The effect of gallium nitride on long-term culture induced aging of neuritic function in cerebellar granule cells

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Abstract

Gallium nitride (GaN) has been developed for a variety of microelectronic and optical applications due to its unique electric property and chemical stability. In the present study, n-type and p-type GaN were used as substrates to culture cerebellar granule neurons to examine the effect of GaN on cell response for a long-term culture period. It was found that GaN could rapidly induce cultured neurons to exhibit a high phosphorylated Akt level after 20 h of incubation. It was assumed that the anti-apoptotic effect of Akt phosphorylation could be correlated with cell survival, neurite growth and neuronal function for up to 35 days of incubation. Morphological studies showed GaN induced larger neuronal aggregates and neurite fasciculation to exhibit a dense fiber network after 8 days of incubation. Western blot analysis and immunocytochemical characterization showed that GaN still exhibited the expression of neurite growth and function, such as high levels of GAP-43, synapsin I and synaptophysin even after 35 days of incubation. In addition, survival of cerebellar granule neurons on GaN was improved by the analysis of lactate dehydrogenase (LDH) release from damaged cells. These results indicated that neuronal connections were formed on GaN by a gradual process from Akt activation and cell aggregation to develop neurite growth, fasciculation and function. Therefore, GaN offers a good model system to identify a well-characterized pattern of neuronal behavior for a long-term culture period, consistent with the development of a neurochip requiring the integration of biological system and semiconductor material.

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Keywords: Gallium nitride (GaN); Cerebellar granule neurons; Long-term culture

1. Introduction

Gallium nitride (GaN) is a so-called III–V compound semiconductor material with unique physical and chemical properties such as chemical stability, hardness and electric properties [1–4]. Thus, GaN has been a subject of intense recent investigation and has emerged as attractive candidate for a variety of microelectronic [1] and optical [5,6] applications. Such advantages make GaN a good candidate in integrating electronics and biological system to develop biochips for different biomedical applications. Recently, the development of

neurochips for stimulation and signal collection from cultured neurons attracts a lot of attention [7,8]. From the technological point of view, tight adhesion of mammalian neurons to an appropriate chip surface is a prerequisite. At present, almost all developed chips for neural system are silicon-based devices [9]. Since the biocompatibility of silicon is poor, coating the surface with bioactive molecules such as poly-D-lysine (PDL) or laminin have been used to promote cell adhesion and survival [10]. However, coating bioactive molecules on the substrate is non-covalent attachment, which may be lost by washing steps, thus, it is a non-permanent modification. More efficient methods of promoting direct cell adhesion to the chip surface would be very valuable. Thus, in our laboratory, we tried to develop more neuron-favorable chip materials to promote cell adhesion to the chip surface. In the previous study [11], GaN has been shown to mediate the response of

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cerebellar granule neurons to promote cell differentiation and neuritic growth during 6 days of culture period. However, the novel function of GaN applied to culture cerebellar granule neurons for a longer period has not been established.

In the present study, GaN was used as a substrate for culture of cerebellar granule neurons for up to 35 days to examine its effect on cell response after a long-term culture period. Similar to the previous study [11], n-type and p-type GaN were used to culture cerebellar granule neurons prepared from 7-day-old Wistar rats. These two types of GaN have different surface compositions. The surface of the p-type GaN surface was enriched with gallium and that of the n-type GaN surface with nitride [12]. For comparison, commercial tissue culture polystyrene (TCPS) and PDL-coated TCPS were also used to culture cells. TCPS is the standard substrate for culturing various cells. PDL is a commonly used coating material in the preparation of neuronal cultures. At first, we investigated whether the specific protein-dependent signaling pathway was responsible for the neuronal survival and differentiation on GaN. Recent evidence indicates that the serine/threonine protein kinase PKB (PKB; also known as Akt) plays an important role in signal transduction [13,14], which regulates cell survival, growth, glucose transport and energy metabolism [15]. In this study, it was found that Akt was rapidly activated by GaN during the first 20 h of cell culture, which is thought to be related to the neuronal behavior after a long-term culture period. After 8 days of incubation, morphological studies of cultured neurons showed that GaN induced the neurite fasciculation and exhibited a dense fiber network from neuronal aggregates. After incubation for 35 days, it was found by Western blot analysis and immunocytochemical characterization that GaN still exhibited the expression of neurite growth and function, such as high levels of GAP-43 [16,17], synapsin I [18,19] and synaptophysin [20]. In addition, survival of cerebellar granule neurons on GaN was improved by the analysis of lactate dehydrogenase (LDH) release from damaged cells. These results indicated that neuronal connections were formed on GaN by a gradual process from Akt activation and cell aggregation to develop neurite growth, fasciculation and function. Therefore, GaN offers a good model system to identify a well-characterized pattern of neuronal behavior for a long-term culture period, consistent with the development of a neurochip requiring the integration of biological system and semiconductor material.

