Generation of Human iPSC-Derived Neurons on Nanowire Arrays Featuring Varying Lengths, Pitches, and Diameters

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Nanowire (NW) arrays interfaced with biological cells have been demonstrated to be potent tools for advanced applications such as sensing, stimulation, or drug delivery. Many implementations, however, have so far only been studied with rather robust basic cell models. Here, the generation of human induced pluripotent stem cell (iPSC)-derived neurons is presented on various types of NW arrays. Specifically, combinations of three NW lengths (1, 3, and 5 µm), three array pitches (1, 3, and 5 µm), and two NW diameters (thin/thick pairs, 270–600 nm/590–1070 nm) are being utilized. The cell/NW interactions range from fakir-like states to NW-encapsulating states depending on the array characteristics. The cultures show equal proportions of neuronal marker-positive cells after 8–9 days of terminal differentiation on the NW arrays (14–15 days in total) compared to planar controls. In addition, the neurons are functional with similar kinetics of the action potentials highlighting the equivalence of the NW arrays for neuronal differentiation. In the future, stem cell research and regenerative medicine might substantially benefit from further functionalized NW arrays enabling the well-established mechanisms such as NW-mediated in vitro gene editing or intracellular delivery of biomolecules to further control and/or to enhance neuronal differentiation.

1. Introduction

State-of-the-art micro- and nanofabrication techniques allow nowadays for engineering advanced biological metamaterials which are being considered promising candidates, for example, to support biomedical translation, to fabricate minimal invasive nanoelectronics, or to be utilized as nanotools in neuroscience and regenerative medicine. In this context, vertically aligned high aspect ratio nanostructures—so-called nanowire (NW) arrays—used as cell culture substrates play an increasingly important role in establishing novel tools for interrogating and stimulating cells on molecular and cellular levels. In recent years, studies testing the unique capabilities of NW arrays have been conducted with a variety of cell types, for instance, basic cell lines such as GPE86, HEK293, and HeLa cells, primary rodent neurons, and (mesenchymal) stem cells (MSCs), to name a few. The impact of such studies on medical applications such as drug screenings and neurodegenerative disease studies might, however, be significantly improved by employing more sophisticated cells, namely, cells derived from human induced pluripotent stem cells (iPSCs).

Human iPSC technologies have changed the way of preclinical research and application by enabling human (patient-specific) in vitro models without restrictions in cell availability. Not only political and ethical controversies raised by using embryonic stem cells (ESCs) are avoided, but also all major cell types including blood-brain barrier models and brain organoids can be generated. For studying neurodegenerative diseases such as Alzheimer’s or Parkinson’s, neurons derived from human iPSCs are of particular interest since adequate models are otherwise scarcely available. Apart from ESCs,
animal models using, for example, neurons from rodents have been employed. Corresponding studies, however, often suffer from high clinical failure rates during the translation to the human physiology and as a result, their pertinence for medical applications is typically rather limited. In the same way, the existing studies demonstrating the advantages of NW arrays in cell culture but only utilizing traditional cell models might be restricted, for instance, in the context of improving neurodegenerative disease studies or pharmacological drug screenings. Nevertheless, stem cell research might substantially benefit from, e.g., improved neuronal differentiation and sophisticated neuronal cell interrogation mediated by (functionalized) NW arrays.

NW arrays used as cell culture substrates elicit a strong interaction between the substrate and the cells by their unique topography. The very interplay between both renders possible a multitude of applications such as sensing, stimulation, and drug delivery. Passive NW arrays have been used, for example, to measure mechanical cell properties to interact with the cell’s nucleus to reorganize actin to stimulate the mechanotransduction machinery or to direct cell polarization such as outgrowth and branching of neurites. Further, the strong coupling between the cell membrane and the substrate can also help to improve electrical recording and stimulation of microelectrode arrays that have been equipped with the nanostructures. Also intrinsic material features of the NWs such as photoelectrochemical properties—either in arrays or free-standing, that is, detached from the substrate—can be used to modulate neuronal or cardiac activity in vitro or as retina implants. Another fundamental advantage of the NW arrays is the capability to support intracellular delivery by stimulated endocytosis in case of hollow NWs—by direct injection into the cell via electroporation. The variety of potential applications renders NW arrays a promising element for next-generation biomedical instruments and nano-enabled neuronal interfaces. However, present studies have also shown that NW arrays can influence basic cell properties such as the viability, movement, proliferation, adhesion, and drug delivery. Passive NW arrays have been used, for example, to measure mechanical cell properties to interact with the cell’s nucleus, to reorganize actin, to stimulate the mechanotransduction machinery, or to direct cell polarization such as outgrowth and branching of neurites.

