3D Bioprinting Human-Induced Pluripotent Stem Cells and Drug-Releasing Microspheres to Produce Responsive Neural Tissues

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3D bioprinting can produce complex human tissue mimics using stem cells (SCs). Herein, cylindrical constructs containing human-induced pluripotent stem cell (hiPSC)-derived neural progenitor cells (NPCs) encapsulated in a fibrin-based bioink containing polycaprolactone (PCL)--retinoic acid (RA) and purmorphamine (puro)-releasing microspheres are bioprinted in a layer-by-layer fashion using the microfluidic-based RX1 bioprinter to engineer responsive neural tissues. The differentiated constructs contain neurons expressing ChAT, GABA, and MAP2, astrocytes expressing GFAP, and oligodendrocytes expressing O4 as indicated by immunocytochemistry and flow cytometry analysis on days 30 and 45. The bioprinted tissues also respond to treatment with acetylcholine (Ach) and gamma-aminobutyric acid (GABA) on days 30 and 45. The use of microsphere-laden bioinks efficiently promotes neural tissue differentiation and maturation in situ using a lower amount of morphogens in comparison with using soluble drugs. This bioprinting strategy serves as a cost-effective solution for engineering humanized neural tissues.

1. Introduction

3D bioprinting, an additive manufacturing process, serves as an exciting new strategy for generating viable and functional tissues in vitro.[1] Previous work has shown how 3D bioprinting can generate different types of tissues, including bone, vascular, neural, and skin.[2–4] These tissues can be used for regenerative medicine applications and serve as preclinical drug screening platforms to identify target compounds, reducing the cost and possibility of drug failure during clinical trials.[5] The bioprinting process requires a 3D printing system capable of dispersing bioinks to generate tissues from a computer-aided design (CAD) file. Groll et al. defined bioinks as a “formulation of cells suitable for processing by an automated biofabrication technology that also contain biologically active components and biomaterials”.[6,7] Currently, two main bioprinting strategies exist. The first method initially 3D prints a cell-free structure followed by cell seeding. While this strategy can create microchannel structures, obtaining consistent distribution of the cells throughout the scaffold can be difficult, and directing the desired differentiation into multiple cell types remains challenging.[8,9] The second strategy incorporates the cells in the bioink prior to printing.[6,10,11] This technique can produce homogeneous or heterogeneous constructs with defined geometries where the cells are distributed as desired within the fibers.[12] This process can also produce constructs containing more than one cell type depending on the level of sophistication of the bioprinter.

The commonly used process of extrusion-based bioprinting relies on pneumatic pressure-driven flow or mechanical forces to continuously deposit the bioink from a nozzle.[14] The length of the nozzle correlates to the time cells are exposed to shear stress, which decreases cell viability.[14,15] The novel microfluidic RX1 bioprinter from Aspect Biosystems has shown promising results for the generation of neural tissues from stem cells (SCs).[3,14,15] Its polydimethylsiloxane (PDMS) microfluidic printhead called the Lab-on-a-Printer (LOP) consists of multiple microchannels where different bioinks and their crosslinker can be loaded and then dynamically bioprinted. A protective sheath surrounds the cell-laden bioink during extrusion, reducing shear stress during printing and thus increasing cell survival and long-term functionality of the tissues.[16] This feature also allows for the initiation of bioink polymerization within the nozzle, resulting in more stable constructs.[3,13] Bioinks that mimic the native...
extracellular matrix (ECM) can improve cell survival and function during bioprinting.[11] As such, the composition of the bioink determines its functionality. The bioink must be printable while also possessing the mechanical properties and pore structure that allow for the delivery of biological cues that promote the formation of neural tissue in a controlled manner.[27,18] Natural biomaterials are favored over synthetic biomaterials due to their intrinsic bioactivity, biocompatibility, natural degradation kinetics, and chemically tunable properties when engineering neural tissue.[19] Synthetic materials are generally biologically passive and are thus inadequate for representing the complex interactions that regulate tissue function and repair.[17] Commercially available bioinks emphasize printability over biological properties, restricting the type and complexity of tissues being printed. The novel bioink used in this study consisted of a combination of the natural biomaterials fibrin, alginate, and chitosan, which provide a suitable environment for human-induced pluripotent stem cell (hiPSC)-derived neural cells to survive with high levels of viability and differentiate into mature neural tissues.[1,15]

Fibrin hydrogels result from the enzymatic reaction between thrombin and fibrinogen, with Ca²⁺ serving as a key cofactor in the conversion.[17] Fibrin hydrogels have been widely used in the field of tissue engineering in combination with other natural biomaterials. For example, Attalla et al. combined alginate and fibrin to bioprint hollow tubules to engineer artificial arteries and veins.[20,21] King et al. used injectable fibrin and fibronectin in rats with spinal cord injury (SCI) showing good integration and formation of neural tissue in a controlled manner.[17,18] Natural biomaterials also possessing the mechanical properties and porosity, role in wound healing, and its ability to build biomimetic nerve tissue.[19] Synthetic materials are generally biologically passive and allow for the pattering of the hindbrain and spinal cord.[44] Another study bioprinted fibrin and hyaluronic acid hydrogels to build biomimetic nerve fibers containing neurons and Schwann cells to promote neurogenesis and myelination.[21,24] Fibrin-based scaffolds have been shown to promote neuronal differentiation and proliferation due to its favorable mechanical properties and porosity, role in wound healing, and its ability to promote angiogenesis along with cell adhesion and growth by binding integrins, growth factors, fibronectin, and protease inhibitors.[17,18,24–26] The rapid degradation of fibrin, however, is unfavorable for long-term cultures. Robinson et al. have shown that the addition of genipin, a plant-derived crosslinking agent with neurotogenic and neuroprotective properties, during the polymerization process alters the physical characteristics of fibrin scaffolds, allowing for a decreased degradation rate.[25]

Extrusion-based bioprinting of alginate has been widely explored due to its superior mechanical properties, biocompatibility with central nervous system (CNS) tissue, and versatile cross-linking methods.[26] Alginate, an algae-derived salt of alginic acid that rapidly crosslinks upon exposure to divalent cations such as Ca²⁺, has been studied as a scaffold to encapsulate neural stem cells (NSCs).[17,26,27] Alginate hydrogels have been shown to provide physical guidance cues for NSCs, with alginate’s elastic modulus being a key determinant of neural proliferation, differentiation, and expression.[28] It has also been shown that NSCs encapsulated in alginate secrete neurotrophic factors that support neuronal survival and plasticity.[29] However, alginate is relatively inert as it does not possess bioactive sequences that cause cell adhesion.[17]

Chitosan possesses neuroprotective properties, creating a cellular microenvironment encouraging cell adhesion and also promoting axonal regeneration, anti-inflammation, and successful delivery of neurotrophic factors.[30] Yang et al. combined fibrin with Sonic Hedgehog (SHH)-loaded chitosan microspheres to provide protection and regeneration in rats with SCI.[21,31] A study examining a chitosan-alginate hydrogel that was highly hydrophilic with a porous inner structure showed good proliferation of NSCs.[32] We have created a suitable bioink by combining the biomaterials (fibrin, alginate, and chitosan) described earlier that can be extruded using the RX1 bioprinting system and be an optimal bioink formulation for maintaining the viability and promoting differentiation of hiPSC-derived NPCs.

