Current Tissue Microenvironment Reports (2020) 1:41–47
https://doi.org/10.1007/s43152-020-00003-y

CELL BEHAVIOR MANIPULATION (S WILLERTH, SECTION EDITOR)

Recent Advancements in Engineering Strategies for Manipulating Neural Stem Cell Behavior

Brian J. O'Grady[1] · Ethan S. Lippmann[1,2,3,4]

Published online: 3 April 2020

Abstract

Purpose of Review Stem cells are exquisitely sensitive to biophysical and biochemical cues within the native microenvironment. This review focuses on emerging strategies to manipulate neural cell behavior using these influences in three-dimensional (3D) culture systems.

Recent Findings Traditional systems for neural cell differentiation typically produce heterogeneous populations with limited diversity rather than the complex, organized tissue structures observed in vivo. Advancements in developing engineering tools to direct neural cell fates can enable new applications in basic research, disease modeling, and regenerative medicine.

Summary This review article highlights engineering strategies that facilitate controlled presentation of biophysical and biochemical cues to guide differentiation and impart desired phenotypes on neural cell populations. Specific highlighted examples include engineered biomaterials and microfluidic platforms for spatiotemporal control over the presentation of morphogen gradients.

Keywords Neural stem cell · Stem cell differentiation · Biomaterial · Morphogen gradient

Introduction

Stem cells are widely appreciated for their ability to expand in an undifferentiated state and their potential to be differentiated into specific cell lineages. Stem cells have revolutionized studies of mechanistic biology, disease modeling, drug discovery, and regenerative medicine. It is well-accepted that stem cells can be instructed towards fate commitment through both biophysical cues and biochemical cues [1–3]. However, the ability to control differentiation through spatiotemporal presentation of diverse biophysical cues [4–6] (e.g., matrix architecture, stiffness, adhesion motifs) and biochemical signals [7–9] (e.g., morphogen presentation) remains challenging.

The biomedical research community has long been interested in using engineering strategies to control the differentiation of stem cells into specific lineages. Historically, stem cell differentiation procedures have been conducted on noncompliant polystyrene well plates coated with a limited number of extracellular matrix (ECM) components to facilitate cell adhesion. Further, media changes in static culture typically occurred on a 24-h time scale without intermediate control over the soluble milieu (including exogenously added and endogenous cell-secreted growth factors and small molecules). Over the last decade or so, there has been a growing appreciation for how engineering principles can be applied to improve these workflows. In this review, we cover recent examples for how the controlled presentation of biophysical and biochemical cues can be harnessed to influence the differentiation of stem cells, including primary neural stem cells (NSCs) and pluripotent stem cells (from embryonic and induced sources), to specific neural lineages. We begin by discussing various ways to manipulate stem cell fate through ECM stiffness and composition. We then discuss current and

This article is part of the Topical Collection on *Cell Behavior Manipulation*

✉ Ethan S. Lippmann
ethan.s.lippmann@vanderbilt.edu

[1] Department of Chemical and Biomolecular Engineering, Vanderbilt University, PMB 351604, Nashville, TN 37235-1604, USA

[2] Department of Biomedical Engineering, Vanderbilt University, PMB 351604, Nashville, TN 37235-1604, USA

[3] Vanderbilt Brain Institute, Vanderbilt University, PMB 351604, Nashville, TN 37235-1604, USA

[4] Interdisciplinary Materials Science Program, Vanderbilt University, PMB 351604, Nashville, TN 37235-1604, USA

emerging strategies to exert control over morphogen presentation in three-dimensional (3D) culture to recapitulate the developmental patterns of the neural tube.