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To make use of the NW array-associated advantages such as the improved electrical junction or the intracellular drug delivery, it thus is essential for the prospective applications that the neuronal differentiation of human iPSCs is maintained especially on passive NW arrays compared to planar controls. The equivalence would permit that well-established differentiation protocols tested in regular Petri dishes can be applied in the future. In previous studies, we showed the general feasibility of generating human iPSC-derived neurons on NW arrays, but the utilized substrates featured only about 1 μm long NWs. As a result, their rather basic topographies just induced a limited amount of deformational stress toward the cells. The impact of NW array geometries—for instance, with longer NWs—on the neuronal differentiation is still unclear and thus has been addressed in this study.

Here, we present the generation of functional human iPSC-derived neurons on NW arrays featuring multiple combinations of three different NW lengths (1, 3, and 5 μm), three different array pitches (1, 3, and 5 μm), and two different NW diameters (thin/thick pairs, 270–600 nm, 590–1070 nm) after only 14–15 days of differentiation. The extensive variation of the NW arrays characteristics went along with fundamental changes in the interaction between the neurons and the NW arrays. Specifically, the interaction regimes ranged from fakir-like states on dense NW arrays where the cells were only in contact with the nerve tips to NW-encapsulating states on sparse NW arrays where the cells enwrapped entire NWs. Severe deformations and indentations of the neurons including their nuclei were observed particularly with 5 μm long NWs. Despite the varying interactions with the NW arrays, the proportion of neuronal marker-positive cells (MAP2, NeuN, TH) after 8–9 days of terminal differentiation on the NW arrays (14–15 days in total) was similar to planar controls. In addition, similar electrophysiological properties including the functional character of the generated neurons confirmed the quality of the neuronal differentiation on the NW arrays. By highlighting the equivalence of the culturing substrates for the neuronal differentiation, the results pave the way for enabling the unique applications of—then functionalized—NW arrays for human iPSC-derived neurons.

We believe that stem cell research and regenerative medicine might substantially benefit in the future from the well-established NW array-mediated mechanisms such as in vitro gene editing or the intracellular delivery of biomolecules, for example, to further control and/or enhance neuronal differentiation.

2. Results

The NW arrays featuring different NW lengths, array pitches, and NW diameters were prepared from plain silicon using reactive ion etching (RIE) and a hard mask of rectangularly arranged dots defined by electron beam lithography (EBL) (Figure 1). Specifically, three NW lengths of 1, 3, and 5 μm (L1, L3, and L5), three array pitches of 1, 3, and 5 μm (P1, P3, and P5), and two diameters (D+/D−, thin/thick pairs from 270–600 nm and 590–1070 nm diameter) were used—resulting in 18 different types of NW arrays (summarized in Table 1). The wide range of NW diameters within the D+/D− categories was caused either by the nature of the RIE process or the pitch of the arrays as described in the following. The D− diameters correspond to the minimal value that was compatible with the RIE process (~270–600 nm). Longer wires (L3 and L5) and larger pitches (P3 and P5) required gradually increased diameters to compensate for the tapering occurring toward the base of the NWs (clearly visible for L5 P3 D− and L5 P5 D−, Figure 1). For D+ NW arrays, the diameter was arbitrarily set to a rather large value of ~1000 nm. Note, for P1 D+ NW arrays, the diameter was only about 600 nm because else the NWs essentially would have been tangent with each other and concomitant, the geometrical nature of a NW array would have been lost.

