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NS21: Re-defined and Modified Supplement B27 for Neuronal Cultures

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Abstract

In vitro culturing of primary neurons is a mainstay of neurobiological research. Many of these culture paradigms have taken advantage of defined culture media rather than serum additives that contain undefined survival factors to facilitate experimental manipulations and interpretation of the results. To culture neurons in the absence of serum, defined supplements such as B27 are now widely used. However, commercially available supplements exhibit large variability in their capabilities to support neurons in culture. We re-optimized and modified earlier published formulations of B27 using 21 different ingredients (NS21). NS21 supports neuronal cultures of high quality as manifested by their morphological characteristics, formation of synapses, and postsynaptic responses. Much of the variability in the quality of B27/NS21 was due to variability in the quality of different sources of bovine serum albumin. Furthermore, we found that holo-transferrin used in NS21 is preferable over apo-transferrin used in B27 for the quality of neuronal cultures.

Keywords

B27 Supplement; NS21 Supplement; Dorsal Root Ganglion Cells; DRG; Primary Hippocampal Cultures; Hippocampal Neurons; Neuronal Cultures; Retinal Ganglion Cells; RGC

INTRODUCTION

Neuronal cultures are widely used to study neuronal development including neurite and synapse formation, neurotransmitter release, subcellular distribution and trafficking of neuronal proteins such as neurotransmitter receptors, and homeostasis of electrical signaling. Initially those cultures depended on the use of sera for factors that are critical for cell survival and growth. Media supplements such as B27 were developed with defined components that eliminate the need for supplementation with serum (Bottenstein and Sato, 1979; Romijn, 1988; Romijn et al., 1984). Such supplements were widely welcomed. Particularly B27 is used by many investigators for a number of different neuronal culture systems (Christopherson et

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al., 2005; Colledge et al., 2003; Craven et al., 1999; Deisseroth et al., 1996; El-Husseini et al., 2000; Mi et al., 2004; Passafaro et al., 2003; Pratt et al., 2003; Roche et al., 2001; Sans et al., 2005; Schluter et al., 2006; Stellwagen and Malenka, 2006; Tai et al., 2007; Thiagarajan et al., 2002; Tomita et al., 2004; Tsui and Malenka, 2006; Ullian et al., 2001).

In theory the use of defined supplements reduces the variability of the culture conditions. It thereby limits the potential for detrimental effects of components that could affect the health of cultures. However, a number of laboratories have experienced large differences in their neuronal cultures over the last 4-5 years when using commercially available supplements (see below and e.g., (Schluter et al., 2006; Tsui and Malenka, 2006)). Commercial supplements available earlier including B27 supported neuronal cultures of excellent quality including neurons derived from hippocampus, retinal ganglia (RGCs), and dorsal root ganglia (DRG) cells. However, more recently supplements available in the United States have largely failed to reliably promote healthy neuronal cultures.

The reason for this variability is likely due to the fact that several components such as bovine serum albumine and transferrin are isolated from biological sources. As sources and isolation procedures differ to some degree they introduce variability in those biological components. We re-evaluated published formulations (Brewer and Cotman, 1989; Brewer et al., 1993; Romijn, 1988; Romijn et al., 1984). We found that the exact source and vendor for some components are critical. Although based on the original B27 formulation, our formulation varies from commercially available B27 by using components of explicitly defined origin (vendor, precise product) and by the use of holo- rather than apo-transferrin. To ensure that the formulation is not confused with the currently widely used commercially available B27 we call this formulation NS21 (Neuronal Supplement 21). We chose this name because we optimized and evaluated this supplement for neuronal cultures and because it has 21 ingredients. The precise composition of NS21 is given in Table 1.