Influence of ECM Stiffness on Neural Fate

The ECM of the human brain is composed of various glycosaminoglycans and proteoglycans [10, 11] that contribute to the low viscoelastic properties of the brain, leading to a 1–10 kPa stiffness that is optimal for many different neural cell subtypes. Changes to the mechanics of the ECM can lead to subsequent alterations in signal cascades (e.g., focal adhesion kinase and Rho-associated protein kinase cascades) during development and disease. One prominent example is during neural tube formation [12]. The ECM was once thought to be strictly a scaffold to support this growing neural structure [13], but its stiffness has been shown to be dynamic and plays a key role in differentiation [13]. The stiffness of the ECM can also influence pathogenic responses in the brain. For example, although not stem cells, microglia are essential innate immune cells of the central nervous system [14] and act as a major regulator of inflammation. When presented with pathogens and/or injury, microglia rapidly change morphology and migrate to the site of injury where they secrete cytokines, phagocytose pathogens, and remove damaged cells [15, 16]. Yet, microglia also exhibit pathogenic responses to implanted materials that depend on the stiffness of the material [17]. Tissue stiffness alterations with age also inhibit the function of oligodendrocyte progenitor cells [18]. Based on these recent investigations, there is a growing appreciation for the sensitivity of neural cells to underlying mechanical cues.

Such realizations have motivated the development of numerous engineered hydrogels of varying stiffnesses to elucidate the interplay between substrate mechanics and neural cell behavior and to guide differentiation fates. Early studies in two-dimensional culture utilized a variety of biomaterials with varying stiffness to demonstrate that NSC fate could be biased via modulation of substrate modulus [19–21]. Later studies performed with human pluripotent stem cells revealed that biomaterials with soft moduli could promote specification to neuroectoderm [22, 23]. These studies paved the way for examining stiffness effects in three-dimensional hydrogels, where one of the most common ways to tune stiffness is through methacrylation of the ECM backbone (Fig. 1). This technique adds methacrylic anhydride to many side chains on the ECM. These modified ECMs can then be mixed with cells and crosslinked in the presence of UV light and a photoinitiator [24, 25]. The stiffness of the hydrogel can be tuned by varying the UV exposure time and/or intensity, giving way to user-defined physical properties [26]. Some common examples of methacrylate-modified biomaterials are hyaluronic acid and gelatin (HAMA and GelMA, respectively). Hydrogels built from these biomaterials have excellent biocompatibility [27•, 28•] and have been used to study NSC responses. For example, one study established a 3D in vitro model of human-induced pluripotent stem cell (iPSC)-derived NSC differentiation using soft and stiff HAMA hydrogels to evaluate the spontaneous differentiation in response to the mechanical rigidity of the ECM. Here, encapsulation of NSCs in the hydrogel caused cells to spontaneously migrate and accumulate into a cluster, followed by neurite outgrowth. In the soft hydrogels, NSCs showed more extensive differentiation over 28 days compared to the stiff HAMA, which restricted the spontaneous differentiation and better maintained the progenitor properties of the NSCs [29]. These findings were mirrored in a separate publication demonstrating that iPSC-derived NSCs cultured in soft HAMA hydrogels could be differentiated into neurons that were more functionally mature with respect to neurons differentiated on planar substrates [30]. Overall, these results suggest that the mechanotransducive signaling of stiffness can dictate the behavior and fate of neurons.

Beyond ECM stiffness, other biophysical features such as patterning and topography can influence ECM presentation and alter cell behavior. For example, alterations to topographic patterns at the nano- and micro-scale can alter primary adult NSC differentiation and fate commitment through MAPK/ERK signaling [31]. More recent studies starting with pluripotent stem cells (i.e., a more embryonic state) have revealed that nanotopography can regulate differentiation into more specialized fates such as motor neurons [32]. Others have covered variations of these topics in extensive reviews that can be referred to as desired [33–35].

Influence of ECM Composition on Neural Fate

The composition of the ECM also exerts influences on cells that can be independent of stiffness-mediated effects. Many natural materials have been used to direct neural cell behavior based on composition and functionality. As an example, gelatin, a simple biomaterial that was mentioned earlier, has endogenous peptide sequences that facilitate cell attachment and matrix metalloproteinase (MMP) degradation [36], and it has numerous side chains (e.g., -OH, -COOH, $-NH_2$) that are available for chemical modification with exogenous cues to pattern neural cells [28•, 32, 33]. In one more recent study, GelMA was chemically grafted with the neurotransmitter dopamine via covalent bonding of the amino group on dopamine to the carboxyl groups present on the backbone of GelMA (GelMA-DA) [28•]. This new biomaterial was then 3D-printed using stereolithography, followed by culture of NSCs on the scaffold. Over time, the NSCs continually grew and were not spontaneously differentiating in response to the ECM dopamine signal. When the NSCs on the GelMA and