In addition to the bioink, 3D bioprinting requires other components that must be strategically chosen to create the desired type of tissue, such as the types of cells and biochemical factors used to promote differentiation and survival of the tissues in vitro.[10,11,33] The use of pluripotent SCs can address the limitations associated with primary neural cell culture, including limited availability and passaging limitations.[11] Pluripotent can be derived from the inner mass of a human embryo or reprogrammed from somatic cell-hiPSCs.[34] Differentiation of hiPSCs into neural tissue has been widely studied and used in the field of tissue engineering.[35–37] Specifically, hiPSCs can be differentiated in a reproducible fashion into a high purity population of motor neurons (MNs), the cells present in the spinal cord in charge of voluntary movement.[17] hiPSC-derived cells have also been bioprinted into neural tissues using a variety of bioinks and biochemical factors that support their differentiation, growth, and maturation.[9,15,38]

Microspheres can deliver biochemical factors at a controlled rate and over a period of time.[39] Drug-loaded microspheres, often made from polycaprolactone (PCL) or poly-lactic-co-glycolic acid (PLGA), can deliver water insoluble drugs, proteins, and small molecules.[40] In particular, retinoic acid (RA)- and purmorphamine (puro)-loaded microspheres efficiently differentiate hiPSCs into neuronal subtypes.[41,42] The major advantages of using drug-loaded microspheres to engineer tissues include localized and controlled delivery of morphogens over time, and its ability to serve as a cost-effective morphogen delivery method when compared with constantly changing media containing significant amounts of morphogens.[42,43] RA is a derivative of vitamin A, which acts as a bone morphogenic protein (BMP) inhibitor. It influences a set of genes that are involved in neural development—the homeobox (HOX) genes. RA is secreted by the mesoderm and endoderm once the neural tube has been formed. The exposure of RA at different levels of the neural tube occurs as a concentration gradient and allows for the patterning of the hindbrain and spinal cord.[44] Puro is a small molecule that acts as a SHH agonist and promotes the activation of protein kinases and transcription factors involved in the differentiation of MNs.[44,45] A number of protocols have shown the differentiation of hiPSCs and NPCs into MNs by the addition of both puro/SHH and RA.[36,37] Moreover, our group demonstrated the successful differentiation of neural tissues from hiPSCs using a combination of both puro- and RA-loaded microspheres leading to the formation of HB9 + MNs by day 35.[43,42] For these reasons, RA was chosen as a second small molecule to be used in this study.

This study builds on our previous work where we showed the efficient differentiation of hiPSCs into neuronal subtypes and bioprinting of hiPSC-derived neural progenitor cells (NPCs) using a fibrin-based bioink.[3] Here, we show the successful differentiation of neural tissue in 3D constructs using our bioink
containing puro- and RA-loaded microspheres in combination with hiPSC-derived NPCs, leading to the presence of mature spinal motor neurons (SMNs) and other relevant types of cells present in the CNS.

Similar cylindrical macrostructures could be fabricated using conventional molding, however bioprinting creates a microarchitecture of thin fibers to allow media penetration to the inner layers of the construct. These constructs possessed an even distribution of both cells and microspheres inside the printed fibers, creating 3D mini neural tissues that can replicate the physiological environment of the native tissue. The bioprinted constructs were cultured for up to 45 days to promote neural differentiation and maturation. hiPSC-derived mature SMNs were also bioprinted as a control tissue to compare cell viability, cell marker expression, and membrane potential. All treatment groups showed ≈80% viability on days 0 and 1 postprinting. The bioprinted constructs containing drug-loaded microspheres had 82% expression of the homeobox protein 9 (HB9) after 15 days. Expression of astrocytic marker (GFAP), neurons—microtubule-associated protein 2 (MAP2), gamma-aminobutyric acid (GABA), choline acetyl transferase—(ChAT), and oligodendrocytes—(O4) was observed on days 30 and 45. Electrical properties were characterized by an increase in membrane potential upon exposure to acetylcholine (Ach) and decreased membrane potentials after exposure with GABA. In future work, these bioprinted tissues could be further optimized and matured for drug-screening applications.

2. Results

2.1. Bioprinted Microspheres and Cells Were Distributed throughout the Bioprinted Constructs

The RX1 microfluidic-based bioprinter produced cylindrical constructs consisting of hiPSC-derived NPCs (1 × 10⁶ cells mL⁻¹ bioink) encapsulated in our neurobioink (Figure 1). Red and green fluorescently labeled microspheres with similar size to other types of cells (15 μm) were bioprinted in combination with NPCs and with drug-loaded microspheres to visualize their distribution within the construct (Figure 2, S1, and S2, Supporting Information). The group containing NPCs, in addition to drug-loaded and fluorescent microspheres, showed an even distribution of the NPCs as observed by 4',6-diamidino-2-phenylindole (DAPI) nuclear staining and fluorescence imaging (Figure 2c,d). Figure 2e,h shows the bioprinting of fluorescent microspheres with drug-loaded microspheres. The red arrows indicate a few of the drug microspheres distributed along the constructs. Analysis of pixel intensity measurements (n = 80 lines per construct) demonstrated that little variation was observed in the intensity between the sampled regions for both the microspheres and cells—suggesting a homogeneous distribution for both. The red microsphere distribution was 8.6 ± 1.3 μm², the green microsphere distribution was 5.3 ± 1.9 μm², and the DAPI stained NPCs distribution was 9.8 ± 2.1 μm² (Figure S1, Supporting Information). Qualitative analysis indicated that the bioprinting of two types of fluorescent microspheres red/green (2:1) and bioprinting green/drug-loaded microspheres (1:1) appeared similar in homogeneous distribution with the construct (colocalized Figure S2e,h, Supporting Information).

2.2. Bioprinted Tissues Exhibit High Levels of Cell Viability

Four groups were bioprinted: (i) bioprinted NPCs (N), (ii) bioprinted NPCs treated with soluble puro and RA (P), (iii) bioprinted NPCs with PCL-loaded-puro and RA microspheres (LM), and (iv) bioprinted NPCs with unloaded PCL microspheres (UM) (Table 1). Cell viability of the bioprinted NPCs was quantified on days 0 and 1 for all groups (Figure 3a,b). On day 0, the groups without microspheres showed higher viability with N at
88 ± 1% and P at 87 ± 1%, whereas LM was 78 ± 2% and UM was 75 ± 3%. On day 1, cell viability was higher for both groups containing drug-loaded and unloaded microspheres, with the highest levels of cell viability observed in LM with 98 ± 1% and UM with 84 ± 1%. N was 79 ± 3%, which was lower in comparison with day 0, whereas P viability was reduced to 80 ± 3%. SMNs were also bioprinted using the fibrin-based bioink and RX1 bioprinting system. Viability of the bioprinted SMNs was 97 ± 1% on day 0 that decreased to 85 ± 3% on day 1 (Figure 3c).