2. Materials and methods

2.1. Sample preparation

The substrates used in this study included n-type and p-type GaN (Huga Optotech Inc., Taiwan). All samples with a size of approximately 1 cm² and a thickness of 1 ± 0.1 mm were cleaned by sonification in water and 70% alcohol, sterilized with autoclave and then rinsed extensively with phosphate buffer solution (PBS). Subsequently, samples were placed in 24-well TCPS plates (Corning, New York, USA). The empty and PDL-coated wells where no sample was placed were used as controls. PDL was purchased from Sigma and diluted to 10 µg/ml in PBS. Before cell culture, wells were covered with 1 ml of PDL diluted solution and incubated for 4 h, after which time excess solution was removed by suction and dried for another hour.

2.2. Cell culture

Cerebellar granule neurons were prepared from 7-day-old Wistar rats according to Levi et al. [21] with slight modification. Briefly, neurons were dissociated from freshly dissected cerebella by mechanical disruption in the presence of trypsin and DNase. Subsequently, neurons were added to the culture wells at a density of 1×10^6 cells/well [22] in basal Eagle medium (BME; Gibco) supplemented with 10% fetal calf serum (unless specified), 25 mM KCl, penicillin G (100 IU/ml) and streptomycin (100 mg/ml). Cultures were maintained at 37 °C in a humidified atmosphere of 95% air–5% CO₂. Cytosine arabinoside (10 mM) was added to the culture medium 1 day after plating to prevent replication of non-neuronal cells.

2.3. Cell morphology

For morphological observation, the cells adhering to the sample were washed with PBS and then fixed with 2.5% glutaraldehyde in PBS for 1 h at 4 °C. After thorough washing with PBS, the specimens were dehydrated by graded ethanol changes and then examined by a metallography microscope (Olympus BX51M, Japan) for GaN substrates or an inverse phase contrast microscope (Olympus IX71, Japan) for TCPS and PDL substrates. Detailed morphological analysis was performed on aggregated neurons by using a Hitachi S-800 scanning electron microscope (SEM), after specimens being critical point dried and gold sputtered in vacuum.

2.4. Immunofluorescence microscopy

For immunocytochemical characterization, cultured neurons were fixed in ice-cold 4% paraformaldehyde in PBS for 25 min and washed three times in PBS after 35 days of incubation. Following fixing, neurons exposed to

Fig. 1. Substrate-induced Akt phosphorylation in cerebellar granule neurons. Western blots were performed with anti-phosphorylated Akt and anti-Akt antibodies for cells cultured on n-type GaN, p-type GaN, TCPS, and PDL after 20 h of incubation. The bottom panel indicates the relative intensities of phosphorylated Akt levels, determined by band densitometry analysis. The ratio of the band intensities was expressed as a percentage of TCPS control.

primary antibody (diluted in 10% BSA/PBS) overnight at 4 °C. The primary antibodies and their dilution used in this study were rabbit anti-microtubule-associated protein 2 polyclonal antibody (anti-MAP-2; 1:500; Chemicon), mouse anti-synapsin I protein monoclonal antibody (1:500; BD), and mouse anti-synaptophysin protein monoclonal antibody (1:500; BD) [23,24]. FITC- and rhodamine-conjugated secondary antibodies were used to visualize the signal by reacting with cells for 30 min at room temperature. The secondary antibodies and their dilutions were FITC-conjugated donkey anti-rabbit IgG (preabsorbed with rabbit and rat serum protein; 1:100; Chemicon, Temecula, CA) and rhodamine-conjugated goat anti-mouse IgG (preabsorbed with rabbit and rat serum protein; 1:100; Chemicon, Temecula, CA). These immunostained cells were visualized by indirect fluorescence under the fluorescent microscope (Axiovert 100TV, Germany).