The interaction of the human iPSC-derived neurons with the NW arrays was investigated by 3D confocal laser scanning microscopy (CLSM) imaging (Figure 2, top-views and cross sections). Neurons (green) were identified by the neuronal marker microtubule-associated protein 2 (MAP2) and their nuclei (red) were stained with DRAQ5. Note, the NW tips'
reflections appeared green. Overall, three interaction regimes were found, namely, fakir-like, intermediate, and encapsulating regimes. In general, the induced topographical stress on the cells (e.g., deformation of the nucleus) increased with the NW length. The specific settling regimes of the neurons were predominantly governed by the array pitch. The fakir-like states where the cells rest on a “bed-of-nails” only being in contact with the very NW tips were found for almost all P1 NW arrays. However, for L1 P1 D− NW arrays, intermediate states were also observed. Note, for L3/L5 P1 D− NA arrays, the neurons were fakir-like but more entanglement with the NW tips was observed. The interactions of the neurons with the P3 and P5 NW arrays were more complex. On P3 D+ NW arrays, to start with, the neurons often showed encapsulating settling states. However, in case of the longest NWs (L5 P3 D+, Figure 2) the cells were likely to lose contact to the bottom of the substrate and were thus mostly in intermediate states. In case of the corresponding D− NW arrays, in contrast, the imaged cells were more often in intermediate or even fakir-like states whereat with P3 L3 D+ NW arrays (Figure 2) the yield of the intermediate state was particularly low (3 of 11 imaged neurons, else fakir-like). For P5 NW arrays, the majority of the neurons completely encapsulated the NWs and were hence also in contact with the bottom of the substrate. As a result, the variation of the NW lengths using P5 NW arrays had a distinct influence on the cell/NW interface. Cells on the L1 P5 NW arrays were only scarcely visibly deformed by the short NWs. Using L3 P5 NW arrays, the NWs showed a larger impact on the somata. The nuclei, however, were usually unaffected. For L5 P5 NW arrays, in contrast, not only the somata were severely deformed but also the nuclei were indented by the NWs. The diameters of the NWs played a subordinate role for which resting state was observed. Nevertheless, the different diameters altered the amount of cell material, that is, cell membrane and nucleus, being displaced by the NWs. The analysis of cells by CSLM already showed that MAP2-positive neurons were generated on the NW arrays despite to some extent severe appearing topographical challenges. In a next step, the overall yields of the neuronal differentiation were analyzed by additional neuronal markers.

The yields of the neuronal differentiation on the NW arrays were determined from immunocytochemistry (ICC) double-stainings and compared to planar controls (Figure 3). On the one hand, the cells were labeled with MAP2 and neuronal nuclei (NeuN) (Figure 3a, examples of L1 P1 D−, L3 P3 D+, and L5 P5 D− NW arrays, all others and controls in Figure S1, Supporting Information) and on the other hand, with MAP2 and tyrosine hydroxylase (TH) (Figure 3b, examples of L1 P1 D+, L3 P3 D−, and L5 P5 D+ NW arrays, all others and controls in

Table 1. Overview of the geometry of the utilized NW arrays. The entries of the table follow the nomenclature of length (L)/pitch (P)/diameter (D) where the approximate values are given in µm/µm/nm. The diameters were measured at the thickest section of the NWs.

| L  | P1  | P3  | P5  |
|----|-----|-----|-----|
|    | D−  | D+  | D−  | D+  | D−  | D+  |
| L1 | 1/1/270 | 1/1/590 | 1/3/280 | 1/3/900 | 1/5/280 | 1/5/890 |
| L3 | 3/1/290 | 3/1/610 | 3/3/380 | 3/3/970 | 3/5/460 | 3/5/1020 |
| L5 | 5/1/360 | 5/1/610 | 5/3/540 | 5/3/1000 | 5/5/600 | 5/5/1070 |
Figure S2, Supporting Information). For quantification, cells were first identified by Hoechst-counterstaining (nuclei). Subsequently, viable cells (about 50%, Figure S3, Supporting Information) were cross-correlated to the respective ICC color channels. The proportion of MAP2-positive cells on the NW arrays was about 65% and comparable to the controls (planar Si, glass; Figure 3c). With regard to the MAP2-positive cells, that is, neurons, about 65% were also NeuN-positive (Figure 3d, similar to the controls). A smaller proportion of the neurons was also TH-positive (about 2.5%, Figure 3e, comparable to the controls). Overall, the differentiation yields on the NW arrays were similar to the planar controls. Another essential measure of the quality of neuronal differentiation is the development of characteristic ion channels and the concomitant ability to process action potentials (APs), that is, the functionality of the neurons. The functionality of the neurons generated on the NW arrays was tested by patch clamp measurements and compared to planar controls (Figure 4). The patch clamp procedure was generally feasible on the NW arrays but noteworthy, the cells’ visibility on the nanostructured substrates was hindered considerably compared to the planar controls (Figure 4a, examples of L5 P1 D+, L1 P3 D−, and L3 P5 D+ NW arrays, controls in Figure S4a, Supporting Information). The generated neurons were functional on all types of NW arrays shown by recordings