2.3. Bioprinted iPSC-Derived NPC Tissues Express HB9 and OLIG2 hiPSC-Derived Markers on Day 15

Immunocytochemistry (ICC) analysis was carried out on the bioprinted NPCs for beta tubulin III (βT-III), a microtubule protein present in neurons, the oligodendrocyte marker 2 (OLIG2), which determines MN and oligodendrocyte fate, and the mature MN marker (HB9) on day 15 (Figure 4). Expression of βT-III was observed in all groups (Figure 4a,f,k,p). OLIG2 expression was mainly observed in P, LM, and UM groups, similar to the expression of HB9 (Figure 4b,g,l,q). Quantification of cell marker expression was also conducted for the bioprinted NPCs using flow cytometry analysis on day 15 (Figure 5). NESTIN expression remained high for all groups, with LM showing the highest level of expression of 92 ± 1%, followed by N with 76 ± 10%, P with 61 ± 8%, and UM with 50 ± 5%. OLIG2 expression was 31 ± 7.1% for LM followed by 23 ± 2% for UM, 20 ± 3% for P, and 15 ± 2% for N. Statistical differences were observed for N and LM along with P and LM (Figure 5b). Expression of HB9 was also quantified at this time point with all the groups showing >56% of expression: LM at 83 ± 6%, P at 80 ± 1%, N at 77 ± 11%, and UM at 57 ± 4% (Figure 5c).

2.4. Bioprinted Tissues Express Mature MN and Astrocytic Markers on Day 30

The bioprinted tissues were stained on day 30 for glial fibrillary acidic protein (GFAP)—an intermediate filament protein expressed in astrocytes, βT-III, and ChAT—the enzyme in charge of synthesizing the production of the neurotransmitter Ach (Figure 6). GFAP expression was highly expressed for all groups (Figure 6a,g,m,s). βT-III in the LM group showed higher expression in comparison with N and P (Figure 6b,h,n,t). ChAT expression was mainly observed for P and LM (Figure 6c,i,o,u). Furthermore, expression of the oligodendrocyte marker (O4) was observed on day 30 for N, LM, and UM (data not available for P) (Figure 7a,e,i). The inhibitory neuron marker GABA was observed mainly for N and LM (Figure 7b,f,i). Quantification of cell marker expression on day 30 was analyzed for HB9, the

Table 1. Description, acronym, and components of the bioprinted groups.

| Condition description                                      | Acronym | Components                     |
|------------------------------------------------------------|---------|--------------------------------|
| Bioprinted NPCs with no added drugs                        | N       | NPCs                           |
| Bioprinted NPCs with soluble drugs added to the media      | P       | NPCs, Soluble puro, Soluble RA |
| Bioprinted NPCs with loaded microspheres                   | LM      | NPCs, puro microspheres, RA microspheres |
| Bioprinted NPCs with unloaded microspheres                 | UM      | NPCs, Blank microspheres (no drug) |

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Figure 2. NPCs, fluorescently labeled, and drug-loaded microspheres distribution within the bioprinted construct. a–d) red and green fluorescent microspheres bioprinted with NPCs stained with DAPI and d) merged figure of red and green fluorescent microspheres with DAPI-stained NPCs. White dashed borders show the construct boundary. e,g) puro-loaded microspheres bioprinted in combination with red fluorescent microspheres and f,h) RA-loaded microspheres bioprinted in combination with green fluorescent microspheres. Red arrows indicate the presence of microspheres. All figures taken on day 0 after bioprinting. Figure a–d) taken at 1.25 x, small images have a 3 x-digital zoom. Scale bar for a–d) is 10 mm. e–h) taken at 4 x, scale bar is 300 μm.
transcription factor Islet-1 (ISL-1) that is required for survival and specification of MNs, and ChAT (Figure 8). The N group showed the highest level of HB9 expression at 73.9 ± 8.7%, followed by P with 72.4 ± 7%, LM with 68.7 ± 3%, and UM with 53.2 ± 5.3% (Figure 8a). ISL-1 expression was 59.7 ± 6.4% for N, 46.6 ± 3.4% for P, 19.9 ± 8.3% for LM, and 12.29 ± 3.6% for UM (Figure 8b). ChAT expression was 59.7 ± 6.4% for N, 46.6 ± 3.4% for P, 19.9 ± 8.3% for LM, and 12.29 ± 3.6% for UM (Figure 8b). ChAT expression was 53 ± 2.3% for N, 52 ± 0.6% for P, 29.8 ± 7.3% for LM, and 29.2 ± 3.7% for UM (Figure 8c).

2.5. Neurons, Astrocytes, and Oligodendrocytes are Present in the Bioprinted Tissues by Day 45

Cell marker expression was quantified on day 45 for MAP2, ChAT, GFAP, and O4. Expression of MAP2 was the highest for LM with 13.3 ± 0.8%, followed by 11.5 ± 1.2% for N, 7.8 ± 0.5% for P, and 2.9 ± 0.3% for UM (Figure 9a). Expression of ChAT was the highest for N with 25.0 ± 3.8%, followed by LM with 19.1 ± 1.7%, UM with 6.9 ± 1.4%, and P with 4.3 ± 0.2% (Figure 9b). GFAP expression was 11.1 ± 1.3% for LM, 10.9 ± 1.7% for N, 8.9 ± 0.3% for P, and 4.9 ± 1.7% for UM (Figure 9c). Expression of O4 was 15.2 ± 0.8% for N, 12.9 ± 0.6% for LM, 8.4 ± 0.6% for P, and 6.4 ± 0.2% for UM (Figure 9d).

2.6. Quantification of Membrane Potential for the Bioprinted NPCs on Days 30 and 45

Resting membrane potential and stimulation using Ach of the bioprinted tissues was quantified on days 30 and 45 for the bioprinted NPCs and on days 0, 1, and 7 for the bioprinted SMNs (Figure 10). All cell culture treatments were responsive to the excitation treatments as observed by an increase in the membrane potential. On day 30, statistical differences were observed for all groups at rest versus excitation. On day 45, the tissues were also exposed to the neurotransmitter GABA after stimulation. All groups showed statistical differences after excitation and after inhibition except for LM, which showed a large standard deviation that reveals a variation in the excitability. Considering this variation, LM readings from day 45 were larger in comparison with day 30, showing an increase in excitability overtime. For the bioprinted SMNs, an increase in membrane potential was observed when exposed to Ach for all time points. Statistical differences were observed after excitation on day 0.
3. Discussion

Bioprinting SCs along with small molecule-releasing microspheres enables the bioprinted tissues to be differentiated in situ using the efficient delivery of a variety of morphogens. We successfully bioprinted drug-loaded microspheres, fluorescent microspheres with similar size to other types of cells, and hiPSC-derived NPCs using our neurobioink in combination with the RX1 microfluidic bioprinter. Distribution of the DAPI-stained NPCs, fluorescent microspheres, and drug-loaded microspheres was analyzed to determine their arrangement. The microsphere-laden bioink contained hiPSC-derived NPCs, which were successfully differentiated into neural tissues after 45 days and analyzed using cell viability analysis, flow cytometry, ICC, and quantification of membrane potential using a voltage-sensitive dye. These neural tissues showed cell maturation and responsiveness through characterization of their cell marker expression and membrane potentials upon excitation and inhibition.

