, Davidson, & Shook, 2003) as well as in the adult organism (Levental, Georges, & Janmey, 2007). Due to their influence on the development of the CNS (Bayly, Okamoto, Xu, Shi, & Taber, 2013; Iwashita, Kataoka, Toida, & Kosodo, 2014) these factors thereby contribute to regional differences in the adult brain (Johnson et al., 2016; Schwarb, Johnson, McGarry, & Cohen, 2016). Several neurological disorders go along with changes in the ECM and, thus, viscoelastic properties in the microenvironment. This phenomenon has been reported for chronic inflammation (Millward et al., 2015; Streitberger et al., 2012), stroke (Freimann et al., 2013), or dementia (ElSheikh et al., 2017). Furthermore, glia scar formation is propagated by changes in elasticity of the affected tissue (Moeendarbary et al., 2017). Interestingly, previous data suggest that NSC as the key players during CNS regeneration may adapt their inherent elastic properties to the ones of the surrounding extracellular milieu (Keung, de Juan-Pardo, Schaffer, & Kumar, 2011) but the effects of elasticity on NSC in their microenvironment to date remain to be characterized.

Choosing different compositions of PDMS-substrates, we mimicked a wide range of physiologically relevant elastic moduli, which enabled us to assess the sole effect of substrate elasticity, independent of other substrate properties like surface topology or wettability. As most scientific work in cell culture is performed on rigid glass substrates, we additionally compared our results to such substrates. However, since glass and PDMS do not only differ by elasticity but also by various surface properties, caution is advised in the interpretation of these results (Coltro, Lunte, & Carrilho, 2008).

We here report that primary rat NSC in homogenous monolayer culture were significantly affected by the elasticity of the substrate they grow on. With softer substrates, cell numbers decreased, however, not due to impaired viability but due to reduced proliferative activity going along with a quiescent state that is physiological to NSC in the brain (Gage, 2000; Johansson et al., 1999). Moreover, elasticity values in the range of the living brain promoted neurogenesis upon NSC differentiation, accompanied by accelerated maturation as assessed by neurite formation.

In line with our results, Teixeira et al. reported decreased numbers of primary rat NSC on PDMS substrates of any elasticity, compared to standard cell culture plates, but did not investigate the reason for this negative impact on cell number (Teixeira et al., 2009). Other groups observed slightly increased NSC numbers on soft substrates, albeit in different culture systems such as neurospheres grown on methacrylamide chitosan (Leipzig & Shoichet, 2009), or adult NSC grown on variable moduli interpenetrating polymer networks (Saha 2008). Moreover, proliferative activity of the respective cells was not directly assessed in these studies (Saha 2008, Leipzig 2009). Keung et al. found adult NSC proliferation unaffected by elasticities between 0.7 and 75 kPa, but did not investigate stiffer substrates such as glass as a control condition (Keung et al., 2011). In our model of fresh primary fetal rat NSC grown as homogeneous monolayer culture, we observed unimpaired cell viability but decreased proliferation of NSC as an elasticity-dependent effect of PDMS, as compared to stiffer PDMS-substrates, as well as standard culture conditions on glass. This decrease in proliferation along with unimpaired cell viability suggested NSC to exit from the cell cycle when cultured on soft substrates. Several external cues regulate the length of the G1-phase, thus affecting the balance between cell-cycle progression and proliferation on the one hand, and escape of the cell-cycle with

consecutive differentiation to specialized cell types on the other hand (Caviness, Takahashi, & Nowakowski, 1999; Lange & Calegari, 2010). The cyclin-dependent kinases (CDK) determine cell cycle progression, a process strictly regulated by CDK-inhibitors (CDKI; Besson, Dowdy, & Roberts, 2008). Several CDKI have been identified, of which the Cip/Kip family and especially p21 and p27 have been implicated as the most relevant regulators in NSC (Nakayama & Nakayama, 1998; Nguyen et al., 2006). In particular, $p27^{Kip1}$ is central to keeping NSC in the adult hippocampus out of the cell cycle and in a quiescent state (Andreu et al., 2015). Quiescence allows NSC in the brain to exist in an unaltered state for long periods of time, thus maintaining their potential to react to, e.g., damage or degeneration (Wang, Plane, Jiang, Zhou, & Deng, 2011). $P27^{Kip1}$ was additionally shown to promote neuronal differentiation in the fetal mouse cortex (Nguyen et al., 2006), and a lack of p27 leads to a reduction of neuroblasts in adult mice (Doetsch et al., 2002), while this effect was not observed with other members of the Cip/Kip family of CDKI (Nguyen et al., 2006). We here present evidence that external mechanical forces interfere with cell cycle regulation through upregulation of $p27^{Kip1}$.