Figure 2. Interactions of the neurons with the NW arrays depending on the NW length, array pitch, and NW diameter. Neurons were stained with MAP2 (green) and nuclei were labeled with DRAQ5 (red). The NW tips’ reflections appeared in green (note, for D− NW arrays, the tip reflections had substantially less intensity—to highlight the settling regimes, the NW tips were indicated in the cross sections by dotted lines). The main panels show top-views of the neurons whereas the panels below depict the corresponding cross-sectional views (z-stacks prepared by CLSM). The numbers in cross-sectional views indicate the proportion of the imaged cells which showed fakir-like (F), intermediate (I), or encapsulating (E) interaction with the NW arrays where the underlined entry is presented in the figure. P1 NW arrays mostly elicited a fakir-like resting state where the cells only interacted with the NW tips (independent of the NW length). Larger array pitches, especially P5 NW arrays, promoted the encapsulation of the NWs where with increasing NW length, the somata and nuclei were exposed to more severe deformations/indentations. Note, states were called intermediate where the cells started to enwrap the NWs but were not in contact with the bottom of the substrate. General note regarding the image composition: For the fakir-like states and the L1 NW arrays, the main panels consist of an overlay of two separate images where one is focused on the NW tips and the other in the cell. Other main panels: Only one image with focus at the NW tips.
of APs (Figure 4b, traces of controls in Figure S4b, Supporting Information). The amplitudes of the APs were about 70 mV and similar to the controls (Figure 4c). The AP thresholds and AP widths were ≈−25 mV and 3.6 ms, respectively, similar to the controls (Figure 4d,e). In addition to the functional analysis, we characterized further electrophysiological parameters of the neurons such as the resting membrane potentials (RMPs), membrane capacitances (MCs), and voltage-dependent membrane currents (Figure 5). Specifically, the RMPs and MCs were about −50 mV and 12 pF, respectively, comparable to the controls (Figure 5a). Lastly, the early-inward and late-outward currents were measured by applying voltage steps from −70 to +30 mV to the cells (Figure 5b, shown here: L1 P1 D−, L3 P3 D+, and L5 P5 D−; all other NW arrays in Figure S5a, Supporting Information, Si control in Figure 5d, glass in Figure S5b, Supporting Information). The recorded traces showed early negative peaks (inward currents, insets of respective panels) and late positive constant currents (outward currents) depending on the applied voltages. The peak values of the early-inward and late-outward currents normalized to cells’ MCs plotted against the applied voltages showed a negative trend (inward currents, insets of respective panels) and a positive trend (outward currents) depending on the applied voltages. The peak values of the early-inward and late-outward currents normalized to cells’ MCs plotted against the applied voltages showed minima of the inward currents at about 0 mV and an increase of the outward current at voltages higher than 0 mV (Figure 5c, Si in Figure 5d, glass in Figure S5c, Supporting Information).