Homogeneous distribution of the cells and drug-loaded microspheres within the fibers was observed, with the fluorescent microspheres having a similar size to other types of cells. While the composition differs from other elements, the bioprinting in this study allows for the visualization of their distribution within the construct and provides a proof of concept for bioprinting similarly sized components, such as different types of cells in coculture, in future studies. Therefore, we characterized the distribution of NPCs, fluorescent, and drug-loaded microspheres in quantitative and qualitative ways (Figure 1, 2, and Figure 2, Supporting Information). The bioprinted, fluorescently labeled, and/or drug-loaded microspheres in the presence or absence of NPCs showed a homogeneous distribution in our tissues. Similarly, we analyzed the distribution of fluorescent and drug-loaded microspheres by colocalizing the images and show that it was homogeneous. An interesting feature of the RX1 bioprinter is that it allows for different materials to be printed within the same construct. By forming continuous fibers and changing the materials on-the-fly, the materials are deposited in precise locations within the construct. The ability to form continuous fibers using this technology is of great importance as it provides a temporary and continuous environment for neighboring cells to connect and migrate while precisely distributing active molecules that support the differentiation and formation of their own specialized microenvironment. Future work using this technology could produce heterogeneous constructs while still maintaining homogeneous cell/microsphere distribution within the fibers or the ability to bioprint different types of cells with specific drug-releasing microspheres to achieve a coculture. Concentration gradients and localized drug delivery of different drug-loaded microspheres within the same construct can promote differentiation into different types of cells.

Maintaining cellular viability during the bioprinting process remains challenging as cells tend to be exposed to high shear tran...
Figure 5. Quantification of cell marker expression of the NPCs 15 days after bioprinting constructs. Markers quantified were a) NESTIN, b) OLIG2, c) HB9 (n = 3 for all, each sample had an average of 24,003 ± 6 cells). One-way ANOVA and Tukey post-hoc analysis was carried out for statistical analysis using a confidence level of 95% (p < 0.05). * represents p < 0.05, **p < 0.01.

Figure 6. Immunocytochemical analysis of the bioprinted constructs 30 days after bioprinting with NPCs. Expression of GFAP-red, βIII-green, and ChAT-blue is observed. a–f) N, g–l) P, m–r) LM, and s–x) UM. a–d,g–j,m–p,s–v) are at 10× + 4× digital zoom, with scale bar of 50 μm. e,k,q,w) at 5× digital zoom from merged column (d,j,p,v), scale bar is 10 μm, f,l,r,x) at 9× digital zoom from merged column (d,j,p,v), scale bar is 5 μm.
Figure 7. Immunocytochemical analysis of the bioprinted constructs 30 days after bioprinting NPCs. Expression of O4-red, GABA-green, and DAPI-blue is observed. a–d) N, e–h) LM, and i–l) UM. Figures are taken at 20× + 5× digital zoom, scale bar indicates 20 μm.

Figure 8. Quantification of cell marker expression of the bioprinted tissues 30 days after bioprinting. Markers quantified were a) HB9, b) ISL-1, c) ChAT (n = 3 for all, each sample had an average of 20,638 ± 1310 cells). One-way ANOVA and Tukey post-hoc analysis was carried out for statistical analysis using a confidence level of 95% (p < 0.05). * represents p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
forces during extrusion. Here, we demonstrate that the LOP soft nozzle of 28 mm long results in a cell viability >79% after being bioprinted on day 1. Our previous study also showed an increase in cell viability of >81% after 7 days of being bioprinted, using our neurobioink and the LOP. In this study, the majority of cells maintained their viability post printing and the progenitor cells were able to continue replicating as demonstrated by the increase in viable cell percentage after day 0. Interestingly, our drug-loaded microsphere group (LM) showed the highest percentage of cell viability on day 1 in comparison with the other groups, suggesting that either the controlled release of the small molecules or the physical properties of the construct could be promoting cellular proliferation for the constructs containing drug-loaded or unloaded microspheres. However, the differences between LM and UM groups observed on day 1 suggest that controlled drug release leads to cellular proliferation and therefore an increase in the number of viable cells after being bioprinted. Mature SMNs were also bioprinted under the same conditions using the fibrin-based bioink to analyze the viability post printing of mature neural cells. Viability levels reduced to 85% after day 1 of bioprinting but remained higher than most of the other bioprinted groups except for the LM group.

As previously reported, the continuous release of puro and RA from drug-loaded microspheres can efficiently differentiate hiPSCs into neural cell types, lower the amount of drug required compared with using dissolved drugs added into the media, and also reduce the need for media changes to add more drug, consequently reducing human intervention and room for error. Based on the 7-day release study (Figure S3, Supporting Information), on day 1, an initial burst of 25% (0.21 μg of puro) was released and by day 7, a total of 45% of the initial drug encapsulated was released. The average puro release per day from day 2 to day 7 was \(3.37 \times 10^{-2} \mu g\). At this rate, the microspheres released puro for a total number of 31 days including the initial burst on day 1. Given this release profile, a total of 0.13 μg of puro mL\(^{-1}\) of bioink was used to differentiate the bioprinted NPCs into mature neural tissues. For P, where the dissolved puro was constantly added to the media, an average of 0.06 μg of puro was added each media change and a total of 3.7 μg of puro mL\(^{-1}\) bioink was used for the 45 days of culture in vitro. The total amount of RA used for PR was 4.7 μg mL\(^{-1}\) of bioink.

Here, we show again that the continuous slow release of these two morphogens promotes the efficient production of mature neuronal subtypes from hiPSCs. The constant exposure of the NPCs to the drugs within the bioprinted construct provides an efficient and less labor-intensive way to differentiate and mature the tissues. The high expression of the mature MN marker HB9 for P and LM by day 15 is similar to other protocols where hiPSC-derived MNs were cultured in 2D and to our previous study where different bioprinted NPCs groups were treated with dissolved puro and RA. We hypothesize that the expression of OLIG2 (a MN progenitor and oligodendrocyte progenitor marker) for both P and LM led to the further differentiation into oligodendrocytes and expression of O4 at later time points. The constructs expressed high levels of the intermediate filament marker NESTIN after 15 days of culture. The elevated expression of this marker was attributed to the bioink degradation rate and

**Figure 9.** Quantification of cell marker expression of the NPCs 45 days after bioprinting. Markers quantified were a) the microtubule-associated protein 2 (MAP2), b) ChAT, c) GFAP, and d) oligodendrocyte factor 4 (O4) (\(n = 3\) for all, each sample had an average of 23,999 ± 1 cells). One-way ANOVA and Tukey post-hoc analysis was carried out for statistical analysis using a confidence level of 95% (\(p < 0.05\)). * represents \(p < 0.05\), ** \(p < 0.01\), *** \(p < 0.001\), **** \(p < 0.0001\).
ECM remodeling as observed by Madl et al. It is possible an initial population of NPCs maintained their self-renewal capabilities, continued to proliferate, and did not begin to differentiate until maturation media was added on day 15. This effect could be supported by the results observed in our previous study, where increased levels of cellular viability (>81%) was observed by day 7 after bioprinting NPCs using the fibrin-based bioink and culturing under the similar conditions. Similar to our results on day 15, other studies have shown the coexpression of NESTIN and OLIG2 in NSCs. Mature neurons coexpressing NESTIN and ChAT have been found in the adult brain in the basal forebrain region, the main region for cholinergic output in the brain. The high expression of NESTIN on the bioprinted constructs on day 15 could also be attributed to the nature of the cholinergic neurons present in the bioprinted tissues.