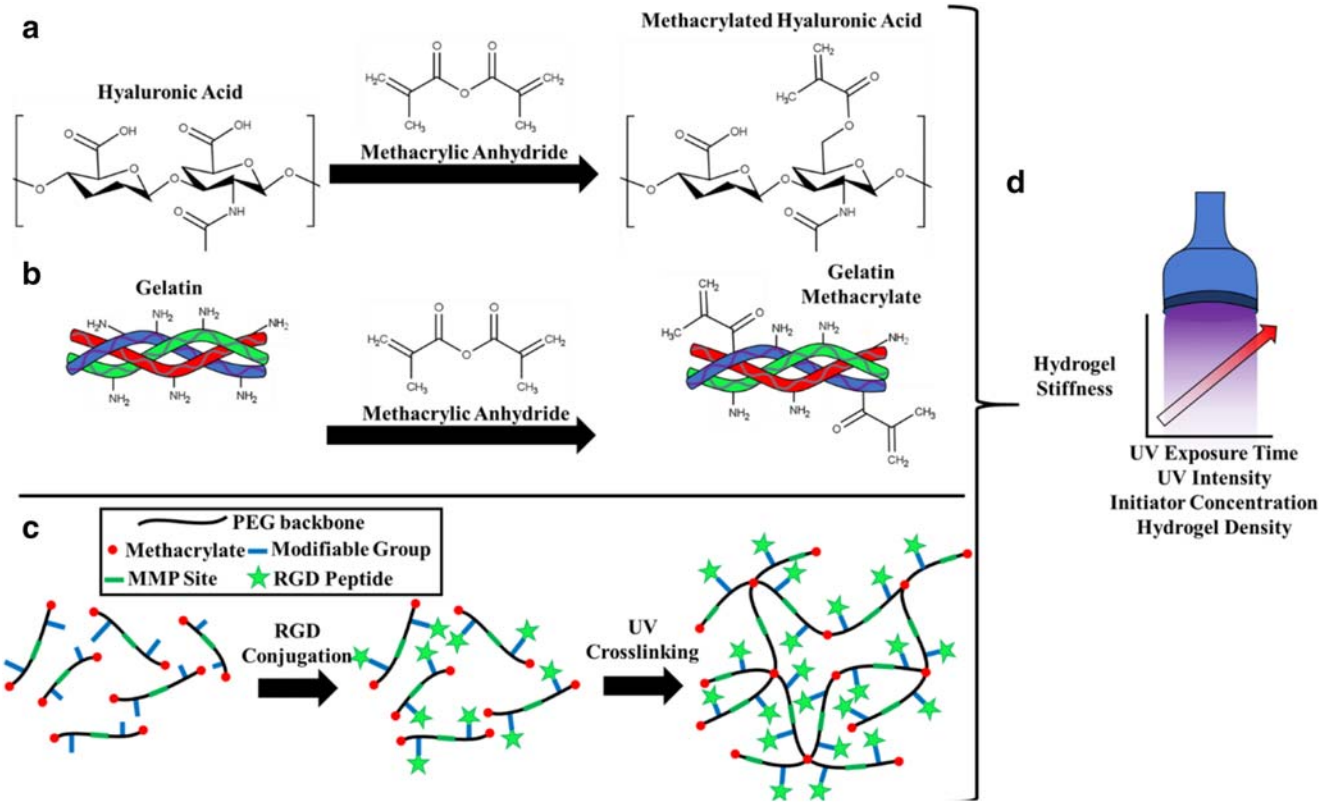

Fig. 1 Chemical synthesis of methacrylated hyaluronic acid (A), gelatin methacrylate (B), and methacrylated PEG hydrogel (C) functionalized with matrix metalloproteinases (MMP) cleavage sites and arginine-glycine-aspartate (RGD) peptide motif. The mechanical properties of the hydrogel (e.g., stiffness and porosity) can be controlled by varying combinations of UV exposure time, UV intensity, initiator concentration, and hydrogel density (D)