3. Discussion

The fabrication of NW arrays by RIE in a top-down process from bulk material such as Si is well-established in the field.[53] By defining the hard mask, that is, the array pitches and NW diameters, via EBL wafer-scale sample fabrication with high-precision is enabled.[54] In general, the utilized NW lengths and array pitches match the values frequently found in the literature.[20] The D− diameters achieved by RIE rather cover the upper range of NW diameters used in the field—especially for the L5 NW arrays. Nonetheless, for applications such as solar cell NWs diameters around 300–400 nm would be acceptable.[55,56] Employing even thicker NWs (D+) extends the universality of the study since, for example, also NWs with a diameter of about 1 µm influence cell spreading or facilitate cell transfection.[57,58] Moreover, such larger diameters fill to some extent the gap between NW and micropillar arrays used in cell culture.[59,60] All NW arrays—with NW lengths up to 5 µm—were prepared in a single-step RIE process. Such long NWs are usually achieved in multi-step deep RIE (DRIE) processes,[61] whereas NW arrays prepared by regular RIE typically reach shorter lengths of about one micron due to a critical degree of underetching.[62] The ensemble of the top-down approach via RIE and a hard mask defined by EBL ensures a high reproducibility of the NW array fabrication virtually without any defects such as missing or askew NWs which is common in bottom-up approaches.[51-55] A defect-free nature of the NW arrays, however, was crucial to study the general impact of the NW arrays on the neuronal differentiation. Single missing NWs, for example, in P5 NW arrays would appear as P10 NW arrays to individual cells—or even as a flat since the cells might fit completely in between the NWs without any further interaction. Nevertheless, we also tested the neuronal differentiation on bottom-up fabricated NW arrays since the NW growth goes
along with enhanced material properties such as improved surface states, fewer material defects, and the capability to add functionality by integrating hetero-junctions by varying the doping during the growth, for example, to build NW solar cells to stimulate cells grown on top.[66,67] Specifically, we prepared L2 P3 D−, L2 P3 D+, L4.5 P1 D+, L4.5 P3 D+, and L4.5 P5 D+ GaAs NW arrays (Figure S6a, Supporting Information) and validated the generation of human iPSC-derived neurons on these NW arrays by MAP2 and NeuN stainings (Figure S6b, Supporting Information). However, employing Si for the NW fabrication—as used herein for the comprehensive analysis—is also reasonable since nanostructured Si is considered a promising material to overcome fundamental limits of current biomedical devices.[68]

The interface between the human iPSC-derived neurons and the NW arrays was marked by a variety of cellular settling states of which the fundamental dependency of the states with regard to the NW lengths, array pitches, and NW diameters was in accordance with a model developed in Buch–Månson et al. [69] In general, this model describes that fakir-like states are favored by cells in the case of long NWs, small array pitches, and thin diameters. In contrast, NW encapsulation is more likely for short NWs, large array pitches, and thick diameters. Overall, for P1 NW arrays, we observed the fakir-like state which is in accordance to the model and other studies with high-density NW arrays.[43,70–72] With increasing pitch, the neurons began to encapsulate the NWs. For the P3 NW arrays, the yields for partial NW encapsulation were reduced in the case of the D+ NW arrays which is contradictory to the expectations derived from the model. One reason might be that in the case of P3 D+ NW arrays the effective distance between the NWs (i.e., pitch minus diameter) falls below the threshold for NW encapsulation. A similar phenomenon has been observed in Hanson et al., where for a constant pitch of 1 μm and a NW length of 500 nm the cells encapsulated NWs featuring a diameter of <200 nm but were fakir-like for 500 nm thick NWs because of the decreased effective array pitch (500 vs 800 nm).[73] This also explains, why for L1 P1 D− not only fakir-like but also intermediate states were observed. For the P5 NW array, we mostly observed complete encapsulation of the NWs which is in accordance with other studies using low arrays densities.[43,74] The strong interaction of the NWs with the nuclei in the encapsulating states is also reported in the literature.[25] In direct comparison to a prior study of us with undifferentiated neural progenitor cells (NPCs)—used to generate the neurons herein—the differentiated neurons were more likely to favor the fakir-like state at similar specifications of the NW arrays.[75]

The neuronal differentiation yields on the NW arrays were analyzed after 14–15 days (8–9 on the NW arrays) of culturing by labeling neuron-specific markers such as MAP2, NeuN,
and TH. About 65% of the cells cultivated on the NW arrays were positive for the postmitotic neuronal marker MAP2. Such a value is comparable to other studies conducted in regular well plates.\cite{76} The majority of the identified neurons (≈65%) were also immunoreactive for another mature neuronal marker, namely, NeuN, similar to other studies.\cite{77} A small fraction of neurons was also positive for TH (about 2.5%) indicating the development toward a dopaminergic phenotype. Notably, the proportion for TH was rather small but being a late marker (e.g., a significant TH upregulation after 20 days of differentiation was reported in Gilmozzi et al.) such values are normal for very young cultures as, for example, literature reports also on single-digit proportions for cells cultured even longer than here (30 days).\cite{78} Overall, the numbers reflect normal yields for the generation of human iPSC-derived neurons but remarkably all yields were equally independent on the type of substrate, that is, the strong interaction with the NW arrays including the partly severe deformations of the somata and nuclei had no impact on the neuronal differentiation. Also, the yields compare well considering the short differentiation period of only 14–15 days versus >30 days used in other studies.\cite{76-78} In this context, it is also striking that the neurons, in addition, were functional already. One potential reason for the fast maturation of the neurons might be the early application of DAPT (on day 6) to the maturation medium to enhance the neuronal differentiation.\cite{79}