All the bioprinted tissues showed the presence of astrocytes as observed by the expression of GFAP on day 30. Our ICC analysis revealed that the neurons expressing βT-III seem to be surrounded by the astrocytes. Expression of ChAT was observed in all groups with a slightly higher expression for LM (Figure 6o). This expression was also observed in our previous study, where NPCs were bioprinted with the same neurobioink and treated with the cocktail of small molecules SB431542 (SB), CHIR99021 (CHIR), and LDN193189 (LDN). However, in this study, the NPCs were only exposed to this cocktail from days 0 to 5 as opposed to days 0–15 as previously reported. These results suggest that an early exposure to the BMP inhibitor and WNT pathway agonist are only needed for a few days after bioprinting to ensure that NPCs commit to a neural lineage. ChAT expression of the bioprinted mature SMNs (Figure S4, Supporting Information) was similar to the expression on the bioprinted NPCs on day 30, suggesting that the culture components, including bioink materials, small molecules, and drug-releasing microspheres, create a suitable environment for these cells to differentiate into mature neuronal subtypes such as mature SMNs.

Flow cytometry analysis revealed the expression of HB9, ISL-1, and ChAT, indicating the presence of mature MNs. Moreover, ICC analysis showed the presence of O4-oligodendrocytes and the inhibitory neurotransmitter GABA in the bioprinted tissues after 30 days of culture, suggesting the presence of multiple neuronal subtypes in the matured tissues. Interestingly, the nuclei of

Figure 10. Membrane potential of the bioprinted NPCs at days 30 and 45 and SMNs at day 0, 1, and 7. a) Membrane potential on day 30, b) membrane potential on day 45, and c) membrane potential for SMNs on days 0, 1, and 7. The membrane potential was quantified at rest, followed by excitation with Ach, for all. After excitation, the bioprinted tissues were inhibited with GABA on day 45 (n = 3 for all). Repeated measures ANOVA was carried out for each group at each time point using a confidence level of 95% (p < 0.05). * represents p < 0.05, **p < 0.01.
these cells that were counterstained with DAPI, showed a noticeable smaller size for the N group in comparison with the microsphere-treated groups. According to the study led by McMahon et al., differences in nuclear volume have shown to be an indication of the differentiation state in glial cells. In their study more differentiated populations of glial cells showed larger nuclei in comparison with undifferentiated progenitors. These observations highlight the ability of our microsphere-treated group to maintain and promote cellular differentiation of hiPSC-derived tissues.

The presence of most of the relevant CNS cell types was further supported on day 45 given the expression of MAP2-neurons, ChAT-cholinergic neurons, GFAP-astrocytes, and O4. Interestingly, LM showed lower levels of ChAT expression in comparison with N. However, the standard deviation of N is more than twice than LM, meaning that the overall distribution of LM was closer to the average value in comparison with N. Overall, P showed the lower expression of all the markers relative to LM, suggesting that the controlled release of the morphogens within the 3D-printed constructs is an efficient way to differentiate and mature SCs into neural tissues, achieving the presence of the cell types in the CNS that are important for maintaining homeostasis and neurotransmission.

Next, the membrane potentials of the bioprinted tissues were analyzed on days 30 and 45 using a voltage-sensitive dye to characterize the electrical properties as an indicator of maturity for our bioprinted tissues. To gauge the level of maturation of our bioprinted NPCs, we bioprinted mature hiPSC-derived SMNs to compare their membrane potential on days 0, 1, and 7. The resting membrane potential of mature neurons lies at −70 mV. An action potential occurs when there is an increase in the electrical charge of the membrane that occurs as an all-or-nothing event once the threshold at −50 mV has been reached. Interestingly, the bioprinted mature SMNs showed a resting potential on day 0 of −9 mV and increased for days 1 and 7. The resting membrane potential of our bioprinted and matured PR and LM tissues was below 7 mV on day 30. However, none of the bioprinted constructs showed a resting membrane potential of −70 mV, our bioprinted constructs remained below 7 mV after day 45 for all hiPSC-derived NPCs bioprinted groups. This behavior could be attributed to the lack of full maturation of our bioprinted NPCs and possibly the need of an acclimation period for the SMNs in our neurobioink.

In addition, constructs were stimulated with the neurotransmitter Ach to analyze their responsiveness as an indication of maturation given their differentiation into MNs, as observed by the coexpression of HB9/ISL-1 and CHAT on days 30 and 45. All groups showed to be responsive upon exposure to Ach by day 30, with LM showing the most significant differences after stimulation, suggesting the presence of cholinergic receptors on MNs. This experiment was repeated on day 45 for our bioprinted constructs but in addition, the constructs were exposed to the inhibitory neurotransmitter GABA after stimulation. This step was added to analyze the constructs’ response to different neurotransmitters. At this time point, we saw similar levels of resting membrane potential, followed by an increase after stimulation with Ach, and decrease in membrane potential after exposure to GABA. All groups but LM showed statistical differences after exposure with each neurotransmitter. This could be explained by the large standard deviation in these readings that could be attributed to the nature of the membrane potential. Moreover, the increase in excitability overtime shows the ability of this bioprinting platform to promote and support the maturation of hiPSC-derived neurons.

The SMNs showed a higher increase in the membrane potential, reaching an average of 25 mV on day 0. These results correlate with Robinson et al., where characterizing the resting membrane potential of mature SMNs in 2D showed it to be below ≈10 mV. The SMNs displayed an average of ≈20 mV upon stimulation with Ach. The inability of the SMNs, both in 2D and the 3D bioprinted tissues, to reach a higher membrane potential could be attributed to their level of maturity and acclimation periods in both culture settings.

GABA is one of the major neurotransmitters in the CNS responsible for the 40% of the inhibitory processes. In the past, it has been observed that the exposure of nonactive or immature neurons to GABA can cause depolarization rather than hyperpolarization as a result of an increased Cl-eflux. In this study, inhibition with GABA for all groups led to a lower membrane potential than the initial readings at rest. These results suggest that cells in all groups have effectively differentiated and matured into MNs and are responsive as observed by an increase in the average membrane potential upon stimulation with Ach and reversed when adding the inhibitory neurotransmitter.