2.5. Western blot analysis

Cells were collected by gentle shaking of the wells and washed twice with PBS. Cell lysates were prepared with ice-cold lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 1 mM NaF, 1 mM Na₃VO₄, 1:200 dilution of Protease Inhibitor Cocktail II;

Calbiochem) for 30 min and then were sonicated at 4 °C for 15 s. Lysates were clarified by centrifugation at 10,000 rpm for 30 min at 4 °C and the resulting supernatant was saved for protein analysis and Western blot analysis. Protein concentration was measured by using the commercial protein assay reagent (Bio-Rad, Hercules, CA). For Western blotting, the supernatant was added to an equal volume of Laemmli sample buffer (62.5 mM Tris, pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol) and heated to 95 °C for 5 min. Proteins (50 μg total protein per lane) were separated by SDS-PAGE on 10% polyacrylamide gels and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk in TBST buffer (Bio-Rad, Hercules, CA) and probed with a monoclonal mouse Akt and phospho(Ser⁴⁷³)-Akt antibody (BD; 1:1000), a monoclonal mouse β-actin antibody (Chemicon; 1:2000), a monoclonal mouse GAP-43 antibody (BD; 1:10,000), a monoclonal mouse synapsin I antibody (BD; 1:25,000) and a monoclonal mouse synaptophysin antibody (BD; 1:25,000), and the membranes were incubated in primary antibody at 4 °C overnight. After washing, the blots were incubated with HRP secondary antibodies (1:5000) at room temperature for 2–3 h. Finally, the proteins on the membranes were detected using the ECL Plus chemiluminescence system. Densitometric quantification of Western blots was done using software of AlphaEase FC (version 4.0).

Fig. 2. Photomicrographs of cerebellar granule neurons cultured on (a) PDL, (b) TCPS, (c) n-type GaN, and (d) p-type GaN after 20 h of incubation. (scale bar = 100 μm).

2.6. LDH assay

The toxic effect of substrates on the cultured cells was quantitatively determined by measuring the release of LDH into the culture medium. LDH activity of collected medium was measured by using LDH kit (Roche, USA) according to the protocol. The optical density of the LDH activity was read on an ELISA plate reader at 490 nm and reference wavelength at 630 nm.

3. Results

3.1. The effect on phosphorylation of Akt

To address the possible mechanism of action of GaN on cultured neurons, we investigated whether Akt pathway participated in the regulation of behaviors of neurons cultured on

GaN surface because Akt has been shown to promote cell survival and prevent apoptosis [15]. Akt kinase activity correlated with its phosphorylation, so the Akt activity of cells cultured on n-type and p-type GaN was determined by measuring Akt phosphorylation in Western blot analysis. For comparison, TCPS and PDL-coated TCPS (is simply termed PDL, hereinafter) were also used to culture cells. Anti-phosphorylated Akt antibody shows that n-type and p-type GaN caused a high level of Akt phosphorylation, whereas both TCPS and PDL induced Akt phosphorylation at an extremely low level after 20 h of incubation (Fig. 1). At this time, cells showed extensive single cell adhesion on PDL (Fig. 2a) but showed cell–cell aggregation on TCPS (Fig. 2b). For the case of n-type and p-type GaN, cells exhibited similar adhesion behavior (Fig. 2c, d). Cell aggregates

Fig. 3. SEM pictures of cerebellar granule neurons cultured on (a) PDL, (b) TCPS, (c) n-type GaN, and (d) p-type GaN after 8 days of incubation. Arrows in (c) and (d) indicate neurite growth in the straight path.

and single cells were observed on both the types of GaN simultaneously. These results show that GaN exhibited different influences on the signal transduction and neuronal morphogenesis within a very short culture period.