The electrophysiological analysis via patch clamping showed that on all types of NWs arrays the generated neurons were functional demonstrated by recordings of APs whose kinetics such as the AP amplitude, AP threshold, and AP width were similar to the planar controls. Moreover, the mean values of amplitude (about 70 mV), threshold (about −25 mV), and duration (about 3.6 ms) were comparable to other human iPSC-derived neurons.\cite{80-84} The RMPs (about −50 mV) and MCs (about 12 pF) were not only similar to the controls but also in accordance with the literature.\cite{80-84} Lastly, we measured the early-inward and late-outward membrane currents to test for the characteristic interplay of the sodium and potassium ion channels. Independent of the type of NW arrays, the neurons showed the typical negative peaks and constant positive currents. Here, the normalized maximum inward/outward currents plotted against the applied voltages showed similar voltage-dependencies to the controls and other studies.\cite{79,85} Considering again the tremendous differences observed for the interface between the neurons and the NW arrays, it is remarkable that the functional maturation is equal to the controls as well as to other studies. As a result, the geometrical specifications of the NW arrays may freely be chosen to optimize both the cell/NW interface and the properties of the NW arrays in order to match prospective applications.

**4. Conclusions**

This study demonstrates the generation of functional human iPSC-derived neurons on NW arrays with varying geometrical specifications such as the NW length (1–5 μm), array pitch (1–5 μm), and NW diameter (270–1070 nm) within only 14–15 days of cultivation. The neuronal differentiation was equal to planar controls in terms of both neuronal markers (MAP2, NeuN, TH) and electrophysiology such as AP kinetics, RMPs, and characteristic membrane currents. Such equal outcome of the differentiation is particularly striking...
since strong interactions of the neurons with the NW arrays have been observed which were going along with altering settling states such as fakir-like states or NW-encapsulating states. The equality of the neuronal differentiation despite the topographical challenges paves the way for future applications with functionalized NW arrays and, especially, allows for tailoring the physical specifications of the NWs arrays which might facilitate advanced applications such as sensing, stimulation, or biomolecule delivery in stem cell culture.

Specific applications being invaluable in the context of stem cell technologies and based on the findings presented herein might be, for example, as follows: The neuronal differentiation of human iPSCs might be enhanced by currents/potentials directly generated from solar cell NW arrays analogously to a study by Kwon et al., where the neurogenesis of fetal stem cells benefited from electrical stimuli mediated by NW arrays externally connected to a power supply.\cite{86} Also, therapeutic applications such as retina implants might have an advantage by employing solar cell NW arrays to precisely stimulate the respective nerve cells since the devices can be embedded into translucent polymers and tailored to be sensitive to specific wavelengths.\cite{87} Moreover, stem cell culture might be facilitated by means of NW-mediated cell transfection to enhance, for instance, in vitro gene editing tools such as CRISPR/Cas9.\cite{49,50} NW arrays with hollow NWs might further extend the number of suitable payloads by soluble cargos to render possible, for example, the direct intracellular delivery of neuronal growth factors to stimulate neurogenesis.\cite{51,52}

In conclusion, our results demonstrate the feasibility to combine human iPSC-derived neurons with NW arrays featuring a widespread compilation of geometrical characteristics to enable a likewise diversity of potential applications. Hence, we believe that stem cell research and regenerative medicine will substantially benefit in the future from further functionalized NW arrays for improved cell differentiation and interrogation.\cite{53,54}