Other studies have conducted similar analysis where MNs are generated from SCs. A study led by Zhou et al. used bone mesenchymal stem cells (BMSCs) and NSCs photocrosslinked in various concentrations of GelMA hydrogels to derive oligodendrocytes and neuronal cells for SC regeneration. These GelMA hydrogels produced significantly more neurons and oligodendrocytes as measured using optical density. Others have used combinations of natural biomaterials to 3D bioprint human NSCs using an alginate/carboxymethyl-chitosan (CMC)/agarose bioink leading to the increased presence of various mature neuronal cell types such as GABAergic neurons in comparison with 2D and 3D control cultures as indicated by qPCR analysis of neuronal markers.

Furthermore, the use of small molecule releasing microspheres in 3D bioprinting was recently achieved by our group, leading to the successful differentiation of hiPSC-derived dopaminergic neurons. The cellular composition of these tissues consisted of 15% TuJ1 positive neurons with 8% of the cell staining positive for tyrosine hydroxylase as determined using flow cytometry. Moreover, to our knowledge, this is the first publication using a bioink that contains a combination of natural biomaterials in addition to drug releasing microsphere that can successfully derive hiPSC-MNs and other mature neuronal subtypes with these levels of efficiency. This study brings significant advances to the field of bioprinting and can be further optimized to serve as a cost-effective drug-screening tool for neurodegenerative diseases and SC regeneration. Furthermore, this study supports our previous findings and proves that the controlled and localized delivery of morphogens in bioprinted tissues can promote hiPSC differentiation into a desired cell type and can be adapted toward different types of tissues in the CNS.
4. Conclusion

We have successful bioprinted and differentiated hiPSC-derived neural tissues using a novel bioink containing small molecule-releasing microspheres. Our drug-loaded microspheres were homogeneously distributed within the fibers of the constructs and promoted an efficient differentiation and maturation of MNs. Our tissues also expressed the markers associated with GABAergic neurons, astrocytes as observed by the expression of GFAP, and oligodendrocytes as observed by the expression of O4. Furthermore, our matured MNs were shown to be responsive upon stimulation with ACh, followed by inhibition with GABA and showed similar behavior to bioprinted mature human SMNs. However, in order to fully assess the functionality of the bioprinted tissues, synaptic activity should be evaluated. In the future, our studies will focus on the localization of drug delivery within the fibers of the construct, as well as the creation of concentration gradients to fully differentiate different cell types and obtain a more complex environment in our 3D neural tissues. Furthermore, additional studies can be conducted to determine the minimum amount of each puro and RA-loaded microspheres required to achieve the differentiation of the bioprinted tissues in a cost-effective way.

5. Experimental Section

Preparation of the Neurobioink: The neurobioink was prepared as previously described.[3,14,15] Briefly, fibrinogen (341578, EMD Millipore) was prepared at a concentration of approximately 0.50 mg mL⁻¹ in tris-buffered saline (TBS) solution and sterilized using 0.2 μm syringe filters. The final concentration for fibrinogen was adjusted to 20 mg mL⁻¹ in the neurobioink. Sodium alginate (180 947, 120 000-190 000 g mol⁻¹, M/G ratio 1.56 Sigma-Aldrich) was prepared at 2% w/v by reconstituting in distilled water and sterilized using 0.2 μm syringe filter. The final concentration of alginate in the neurobioink was 0.3 mg mL⁻¹. The crosslinking solution consisted of calcium chloride (CaCl₂) (C1016, Sigma-Aldrich), thrombin (T7009, Sigma-Aldrich), and chitosan (C3646, Sigma-Aldrich). CaCl₂ was prepared at a concentration of 200 μL mL⁻¹ in TBS. Thrombin was reconstituted at a concentration of 1000 μM mL⁻¹ in sterile TBS. The final concentration of thrombin was 1.7 μM mL⁻¹ in the crosslinker. Chitosan was prepared at a concentration of 25 mg mL⁻¹ using 1% acetic acid. The pH was adjusted to 7.4 using β-glycerolphosphate (β-GP) (G9422, Sigma-Aldrich). The final concentration of chitosan in the crosslinker was 0.075% w/v. The crosslinking solution was sterilized by filtering using 0.2 μm syringe filters. For visualization purposes while printing, a solution of phenol red TBS (PR-TBS) at a concentration of 0.5 mg mL⁻¹ PT-TBS was prepared and sterilized using 0.2 μm syringe filter before being added to the bioink.

Bioprinting of Drug-Loaded Microspheres and Fluorescent Microspheres: puro- and RA-loaded PCL microspheres were fabricated as previously described using a single emulsion oil-in-water (o/w) technique.[41,42] Based on our previous reports, the average diameter size of the RA- and puro-loaded microspheres was 3.41 ± 1.6 and of 3.4 ± 1.17 μm, respectively.[41,42] Red FluoroSpheres (F8842, Thermo Fisher), and green FluoroSpheres (F21010, Thermo Fisher) of 15 μm were also used for bioprinting to show the distribution of similar size elements within the fibers of the construct. Several combinations of microspheres were bioprinted to replicate their printability and distribution. A total of 0.25 mg mL⁻¹ of microspheres were bioprinted for every combination. The final ratio was 1:1 for the groups containing red and green fluorescent microspheres. Red microspheres were combined with puro microspheres in a 1:1 ratio, and RA microspheres were combined with green microspheres on a 1:1 ratio. For the groups containing RA, the cell density was 1 x 10⁶ cells mL⁻¹ bioink. The bioprinted constructs containing combinations of drug-loaded and/or fluorescent microspheres were imaged using an Olympus IX-81 inverted microscope using the Metamorph imaging software 7.6.1 from MAG Biosystems.

Visualization of the Bioprinted Constructs and Microsphere Distribution: Bioprinted constructs containing no microspheres or 0.25 mg mL⁻¹ of unloaded microspheres were imaged using the Cytation 5 and the software Gen5 version 3.05 (BioTek instruments). Constructs containing red and green fluorescent microspheres with puro, RA-loaded microspheres, and DAPI-stained NPCs were imaged using the same platform. Fluorescent microsphere and DAPI stained NPCs distribution was obtained by manually drawing 80 lines across each construct and analyzed using plot profile from Image J. This feature displays the average gray value of pixels from each line selection. The average intensity profile of each line across the construct was taken and is shown on Figure S1, Supporting Information. Colocalization of images for Figure S2, Supporting Information, was performed using Fiji software version 2.0.0-rc69/1.52p. Phase contrast images were binarized using the Sauvola method from the Auto Local Threshold plugin and selecting a radius of 20 pixels.[43] This method calculates the thresholds for every pixel and is useful for images with non-uniform backgrounds. Image noise was then removed using the Despeckle plugin. The resulting image was then colocalized with the red and green fluorescent microspheres using the Colocalization Threshold analysis.