3.2. The effect on neurite fasciculation

To analyze the effect of GaN on neurite fasciculation, the morphology of cerebellar granule neurons cultured on PDL, TCPS, n-type and p-type GaN was examined by SEM after

incubation for 8 days. Fig. 3a shows neurons plated on PDL formed neurite networks with the defasciculated feature of the neurite. In contrast, n-type and p-type GaN induced the axonal fasciculation and exhibited a dense fiber network extending in all directions (Fig. 3c, d). These formed neurites on the surface of n-type and p-type GaN tended to grow in the straight path and formed minor bends of less than 17° (arrows in Fig. 3), as measured through fractal analysis, which may be attributed to the stiff properties of microtubule polymer bundles [25]. Generally, dendritic outgrowth does not exhibit the

Fig. 4. SEM pictures of cerebellar granule neurons cultured on (a) PDL, (b) TCPS, (c) n-type GaN, and (d) p-type GaN after 8 days of incubation in the serum-free medium.

long-distance characteristic of axonogenesis, and the dendritic branch angles vary across neuronal cell types [1,26]. Thus, it is reasonable to infer that axonal extension of granule neurons on the surface of n-type and p-type GaN. In addition, cells grew into compact neuronal aggregates on the surface of n-type and p-type GaN and intimate cell–cell contact was observed within the 3-D aggregate structure. A similar cell behavior was observed for neurons cultured on TCPS, but less neurites and neuronal aggregates were observed (Fig. 3b).

To further evaluate the effect of GaN itself on neurite development, cerebellar granule neurons were cultured on PDL, TCPS, n-type and p-type GaN in serum-free medium (Fig. 4). Clearly, neurons cultured on PDL and TCPS only showed individual adhesion after 8 days of incubation. Conversely, GaN not only maintained neuronal adhesion but also neurite outgrowth in the absence of serum, regardless of n-type or p-type. In general, the direct contact between cell and the substrate is the first step for cell adhesion onto the substrate, but it is known that cell–substrate adhesion is generally considered to be a complicated process involving adsorption of extracellular matrix proteins onto the substrate surface, especially for the presence of serum. Therefore, one can argue that GaN contributed to neurite outgrowth patterns via a more complex cell–substrate interaction that was enhanced by the presence of serum. Although such a possibility cannot be completely ruled out, Figs. 3 and 4 show that GaN promoted cell adhesion as well as induced neurite outgrowth simultaneously in the serum-dependent and -independent interactions.

Fig. 5. Western blots were performed with anti-GAP-43, anti-synapsin I, and anti- β -actin antibodies for cerebellar granule neurons cultured on TCPS, n-type GaN, p-type GaN, and PDL after 8 days of incubation. The bottom panel indicates the relative intensities of GAP-43 and synapsin I levels, determined by band densitometry analysis. The ratio of the band intensities was expressed as a percentage of TCPS control.

3.3. The effect on axonal growth and synaptogenesis

To evaluate the effect of GaN on axonal growth and synaptogenesis, cerebellar granule neurons on PDL, TCPS, n-type and p-type GaN after 8 and 35 days of culture were prepared for Western blot analysis. A number of neurotypic proteins have been associated with neuron differentiation and neurite outgrowth. GAP-43 is preferentially distributed in the growth cone and elongating axon of developing neurons, whose expression can be correlated with axonal and neurite outgrowth in culture [27]. Figs. 5 and 6 show that GAP-43 levels were always low for cells cultured on TCPS, regardless of 8 or 35 days of culture. Compared to TCPS, there was considerable expression of GAP-43 for cells cultured on PDL and GaN after 8 days of incubation. This large increase in GAP-43 suggests that differentiation of neurons induced by PDL and GaN was similar and obvious. However, compared to GaN, neurons cultured on PDL could not maintain the strong expression of GAP-43 after 35 days of culture. A similar result was observed for Western blot analysis of synapsin I (Figs. 5 and 6), a pre-synaptic protein involved in synapse formation and synaptic

Fig. 6. Western blots were performed with anti-GAP-43, anti-synapsin I, and anti- β -actin antibodies for cerebellar granule neurons cultured on TCPS, n-type GaN, p-type GaN, and PDL after 35 days of incubation. The bottom panel indicates the relative intensities of GAP-43 and synapsin I levels, determined by band densitometry analysis. The ratio of the band intensities was expressed as a percentage of TCPS control.

transmission as well as preferentially localized in axons, particularly concentrated in the distal axon and growth cone [28]. This indicates that GaN could maintain a neuron-favorable environment for axonal growth and synaptogenesis for a longer period than PDL, a standard substrate in the preparation of neuronal cultures.