5. Experimental Section

Nanowire Array Preparation: The NWs arrays were fabricated from silicon wafers (Si-Mat, P/Bor <100>, 5–10 μc-m) in a top-down process by RIE using a chromium hard mask. The mask was predefined via EBL (Raith Voyager e-beam lithography system) in a 90 nm thick layer of positive photoresists (PMMA, AllResist, AR-P 671.02). Circles with varying diameters (from, for example, 200 nm for the L1 PL D<sup>N</sup> NW arrays, up to, for instance, 700 nm for the L5 P5 D<sup>N</sup> NW arrays) were defined in a rectangular arrangement with pitches of 1, 3, and 5 μm. After developing (1:3 methyl isobutyl ketone (MIBK):isopropyl alcohol (IPA) including 2.7% H<sub>2</sub>O for 30 s and IPA for 30 s), an about 50 nm thick layer of chromium was deposited on a physical vapor deposition (Balzer/Pfeiffer, PLS 500) process. The samples were etched with an inductively coupled plasma (ICP)-RIE (Sentech SI500) using 200 W ICP power, 50 W RF power, 2 Pa pressure, 25 sccm SF<sub>6</sub> and 50 sccm C<sub>4</sub>F<sub>8</sub> at 0 °C electrode temperature. The etch rate was about 300 nm min<sup>-1</sup> and the etch duration was set accordingly to obtain NWs with lengths of 1, 3, and 5 μm. The NW arrays were passivated by a layer of Al<sub>2</sub>O<sub>3</sub> (about 20 nm) deposited by atomic layer deposition in a custom-built system. The samples were imaged with a Crossbeam 550 (Zeiss). To ease the handling of the wafer pieces, the substrates were glued with polydimethylsiloxane (PDMS, SYLGARD 184 Elastomer Kit, Dow Corning) to 12 × 12 mm<sup>2</sup> glass coverslips. For cell culture, the samples were treated with an oxygen plasma, placed in a well of a 12-well plate, sterilized in 70% ethanol for 5 min, and coated with Matrigel (Corning 354263, 1:150 in Knock Out DMEM, Life Technologies) over night at room temperature or 1 h at 37 °C (1 ml per well). The GAAs NW fabrication is stated in the Supporting Information.

Cell Culture: The human iPSC-derived neurons were generated from human iPSC-derived NPCs.\cite{85} The NPCs were maintained in Matrigel-coated (0.5 ml per well) 6-well plates using basic N2/B27 medium (1:1 mixture of DMEM/F12 and Neurobasal medium, 1% penicillin/streptomycin/glutamine, 100X, 1% B27 supplement without vitamin A, 50X, 0.5% N2 supplement, 100X, Life Technologies) supplemented with 100 μM ascorbic acid (AA, Sigma-Aldrich), 3 μM AG-2 (Biocline), and 3 μM CHIR 99021 (Axon MedChem) and split 1:10 to about 8000 cells per well of a 6-well plate every 4–5 days (detachment via accutase, Sigma-Aldrich). The neuronal differentiation of the NPCs (8000 cells per well freshly seeded in 6-well plates) was initiated by changing to a patterning medium (basic N2/B27 medium supplemented with 100 μM AA, 0.5 μM SAG, 1 ng ml<sup>−1</sup> GDNF, PeproTech, 1 ng ml<sup>−1</sup> BDNF, PeproTech) for 6 days. The 6-days predifferentiated NPCs were reseeded into 12-well plates containing the NW arrays and controls. From here on, the differentiation was continued using maturation medium (N2/B27 basic medium supplemented with 100 μM AA, 2 ng ml<sup>−1</sup> GDNF, 2 ng ml<sup>−1</sup> BDNF, 1 ng ml<sup>−1</sup> TGF-β3, PeproTech, 100 μM db-cAMP, Sigma-Aldrich) for another 8–9 days. Note, at the beginning of the neuronal maturing on the NWs arrays, only a small fraction of cells was MAP2-positive (about 15% one day post-plating to the NW arrays and controls, Figure S7, Supporting Information). On day 6, also 10 μM DAPT (Biomol) was added for one day to the maturation medium.\cite{59} The cells were kept at 37 °C with 5% CO<sub>2</sub> in a humidified atmosphere and culture media were replaced every 2–3 days. All experiments were conducted 14–15 days after initiation of the differentiation where for NW arrays the last 8–9 days the cells were on the very substrates. Ethics declaration: All experiments were conducted in accordance with the ethical statement in Reinhardt et al.\cite{85} Confocal Laser Scanning Microscopy: An upright Leica TCS SP8 confocal microscope with a HC PL APO CS2 63x/1.20 water immersion objective and 488/638 nm lasers was used to prepare the z-stacks (slicing distance: 250 nm). Neurons were stained with MAP2 (see "Immunocytochemistry") and nuclei were stained with DRAQ5 (ThermoFisher Scientific, Fluorescent Probe Solution, 5 μM, Cat. No. 62251, 1:1000 in Dulbecco’s phosphatebuffered saline (DPBS)) for 10 min at 37 °C. Processing and analysis of the z-stacks were conducted with the Leica LAS X Core software. For illustration, images were optimized in contrast, brightness, and false color, if applicable. Immunocytochemistry: Samples were rinsed with DPBS, fixed in formaldehyde (4% in DPBS, Sigma-Aldrich) for 10 min, rinsed three more times with DPBS, and stored at 4 °C in DPBS until imaging. Fixed cells were permeabilized and blocked for 45 min with 3% bovine serum albumin (BSA, Carl Roth), 0.1% Tween (Tweeen 20, Sigma-Aldrich), and 0.1% Triton-X (Triton X 100, Carl Roth) in DPBS. Incubation with the primary antibodies (MAP2, NeuN, TH) was conducted in DPBS with 0.1% BSA overnight at 4 °C with concentrations as follows—anti-MAP2 (microtubule-associated protein 2, mouse anti-MAP2 monoclonal antibody [Y113], Invitrogen, Cat. No. 13-1500): 1:500, anti-NeuN (neuronal nuclear antigen, rabbit anti-NeuN polyclonal antibody, Abcam, Cat. No. ab104225): 1:500, anti-TH (tyrosine hydroxylase, rabbit anti-TH polyclonal antibody, Abcam, Cat. No. ab112): 1:1000. Subsequently, the cells were washed twice with DPBS and incubated with Alexa 488/555 fluorophore-conjugated anti-mouse/anti-rabbit secondary antibodies (1:1000 in DPBS with 0.1% BSA, goat anti-mouse/anti-rabbit IgG-Alexa Flour Plus 488/555 polyclonal antibody, Invitrogen, Cat. No. A32723/A32732) for 1 h in the dark. Stained cells were kept in the dark and washed three times with Tween (0.05%) in DPBS for 5 min (second washing step with Hoechst 33342 for counterstaining, 5 mg ml<sup>−1</sup> in H<sub>2</sub>O, 1:1000). If not further noted, the steps were conducted at room temperature. Images were analyzed using a custom analysis pipeline for CellProfiler 4.2.\cite{48} The Hoechst channel was used to identify nuclei. Dead cells were sorted out in the pipeline by discrimination of bright nuclei (apoptotic) and medium bright nuclei (viable).\cite{49} Viable cells were used to mask the MAP2 channel to