Characterization of the Controlled Release of Pumorphamine-Loaded Microspheres from the Bioprinted Constructs: A 7-day release study was conducted to determine the release kinetics of puro from drug-loaded microspheres bioprinted using the fibrin-based bioink, the LOP technology and RX1 bioprinter. The drug was extracted from the remaining microspheres on each sample and quantified using high-performance liquid chromatography (HPLC) to estimate puro concentration. The bioprinted constructs, pattern, and printing parameters used were as previously mentioned in this study. Collection days were 0, 1, 4, and 7 and a media change was carried out for each of these days. All samples (n = 4) contained four cylindrical constructs, for a total of 1 mL of bioink and 0.167 mg of puro microspheres cultured in 4 mL of phosphate-buffered saline (PBS) per sample (i.e., 0.25 mL of bioink and 0.0416 mg of puro microspheres cultured in 1 mL of PBS for each construct replicate). The amount of microspheres chosen for each sample, 0.167 mg, was the same as the amount of puro microspheres used in this study for bioprinting constructs containing hiPSC-derived NPCs. The constructs were placed in petri dishes and incubated at 37 °C. The samples were placed in 15 mL conical tubes and frozen at −20 °C for further processing. For scaffold degradation, each sample was thawed at room temperature then placed in C-tubes and processed as previously mentioned using the MACS Miltenyi Biotech Neural Tissue Dissociation kit and the gentleMACS dissociator. After enzymatic and mechanical dissociation of the scaffolds, the samples were vortexed vigorously until broken apart followed by centrifugation at maximum speed and washing with dH₂O twice. Supernatant was removed and the samples were freeze-dried overnight. Puro extraction from the recovered microspheres was carried out by adding 300 μL of acetonitrile (ACN) and mixed by vigorously pipetting and placing in the vortex mixer (Eppendorf MixMate) at 3000 rpm for 10 min. The samples were then placed at −80 °C for 5 min, followed by centrifugation at 15 000 rpm for 5 min. The remaining supernatant was collected and filtered through 0.2 μm PTFE syringe filters using a 1 mL syringe (Norm-Ject tuberculin) and placed in HPLC amber vials (Agilent). Quantification of puro using HPLC was carried out as previously described.[43]
Bioprinting and Culture of MNs: Each cryovial of hiPSC-derived SMNs from BrainXell (BX-0100) containing 5 x 10^6 cells was removed from the liquid nitrogen and thawed in a water bath at 37 °C. The SMNs were slowly resuspended in Neurobasal Medium (NBM) (21103-049, Life Technologies) at a rate of ~1 drop s⁻¹ and centrifuged at 300 rcf for 5 min. After pellet formation, the supernatant was removed and the SMNs were gently resuspended in the neurobiobio for bioprinting. Printing conditions and design were the same as those used for NPCs. Culture conditions were carried out as recommended by BrainXell.[24] On day 0, the bioprinted SMN constructs were cultured in seeding medium containing 0.5 x of DMEM/F12 (11330-032, Life Technologies), 0.5 x of NBM, 1 x B27 (17500-044, Life Technologies), 1 x N2, 0.5 mM GlutaMAX (35050-601, Life Technologies), and 1 x of Neuron seeding supplement (provided by BrainXell). On day 1, all the media was removed, and the cells were cultured with medium containing 0.5 x of DMEM/F12, 0.5 x of NBM, 1 x B27, 1 x N2, 0.5 mM GlutaMAX, 1 x of Neuron seeding supplement, 10 ng/mL BDNF (PHC0704-Thermo Fisher), 10 ng/mL GDNF (PHC0704, Thermo Fisher), 1 ng/mL TFG-j1 (PHC9214, Thermo Fisher), and 15 μg/mL of Gentrex (A15696-01, Gibco). On day 4, all the media were removed and the bioprinted constructs were cultured with 0.5 x of DMEM/F12, 0.5 x of NBM, 1 x B27, 1 x N2, 0.5 mM GlutaMAX, and 1 x of Neuron day 4 supplement (provided by BrainXell). Media was changed every 3-5 days. On day 7, the bioprinted SMN constructs were cultured in iPS media.

Assessment of Cell Viability: Cell viability for bioprinted NPCs and SMNs was carried out by obtaining a single-cell suspension using the MACS Miltenyi Biotechnique Neural Tissue Dissociation kit and gentleMACS™ disso- ciator (130-093-235, Miltenyi Biotech). This process consists of a combination of enzymatic and gentle mechanical dissociation cycles that degrade the bioink of the bioprinted tissues. Triplicate samples (n = 3) for each group were prepared for cell viability by removing cell media and transferring constructs to a C-Tube. Mix 1 was prepared by adding 1,432 mL of Buffer Z and 37.5 μL of enzyme P. The culture wells were washed with 1470 μL of mix 1 and then placed in the C-Tube with the constructs. After addition of mix 1, the C-tubes were gently inverted twice to ensure that the constructs were located in the rotator area. They were then placed in the gentleMACS™ dissociator and the preprogrammed m_brain_01 program was run twice. The samples were then incubated at 37 °C for 20 min, followed by the addition of 225 μL of mix 2. Preparation of mix 2 was carried out by adding 15 μL of Buffer Y and 7.5 μL of enzyme A. The C-tubes were gently inverted twice, then placed in the gentleMACS™ dissociator and the preprogrammed m_brain_02 program was run twice. The samples were then incubated for 30 min at 37 °C for 20 min. The m_brain_03 was run twice, and 2 mL of PBS were added to each C-Tube. The samples were then strained into 15 mL conical tubes using a 7 μm reversible strainer (27215, STEMCELL Technologies). Finally, the C-tubes were washed with 2 mL of PBS and the remaining solution was strained. The samples were then washed three times by centrifuging at 300 rcf for 5 min and adding 1 mL of PBS. Cell viability was carried out as previously described.[25] Briefly, 20 μL of the cell suspension was mixed with 380 μL of Guava ViaCount reagent (4000-0040, Millipore). For analysis, 100 μL of the mix was added to each well on a 96-well plate (n = 3 per group). Cell viability was determined using the Guava EasyCyt HT flow cytometer (Millipore).

Immunocytochemistry: ICC was performed as previously described by removing cell media and washing the constructs with PBS, followed by fixation with 10% formalin at 4 °C, under agitation at 10 rpm for 2 h.[1] Permeabilization was carried out by adding 0.1% of triton-X (HTS01128, Sigma) diluted in PBS and incubating for 45 min under the same conditions. Blocking was accomplished by adding 5% NGS (NS02L, Sigma) diluted in PBS and incubating at 4 °C under the same conditions. Primary anti-bodies were incubated overnight under the same conditions for the following concentrations: H99 (1:1000, AB1714, Millipore), OLG1 (5 μg/mL⁻¹, AF2418, R&D systems), ChAT (5 μg/mL⁻¹, AF3447, R&D systems), JT-III (1:500, ab18207, abcam), GFAP (1:1000, ab10062, abcam), MAP2 (1:200, ab32454, abcam), O4 (1:500, MAB328, Millipore), and GABA (1:500, AB18207, abcam). Following incubation with the primary anti-bodies, samples were washed
twice with PBS under agitation at 4 °C for 25 min for the first wash and 40 min for the second wash. Secondary anti-bodies were incubated for 2 h under the same conditions: donkey anti-mouse 568 (1:500, ab175700, abcam), donkey anti-goat 405 (1:500, ab175664, abcam), donkey anti-rabbit 488 (1:500, ab150073, abcam), goat anti-rabbit 568 (1:500, ab175471, abcam), and donkey anti-goat 488 (1:1000, 705-545-1477, Jackson immuno). Following incubation, the samples were washed twice with PBS under agitation at 4 °C for 25 and 40 min, respectively. After washing, the samples were stained with DAPI at 0.105 μg/mL [1] (D1306, Molecular probes) under agitation at 100 rpm for 7 min, followed by 2 washes with PBS under agitation at 4 °C for 25 min. Fluorescence imaging was performed using the Olympus IX-81 inverted microscope using the Metamorph imaging software 7.6.1 from MAC Biosystems.