For the next experiment, presynaptic vesicles were visualized by immunofluorescent labeling for the vesicle-associated proteins synapsin I and synaptophysin after 35 days of culture. Figs. 7a, b and 8a, b show that cells on PDL and TCPS were faintly stained for the expression of synapsin I and synaptophysin after a long-term culture period. In contrast, for cells cultured on n-type and p-type GaN, immunostaining for synapsin I (Fig. 7c, d) was concentrated in bright fluorescent puncta around cell bodies and along radially oriented axons. The distribution of synaptophysin for cells cultured on n-type and p-type GaN is shown in Fig. 8c, d. Basically, a similar pattern of localization was observed for the immunostaining expression of synapsin I and synaptophysin. Neighboring neuron aggregates were connected by axons and synaptic puncta distributed along the length of axons. This suggests that GaN maintained neurite network and neuronal function after a long-term culture period. In addition to synapsin I and synaptophysin, the immunostaining for MAP-2, the marker used to define the presence of dendrites [29], was also shown in Figs. 7 and 8. However, our immunocytochemical analysis failed to detect dendrites on both types of GaN,

which were MAP-2-negative. Therefore, the formation of functionally mature presynaptic release sites did not contact a postsynaptic apposition for cerebellar granule neurons cultured on GaN surface. The conclusion that cell–cell contact is not a prerequisite for the formation of a functional presynaptic release site is consistent with the previous finding of culturing rat hippocampal neurons [30].

3.4. LDH assay

Finally, the toxic effect of GaN on the long-term cultured neurons was quantitatively determined by measuring the release of LDH into the culture medium in the same experiments described for the Western blot analyses. LDH is a stable cytoplasmic enzyme and is abundantly present in central neurons [31]. Generally, LDH assay is a precise, fast and simple colorimetric method to quantify cytolysis when damage to the plasma membrane occurs [32]. As shown in Fig. 9, there was no difference in LDH release in the culture medium between neurons cultured on n-type and p-type GaN, but the significant difference was observed when both the types of GaN were compared to the PDL or TCPS ($p < 0.05$) after 35 days of culture. Therefore, similar to the morphological observation, GaN could improve the neuronal survival to reduce the neuronal death compared to the PDL and TCPS.

Fig. 7. Immunocytochemical staining of the presynaptic protein synapsin I and the dendrite marker MAP-2 in cerebellar granule neurons. Fluorescent photomicrographs represent cells on (a) PDL, (b) TCPS, (c) n-type GaN, and (d) p-type GaN after 35 days of culture. Synapsin I and MAP-2 labeling are red and green, respectively (scale bar = 100 μm).

Fig. 8. Immunocytochemical staining of the presynaptic protein synaptophysin and the dendrite marker MAP-2 in cerebellar granule neurons. Fluorescent photomicrographs represent cells on (a) PDL, (b) TCPS, (c) n-type GaN, and (d) p-type GaN after 35 days of culture. Synaptophysin and MAP-2 labeling are red and green, respectively (scale bar = 100 μ m).

4. Discussion

The purpose of this study is to investigate whether the neurite development and function still can be maintained on GaN after a long-term culture period. The substrates used in this study included TCPS, PDL, n-type and p-type GaN. It is known that biomaterial surface characteristics govern cell–substrate interactions. Changes in cell–substrate interactions through the cell surface receptors corresponded to changes in cellular signaling events, as well as changes in cell survival and differentiation. Therefore, the ultimate cellular response may be resulted from the effect of signal transduction at an early stage of cell adhesion process. The anti-apoptosis role of the phosphatidylinositol 3-kinase (PI3-K) pathway has been well documented [14,33]. The importance of PI3-K pathway is to trigger a chain of phosphorylation reactions that include insulin and cell survival signals [34]. Akt is known to be a downstream target of PI3-K and plays an important role in signal transduction [13,14], which regulates cell survival, growth, glucose transport and energy metabolism [15]. In this study, we investigated whether Akt pathway participated in the regulation of survival of neurons cultured on GaN at an early stage and might involve additional events that occurred at later stages. Interestingly, Western blot analysis shows that PDL and TCPS exhibited a very low level of Akt

phosphorylation, whereas both n-type and p-type GaN induced Akt phosphorylation to a high level during the first 20 h of incubation, as shown in Fig. 1. In addition, different cell distribution was observed on different substrates at this same culture period (Fig. 2). Both cell aggregates and single cells were observed on the GaN simultaneously, which was different from single cell adhesion on PDL and only cell aggregates on TCPS. Therefore, elucidation of the substrate-dependent, Akt-mediated differences in cell signaling and survival is important to bridge the gap between GaN semiconductor material and neuronal activities for developing a long-term used neurochip.