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identify MAP2-positive cells. The NeuN/TH channels were masked by the MAP2-positive cells to identify NeuN/TH-positive cells.

**Electrophysiology:** A HEKA EPC 10 USB patch-clamp amplifier with a red star headstage installed to an upright Nikon Eclipse FN1 microscope (objective: Nikon CF1 TU Plan EPI ELWD 50× N.A. 0.60/W.D. 11.00 mm) was used for trace recording.[27] The patch clamp pipettes (resistance: 5–7 MΩ) were manufactured from GB150T-8P borosilicate glass capillary blanks (Science Products) using a P-2000 pipette puller (Sutter) and polished with a CPM-2 microforge (ALA borosilicate glass capillary blanks (Science Products) using a P-2000 pipettes (resistance: 5–7 MΩ)

**Statistical Analysis:** Confocal laser scanning microscopy imaging was conducted on 5–12 neurons per type of NW array. Immunocytochemistry data per type of NW array was collected from seven images with an are of around 1374 × 914 μm² containing in average 1035 ± 418 cells. Patch clamping was conducted on 2–7 cells per type of NW array. Origin (v.2022) was used to analyze the data using a one-way ANOVA with a post-hoc Tukey’s test. The graphs are presenting ± standard deviations. Differences were considered not statistically significant for p > 0.05.

**Supporting Information**

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

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**Conflict of Interest**

The authors declare no conflict of interest.

**Author Contributions**

Si NW preparation, cell culture, immunofluorescence and confocal microscopy imaging, patch clamping, data analysis, and manuscript writing: J.H.; GaAs NW preparation: W.K., A.F.i M., and J.H.; assistance in cell culture: M.S.; ALD: J.H., C.H., and R.Z.; conceptualization: J.H.; funding: A.F.i M., R.Z., and R.H.B.

**Data Availability Statement**

The data that support the findings of this study are available in the supplementary material of this article.

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