Confocal Imaging: Immunofluorescence imaging of the bioprinted tissues was carried out using the Olympus BX61WI confocal microscope and the FluoView-1000 software (Olympus, Toronto, ON). A small sample of the bioprinted tissue was excised using dumont tweezers and McPherson-Vannas microdissecting spring scissors (Roboz Surgical Instrument, Gaithersburg, MD) under a SMZ-168 stereomicroscope (Motic, Richmond, BC). Samples were placed in precleaned superfrost glass slide (Fisher Scientific, Pittsburgh, PA) and cover slipped using Vectashield (Vector Laboratories, Burlingame, CA). Images were acquired with Olympus UPlanSApo objectives at 10×, 40× + 2 digital zoom using a 4, 2, or 1 μm z-step, respectively, and a 1024×1024-pixel frame size. Samples were imaged using the 405, 488, and 568 filters. FJI (Image); National Institutes of Health) was used to create maximum intensity projections from image stacks.

Flow Cytometry Analysis of Bioprinted Neural Constructs: Degradation of the scaffolds was performed as previously described for cell viability using the MACS Miltenyi Biotech Neural Tissue Dissociation kit and the gentleMACS™ dissociator. After the samples were washed three times in PBS, the resulting cell suspension was processed as previously described following the manufacturer’s instructions (R&D systems).[1,2,13] Cell marker expression was quantified on the hiPSC-derived NPCs prior to bioprinting for the sex-determining region Y-box2 (SOX-2) (CS202594, Millipore), and NESTIN (IC1259P R&D). On day 15, quantification was carried out for the expression of Homeobox protein 9 (HB9) (bs-11320R, Bioss anti-bodies), Oligodendrocyte transcription factor 2 (OLIG2) (IC2230P, R&D), and NESTIN. On day 30, quantification was performed for the expression of HB9, Islet-1-Islet-1 (S62547, BD Pharmingen), and Choline Acetyl Transferase (ChAT) (ab22400, abcam). On day 45, cell marker expression was quantified for the microtubule-associated protein 2 (MAP2) (FCMAB318, Millipore), Gial fibrillary acidic protein (GFAP) (NBMP2-3184P, Novusbio), oligodendrocyte marker (O4) (FAB1326P, R&D systems), and ChAT (ab22400, abcam). Isotype controls were Phycoerythrin (PE) (IC015P, R&D systems), and Peridinin Chlorophyll Protein Complex (PerCP) (IC003C, R&D systems). The samples were analyzed on the Guava easyCyte HT flow cytometer using the InCyte software 2.6.

Characterization of Membrane Potential: Characterization of the membrane potential of the bioprinted hiPSC-derived NPCs was carried out on days 35 and 45 using the voltage-sensitive dye FLIPR Membrane Potential Assay kit-blue component diluted in buffer component B (ROB42, Molecular Devices).[13] Characterization of the bioprinted SMN’s membrane potential was carried out on days 0, 1, and 7. The FLIPR blue dye was added to the bioprinted constructs on a ratio of 1:1 of dye to cell culture media. The plates were then covered from light exposure and incubated at 37 °C with 5% CO2 for 45 min. After incubation, the cell culture plate cover was removed and a Microseal B adhesive sealer (MSB-1001, Biorad) was placed on top of the plate under sterile conditions. Fluorescence scans of the constructs at rest were run using the microplate reader as previously described by Robinson et al., with excitation at 530 nm, emission at 565 nm, 25 flashes, and a 5 × 5 reads per well.[13] Background readings were obtained by adding 1:1 dye to cell media in wells containing constructs with only bioink to normalize for N and P and constructs containing 0.25 mg mL−1 of PCL microspheres to normalize for LM and UM. After reading the fluorescence at rest, the bioprinted neural tissues were then excited by adding 100 μM of ACh (A22611, Sigma) and incubated under the same conditions for 25 min. Fluorescence readings of the bioprinted tissues at excitation were then performed as previously described. After excitation of the bioprinted constructs at day 45 with Ach, the tissues were then exposed to GABA (A2129, Sigma) at a concentration of 10 μM and incubated for 25 min under the same conditions.[14] Fluorescence readings were carried out as previously described. Change in fluorescence was normalized following the Equation (1), where F0 is the average background reading. Normalized fluorescence readings were then converted to membrane potential ∆E following Equation (2) as described by Robinson et al.,[13] where R is the gas constant, F is Faraday’s constant, T is the average temperature of the readings, and z is the apparent charge of the external dye concentration.

\[ \Delta E = \frac{F - F_0}{F_0} \]  

Statistical Analysis: All data were reported as the mean ± standard deviation for the bioprinted constructs containing puro-loaded microspheres for in vitro release study (n = 4), release kinetics of puro (n = 3), bioprinted constructs containing combinations of drug-loaded, fluorescent microspheres and NPCs (n = 3), analysis of microsphere and NPC distribution within the bioprinted constructs (n = 80), bioprinted constructs for groups N, P, LM, and UM (n = 3), bioprinted constructs containing MNS (n = 3), assessment of cell viability, flow cytometry, ICC, and characterization of membrane potential (n = 3). Statistical analysis for cell viability was carried out using one-way ANOVA and Tukey post-hoc analysis between groups per day using a confidence level of 95% (p < 0.05), 99% confidence (p < 0.01), and 99.9% confidence (p < 0.001). For the SMNs, unpaired T-test with equal standard deviations was carried out using a confidence level of 99% confidence (p < 0.01). For flow cytometry, one-way ANOVA and Tukey post-hoc analysis was carried out using a confidence level of 95% (p < 0.05), 99% confidence level (p < 0.01), and 99.9% confidence level (p < 0.001). Statistical analysis of the membrane potential was carried out using repeated measures ANOVA for each group at each time point using a confidence level of 95% (p < 0.05), 99% confidence (p < 0.01). Cell viability and flow cytometry data were obtained using the Guava InCyte software and all the statistical analysis was carried out using GraphPad prism 6 statistics software.

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

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Conflict of Interest
Laura de la Vega, Laila Abelseth, and Stephanie Willerth are co-founders of Axolotl Biosciences - a start-up focused on the production of novel bioinks.
Author Contributions

L.D.I.V. designed, set-up, performed experimental procedures, analysis and preparation of manuscript. L.A. assisted on experimental design and procedures, analysis, and editing of the manuscript. J.T.-P. performed confocal microscopy imaging and assisted on writing confocal microscopy methodology. R.S. assisted on preparation of the bioink and bioprinting, M.R. assisted on experimental procedures. S.M.W. provided feedback on the experimental design, analysis and editing of the manuscript.

Data Availability Statement

Data available from the corresponding author upon request.

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