Fig. 3 shows GaN induced larger neuronal aggregates and axonal fasciculation to exhibit a dense fiber network after incubation of 8 days. Changes in the degree of neuron distribution and neurite fasciculation often reflect a balance between two competing forces: the interactions of cell–substrate and cell–cell [35]. Enhancement of cell-to-substrate adhesion produces monolayered cells and neurite branches (e.g., adhesive PDL substrate), while dominance of cell–cell interaction results in neuron aggregation and neurite–neurite fasciculation. Therefore, when cerebellar granule neurons were cultured on relatively low adhesive GaN surface, cell–cell interaction caused cell migration and aggregation as well as neurite fasciculation, resulting in the formation of a dense fiber

	n-type GaN	p-type GaN	TCPS
PDL	*	*	*
n-type GaN		n.s.	*
p-type GaN			*

Fig. 9. LDH assay of cerebellar granule neurons cultured on TCPS, PDL, n-type GaN and p-type GaN after 35 days of incubation. Results represent mean $\pm$ standard error of mean from three independent cultures and determinations. Asterisks indicate significant difference ($p < 0.05$) as determined by one-way ANOVA and post-hoc Duncan test.

network from gradually enlarging neuronal aggregates. In vivo, cerebellar granule neurons are tightly packed in the internal granule layer [36]; thus, neurons on GaN surface adopted physiological aggregated-structure resembling in vivo condition, which were different from the traditional monolayered morphology in vitro. Clearly, it is important to elucidate the interaction of neurons with the underlying substrate surface to regulate neuron adhesion and neurite development. Regardless of n-type or p-type GaN, such a phenomenon still could be observed even in the absence of serum, as shown in Fig. 4. Although the detailed mechanism by which GaN promotes expression of Akt is not clear, the function of Akt pathway also has the protective effect on serum-starved neurons after 8 days of incubation.

Although neurons cultured on GaN and PDL had different neuronal patterns after 8 days of incubation, expression of GAP-43 and synapsin I by Western blot analysis was similar at this time (Fig. 5). Therefore, it is difficult to discriminate the effects of GaN and PDL on axonal growth and synaptogenesis only for a short-term culture period. Different outcome occurred at later stages. Compared to GaN, neurons cultured on PDL could not exhibit the strong expression of GAP-43 and synapsin I after 35 days of culture. However, GaN still maintained a neuron-favorable environment for axonal growth and synaptogenesis over a longer period of time (Fig. 6). Parallel immunocytochemical observations confirmed the presence of the vesicle-associated proteins synapsin I and synaptophysin on both types of GaN after 35 days of culture (Figs. 7 and 8). It is known that neuronal polarity is controlled

by the expression of axon markers such as synapsin I and synaptophysin [37–39] and it is important to develop electrophysiological properties of neuritic network with the acquisition of neuronal polarity for extracellular recording of cultured neurons [40]. This suggests that GaN maintained neuritic network and neuronal function after a long-term culture period. Finally, GaN could improve the neuronal survival to reduce the neuronal death compared to the PDL and TCPS (Fig. 9), which may result from the high expression of Akt phosphorylation at the initial culture period. These results further confirm that the GaN semiconductor material has the potential for developing a long-term used neurochip.

5. Conclusion

Survival and differentiation of cultured neurons are known to depend on the culture substrate. We report here the results of neuronal signal transduction at the early stage and neuronal development after a long-term culture period. In summary, the GaN substrate could activate Akt phosphorylation to a high level during the first 20 h of culture and could maintain neurite development and function after 35 days of culture. Therefore, culturing of cerebellar granule neurons on the GaN semiconductor material represents a feasible method to develop a GaN-based neurochip in the near future.

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