Several groups have previously investigated the differentiation fate of rat NSC on elastic substrates, but most did so in the presence of various morphogenic factors aimed at promoting a specific differentiation fate (Engler et al., 2006; Saha et al., 2008; Teixeira et al., 2009). Others used fetal cortical cells from late gestational stages (E17-19) that were thus already pre-committed to a neuronal or glial fate, respectively (Georges, Miller, Meaney, Sawyer, & Janmey, 2006), or neuronal precursor cells derived from the adult rat hippocampus (Keung et al., 2011). Overall, these studies have suggested a pro-neurogenic effect of lower elastic moduli similar to that of the brain, which is in line with our results on spontaneously differentiating primary rat NSC. Based on the finding by Nguyen et al. that $p27^{Kip1}$ promoted

neurogenesis even independent of its main function in cell cycle regulation (Nguyen et al., 2006), and our result on $p27^{Kip1}$ upregulation in NSC on soft substrates, we speculate that $p27^{Kip1}$ might be – at least in part – responsible for inducing increased neurogenesis on soft substrates. For the first time, we here present longitudinal data on the differentiation fate of NSC, demonstrating a stable ratio of neurons between 7 and 14 days of differentiation. This finding corroborates a recent observation by Rammensee et al. who described a critical time window of mechanosensitivity between 12 to 36 hours of NSC differentiation (Rammensee, Kang, Georgiou, Kumar, & Schaffer, 2017). So far, Panthak et al. were the only group assessing the differentiation fate of fetal NSC of human origin on soft QGel based substrates, and surprisingly detected a decrease in neurogenesis with softer elasticity (Pathak et al., 2014). However, to verify potential species-dependent effects, further studies are warranted. Neuronal maturation, as assessed by the length of the longest neurite 14 days after mitogen withdrawal, increased by 29% with substrate elasticity in our study. Teixeira et al. described a similar effect already 7 days after mitogen withdrawal (Teixeira et al., 2009). However, at a later time point in differentiation, we additionally found neural networks – assessed as the overall sum length of all (interconnecting) neurites – to be increased by almost 30% on soft substrates, cautiously suggesting increased synaptogenesis as additional hallmark of neuronal maturation.

Conclusion:

In conclusion, our data reveal that primary NSC are significantly affected by the mechanical properties of their microenvironment. Culturing NSC on a substrate that mimics the softness of the living brain keeps NSC in their physiological, i.e.,

quiescent state and also increases their neurogenic potential. Thus, to optimally mimic the brain microenvironment in vitro for stem cell research, the use of elastic substrates for NSC cultivation is warranted.

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Data availability: The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

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Figure legends

Figure 1: Soft substrates do not affect neural stem cells (NSC) viability

A) Representative images demonstrate that all primary NSC grown in the presence of the mitogen FGF express SOX2 (green) as marker of undifferentiated cells, both when cultured on glass or on

A') PDMS of 1 kPa. All cells were counterstained with Hoechst (blue; scale bar = 100 µm).

B) When cultivated for 24 h, NSC showed no sign of increased cell death on Polydimethylsiloxane- (PDMS-) substrates of any elasticity compared to those on a glass surface, as monitored by the Live/Dead-assay (values displayed as means + SEM; n.s., n = 3, ANOVA-TukeyHSD). Representative images show dead, propidium iodide- (PI-) positive cells (red), all cells regardless of viability were counterstained with Hoechst (blue) on

B') glass and

B'') PDMS of 1 kPa, mimicking the elasticity of the brain (scale bar = 100 µm).

C) Similar results were obtained with an independent assay of cell viability, photometrically quantifying LDH production. Positive control: cell lysates, negative control: blank culture medium (values display as normalized to positive control + SEM; n.s., n = 3, ANOVA-TukeyHSD).

Figure 2: Soft substrates reduce cell proliferation and promote NSC quiescence by upregulation of cyclin dependent kinase inhibitor (CDKI) 1b ($p27^{Kip1}$)

A) NSC proliferation as measured as Bromodeoxyuridine- (BrdU-) incorporation decreased with substrate elasticity. This effect was not mediated by insufficient cell adherence to soft PDMS, since it was independent of the concentration of fibronectin used for plate coating (values displayed as means + SEM; **p<0.01 as compared to >1 GPa, # p<0.05 as compared to 50 kPa PDMS, n = 5, Kruskal-Wallis Test). Representative images show BrdU+ proliferating cells (green), counterstained with Hoechst (blue) on

A') glass and

A'') PDMS of 1 kPa, mimicking the living brain's elasticity (scale bar = 100 µm).