GelMA-DA hydrogels were subjected to differentiation, there was a noticeable increase in TUJ1 expression in the GelMA-DA group, indicating formation of neurons. Additionally, quantification of the neurite length showed a significant increase in the GelMA-DA hydrogels compared to unmodified GelMA. The authors concluded that the cellular behavior observed in that study could be attributed to the fact that dopamine not only acts as an inducer of neural differentiation but also serves as a site for cell adhesion similar to the canonical RGD-motif. RGD (arginine-glycine-aspartic) is an important adhesion molecule that binds to integrin receptors and is naturally found in collagen, gelatin, laminin, and fibronectin. Studies have shown that this molecule induces a cascade of intracellular events in neurons to alter the cytoskeletal composition [37] and improve focal adhesion in 3D-engineered systems [38].

More complex synthetic materials have also been developed to regulate neural cell fates. In general, synthetic materials offer more control over the ECM backbone and its properties, which allow for more detailed mechanistic-driven explorations. For example, matrices built from elastin-like peptides (EPLs) have enabled effective decoupling of stiffness and functionality for a variety of studies. Early work using EPLs and primary dorsal root ganglia explants demonstrated the influence of cell-adhesion ligand density on neurite outgrowth [39]. More recent work with EPLs revealed that matrix degradability is an important characteristic for maintaining NSCs in an undifferentiated state [40]. A follow-up study further demonstrated that matrix remodeling impacted the differentiation propensity of the NSCs in a Yes-associated protein (YAP) and β-catenin-dependent manner [41]. Another recent study explored the biochemical and biophysical properties of chemically defined ECMs to recapitulate the early stages of neurogenesis [42]. In previous work, neural organoids have been embedded in Matrigel to study neural tube development. However, the aspects of Matrigel that drive spatial organization in 3D aggregates are largely unknown. The authors developed a high-throughput system of hydrogels using a library of synthetic materials to investigate the optimal properties required to recapitulate neural tube patterning. The results in the study showed that a combination of microenvironment characteristics could promote proliferation and apical-basal polarity. Additionally, the authors showed that manipulation of the chemical and physical properties of the matrix was essential for early downstream patterning of dorsoventral polarity. These examples represent only a fraction of

ongoing efforts to engineer stem cell niches [43], and it is likely that more exotic materials will be developed to help advance NSC differentiation paradigms.

Spatiotemporal Platforms for Presenting Soluble Factors to Control Neural Cell Fate

Similar to the ECM, soluble cues are an essential aspect of any biological system. During development, soluble cues (e.g., morphogens) are presented with precise concentrations at exact locations for a specific duration to achieve complex tissue patterning. There are numerous examples of spatiotemporal regulation of morphogens in biology, ranging from cardiac development [44] to intestinal wall organization [45]. However, we again focus here on early development of the neural tube as the subject of many in vitro differentiation studies. The neural tube is formed with multiple, opposing, and complex morphogen gradients [46]. The anteroposterior axis of the brain is shaped by a gradient of Wnt signaling, whereas the anteroposterior axis of the spinal cord is shaped by temporal gradients of Wnt, fibroblast growth factor (FGF), growth differentiation factor (GDF), and retinoic acid (RA). Concurrently, the dorsoventral axis is formed by opposing gradients of sonic hedgehog (SHH) and bone morphogenic protein (BMP) (Fig. 2A). The SHH gradient guides the ventral portion of the neural tube, while BMP is responsible for differentiating dorsally located neural cells; this tightly regulated presentation of each morphogen shapes discrete progenitor domains that further differentiate into specialized neural cells [47, 48]. As the brain and spinal cord develop, additional signaling nodes secrete morphogens to locally refine spatial patterning.

In general, the application of soluble cues can be used to differentiate stem cells into various regionally specified neural fates. This concept has been applied to the generation of neurons from various brain regions [49], although the lack of robust control over the extracellular milieu generally leads to mixed, impure cell populations. As a recent example of more discrete patterning in static well plate cultures, the differentiation of pluripotent stem cells into distinct spinal cord domains, as identified by combinatorial *HOX* transcription factor expression, was accomplished by temporal exposure to saturating concentrations of Wnt, FGF, GDF, and RA [50]. However, due to the precise and controlled nature of morphogen signaling for organized tissue development in vivo, engineered platforms that have tight control over spatial and temporal presentation of morphogens are necessary for building more complex, spatially organized in vitro models.

The construction of patterned tissues with discrete organization of multiple cell types is most often facilitated by microfluidic platforms. Some primary advantages of using microfluidics are the ability to control the delivered fluid at volumes down to the picoliter scale and predict the spatial concentration profiles of soluble factors using fluid mechanics and mass transfer principles [51]. One early approach utilized a microfluidic hydrogel chip to pattern mouse embryonic stem cells [52••]. This system was created with perfusable

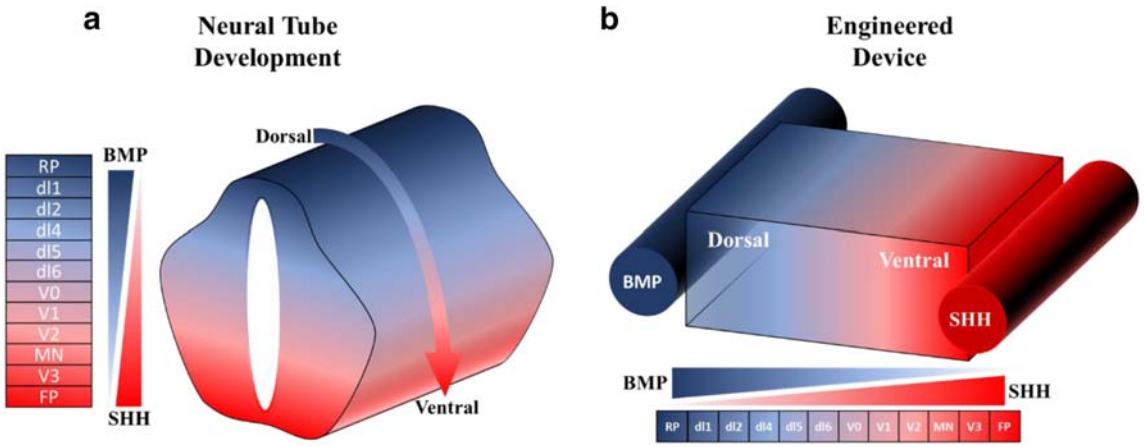

Fig. 2 Graphical overview of in vivo neural tube development and engineered devices. In normal neural tube development, distinct sections of neurons are generated in a spatially organized manner due to multiple opposing signals. SHH is secreted from the notochord and floor plate, located ventrally on the neural tube. The diffusion of this morphogen from ventral to dorsal creates a spectrum of differentiation, leading to discrete domains of neurons. An opposing gradient of BMP is generated from the roof plate on the dorsal side. The diffusion of this inhibitory morphogen from dorsal to ventral establishes the differentiation of neurons into various discrete domains (A). Isolated perfusion channels in the microfluidic device supply nutrients and morphogens to the cell-laden hydrogel. In one channel, SHH is supplied and allowed to continuously diffuse into the hydrogel. This establishes a persistent concentration gradient, mimicking in vivo diffusion of SHH from ventral to dorsal. In the opposing channel, an opposing morphogen concentration gradient of BMP is established. This process mimics the dorsal to ventral differentiation seen in vivo. While the dorsoventral differentiation of the neural tube is represented here, many of the opposing gradients to produce neural subtypes can be generated with this platform (e.g., anteroposterior axis and rostral/caudal differentiation) (B)