B) In NSC, expression of CDKI1b/$p27^{Kip1}$ mRNA as measured by quantitative real-time PCR (qPCR) increased with the softness of the substrate they were cultivated on, suggesting exit from the cell cycle and a quiescent state. Values were normalized to glycerinaldehyd-3-phosphat-dehydrogenase (GAPDH) expression as housekeeping gene, and individual expression levels were normalized to the glass condition as control (values displayed as $2^{(-\Delta\Delta Cq)}$ + SEM; ** p<0.01, ***p<0.001 as compared to >1 GPa, ## p<0.01 as compared to 50 kPa, n = 3, ANOVA-TukeyHSD).

C) Relative expression of CDKI1a/$p21^{cip1}$ in NSC was not regulated by substrate elasticity.

Figure 3: Differentiation of NSC is accelerated on soft substrates

A) In the presence of FGF2 as a mitogen, the majority of NSC expressed SOX2 (green) as a marker for undifferentiated cells; all cells were counterstained with Hoechst (blue) as a nuclear marker.

B) Seven days after mitogen withdrawal, and compared to glass (>1 GPa), only few NSC grown on the soft substrate of 1kPa (B') still expressed SOX2 (green, scale bar = 100 µm for panels A and B).

C) Over 7 days of differentiation, the percentage of SOX2+ undifferentiated NSC decreased under all experimental conditions; however, this process of differentiation was accelerated on softer substrates of 15 or 1 kPa compared to control (values displayed as means + SEM; * $p<0.05$, ** $p<0.01$, ***$p<0.001$ as compared to >1 GPa, # $p<0.05$ as compared to 50 kPa, n = 3, ANOVA-TukeyHSD).

Figure 4: On soft substrates, the fate of NSC differentiation shifts towards neurogenesis

A) After 14 days of differentiation, most NSC had differentiated into GFAP+ astrocytes (red) when grown on a hard glass surface. Grown on PDMS of 50 kPa elasticity (A'), more TuJ1+ young neurons (green) were visible. Neurogenesis was most pronounced on very soft 1 kPa substrates mimicking the brain (A''), on which most NSC had differentiated into TuJ1+ neurons within 14 days of mitogen withdrawal. All cells were counterstained with Hoechst (blue) as a nuclear marker; scale bar = 100 µm.

B) Seven days after initiating differentiation by mitogen withdrawal, quantitative effects of substrate elasticity on the generation of neurons from NSC were detected (n = 5, ANOVA- Games-Howell). This promotion of neurogenesis remained stable for the entire observation period of 14 days (B'; ** $p<0.01$ as compared to >1 GPa, # $p<0.05$ as compared to 50 kPa, n = 3, Kruskal-Wallis Test).

C) In turn, the generation of astrocytes from NSC, as the main cell fate when differentiating on glass, was reduced on 1 kPa PDMS (***$p<0.001$ as compared to >1 GPa, # $p<0.05$ as compared to 50 kPa, n = 3, ANOVA-TukeyHSD).

Figure 5: Substrates of higher elasticity promote neurite outgrowth

A) During the 14 days of differentiation, TuJ1+ neurons (green) derived from NSC grew long neurites as a sign of maturity. Neurites appeared considerably longer when neurons matured on soft PDMS of 1 kPa elasticity (A'). Cells were counterstained with Hoechst (blue) as a nuclear marker; scale bar = 100 µm.

B) Quantification of the longest neurite per neuron revealed significantly longer neurites on 1 kPa PDMS, mimicking the living brain's elasticity, as compared to any other substrate (**p<0.01 as compared to >1 GPa, ## p<0.01 as compared to 50 kPa, ‡ p<0.05 as compared to 15 kPa, n = 4, ANOVA-TukeyHSD).

C) The sum length of all neurites formed on 1 kPa PDMS was longer than on other substrates, suggesting more mature neuronal networks (**p<0.01 as compared to >1 GPa, ‡ p<0.05 as compared to 15 kPa, n = 4, ANOVA-TukeyHSD).

D) Moreover, neural networks built between neurons (green) differentiating from NSC appeared more clustered on soft 1 kPa PDMS as compared to glass, where neurons seemed more scattered (by morphological comparison, D'), 14 days after mitogen withdrawal. All cells were counterstained with Hoechst (blue) as a nuclear marker; scale bar = 100 µm.