microfluidic channels embedded in a hydrogel to deliver morphogens to stem cells on the surface. The authors were able to control the spatial differentiation by generating and manipulating RA gradients. These data suggest that this configuration allowed for tight control over spatial and temporal delivery of biomolecules and laid the groundwork for generating more complex dynamic microenvironments similar to what is seen during embryogenesis. In the area of neural differentiation, a 3D microfluidic chamber was recently developed to recapitulate the multiple opposing morphogen gradients in neural tube development [53••]. In this system, a microfluidic chamber was fabricated to create orthogonal linear gradients of soluble factors within a microscale cell culture chamber, whereby the authors used a computational model to predict spatial and temporal presentation of cues based on concentration and device geometry. The authors were then able to create gradients of morphogens that impacted the differentiation fates of mouse embryonic stem cells, thus recapitulating some aspects of axis patterning (Fig. 2B). In a related study, opposing linear gradients of RA and SHH signaling were created in a 3D cell laden hydrogel [54]. Tuning of these gradients induced mouse embryonic stem cells to differentiate into ventral motor neurons. Overall, these two studies were able to demonstrate a high level of control over both spatial and temporal presentation of morphogens. Thus, microfluidics has been proven to be a powerful tool in manipulating cellular behavior.

More recent studies have built on these design principles, albeit not yet in the neural space. For example, a very recent study fabricated gradient-generating devices that contained molded agarose hydrogel between two reservoirs [55]. One reservoir served as a source of biomolecules and the other as a sink. A thin space between the sink/source reservoirs (100 μm in height) was used to embed a monolayer of human umbilical vein endothelial cells. When one reservoir was filled with a specific concentration of morphogen, the substrate would then diffuse across the surface of the cells to the sink source, thus creating a diffusion gradient across the entirety of the monoculture without external flow. Additionally, using finite element modeling, the authors were able to predict the behavior of morphogen gradients to a two-dimensional surface with high certainty. A separate study engineered a microfluidic device that could emulate the dynamic concentration gradients that are seen in embryonic development and germ layer formation [56]. The system contained four parallel chambers with barriers to confine cells and generate concentration gradients without the development of convective flow. Because this system was developed with defined sizes, geometries, and flow rates, a simple Fickian diffusion model could be employed, whereby computational simulations could then afford the prediction of time-evolving concentration gradients. These models were validated and found to have a high degree of accuracy for the spatial presentation of BMP4 to the embedded stem cells. Additionally, the authors demonstrated the ability to create opposing gradients of antagonists to mimic the asymmetric signaling found in the developing embryo (e.g., "symmetry breaking"). It should be noted that previous studies have shown that physiological levels of convective flow are able to increase stem cell proliferation and are an important regulator of adult NSC fate, which could also potentially be modeled in microfluidic systems [57]. Ultimately, these tools can be used for building more complex signaling environments that are commonly seen in many regions of the body, including the brain.

Conclusions

There have been exciting advances in the use of engineering tools to direct neural cell fates. Recent developments have offered powerful routes for cellular manipulation in three-dimensional systems with applications towards basic research, disease modeling, and regenerative medicine. However, many challenges still must be overcome. Although morphogen presentation using microfluidic devices has yielded success for patterning in embryonic cultures, the size of such culture platforms limits the ability to pattern larger and more complex neural tissues. These problems may be overcome using larger hydrogel systems that facilitate morphogen gradient generation across millimeter distances [58, 59]. Further, the regulation of neural cell behavior within biomimetic hydrogels has been mainly focused on binary fate decisions (e.g., NSC self-renewal versus differentiation, differentiation to neurons versus astrocytes) rather than more complex events such as differentiation and organization of iPSC-derived neuron subclasses into complex, functional circuits. We suggest that biomaterials and engineered cell culture platforms will ultimately need to be integrated to achieve the additional control necessary to carry out such differentiation in future studies.

Funding Information Work in our laboratory related to this review is supported by funding from the Chan Zuckerberg Initiative (grant 2018-191850 to ESL) and the National Science Foundation (grant 1706155 to ESL). BJO is supported by the Interdisciplinary Training Program in Alzheimer's Disease funded by the National Institutes of Health (T32 AG058524).

Compliance with Ethical Standards

Conflict of Interest Dr. O'Grady and Dr. Lippmann have nothing to disclose.

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