MATERIALS AND METHODS

Primary Cultures of Rat Hippocampal Neurons

Low-density cultures of dissociated hippocampal neurons were prepared as given earlier (Lim et al., 2003). In brief, hippocampi were dissected from E18 embryonic rats (Sprague Dawley, Harlan), treated in Hank's balanced salt solution (HBSS; Invitrogen) with trypsin (0.03%) for 15 min at 37°C, washed three times with HBSS, and triturated with a fire-polished pasteur pipette. After pieces of tissue that were not dissociated settled, cells in the supernatant were collected by centrifugation (1,100 rpm, 200×g, 1 min), resuspended, counted, and plated on coverslips (Warner Instruments, Hamden, CT) coated with 0.1% (w/v) poly-L-lysine (Peptides International, Louisville, KY). Cell density was $3\text{--}6 \times 10^3 \text{ cm}^{-2}$ in Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B27 (Invitrogen) or NS21, 0.5mM glutamine and 5% Fetal Bovine Serum (FBS; HyClone Laboratories, Logan, Utah; catalog number: A-2151-D). 4 h later, the medium was replaced with serum-free Neurobasal medium plus B27 or NS21 and 0.5 mM glutamine (Invitrogen). Details of the formulation of NS21 are given in Table 1. Cells were grown in a humidified environment of 95% air/5% CO₂ at 37°C. One third of medium was changed after 4 DIV and weekly thereafter.

Immunofluorescence Microscopy of Hippocampal Cultures

Cultures were washed (PBS), fixed (PBS plus 4% paraformaldehyde and 4% glucose; 15 min), washed (PBS), permeabilized (0.05% Triton X-100; 20 min), blocked (PBS containing 2% glycerol, 0.05M NH₄Cl, 5% FBS, 2% goat serum; 2 h), and incubated with mouse monoclonal anti-CaMKII α at a dilution of 1:1,000 for 1.5 h at room temperature or overnight at 4°C. The anti-CaMKII α antibody is described in (Leonard et al., 1999). Cover slips were then washed,

incubated with Alexa 488 conjugated goat anti-mouse secondary antibodies (Molecular Probes, Eugene, OR; dilution 1:200; 1h, room temperature), washed, and mounted (Prolong Gold Antifade mounting media; Molecular Probes, Eugene, OR). Fluorescence images were viewed with an IX-70 inverted epifluorescence microscope equipped with a 100x, 0.75 NA objective (Olympus), an MAC2002 shutter (Ludle), and a fluorescence filter set (Chroma, Brattleboro, VT) for Alexa 488 (490 nm band-pass excitation, 528 nm long-pass emission). Images were collected with a Hamamatsu (Bridgewater, NJ) ORCA II CCD camera equipped with a frame grabber EDT DV PCI card controlled by Esee software (Inovision, Chapel Hill, NC). To quantify the density of filopodia-like protrusions and spines on dendrites of 11 and 18 DIV cultures, respectively, images were first coded for blind analysis. 50 μ m long segments of dendritic regions near somata were randomly chosen and marked and all protrusions counted manually.

Electrophysiological Recordings from Hippocampal Neurons and mEPSC Analysis

Recordings were obtained from neurons identified morphologically as pyramidal cells by the teardrop-shaped somata and the presence of an apical-like dendrite. Whole cell patch recordings were executed with custom 8520 Patch Glass capillary electrodes with Tip resistances of 5-10 M Ω (Warner Instruments, Hamden, CT). The glass electrodes were filled with 110 mM K-gluconate, 10 mM KCl, 10 mM HEPES, 5 mM EGTA, 3 mM MgATP, 0.5 mM MgGTP, 1 mM CaCl₂, pH 7.35 with KOH (240-254 mOsm). Extracellular solution: 120 mM NaCl, 3 mM KCl, 3 mM CaCl₂, 2 mM MgCl₂, 5 mM Glucose, 0.23 mM Na-Pyruvate, 10 mM HEPES, pH 7.35 with NaOH, 260-270 mOsm. Recordings were performed with an Axopatch 200B amplifier (Digidata 1322A; Axon Instruments, Foster City, CA) at room temperature. Liquid junction potentials were calculated to be 5–6 mV. Voltages were left uncompensated for the junction potential. Seal resistances were 3 G Ω . Cells had resting potentials between -50 and -60 mV. The sampling rate was 10 kHz and lowpass filter frequency 1 kHz. AMPA receptor mEPSCs were recorded in 10-12 DIV neurons clamped to -70 mV with picrotoxin (50 μ M), MK801 (50 μ M), and tetrodotoxin (1 μ M) in the extracellular medium. pClampex and pClampfit 9.01.07 (Axon Instruments) were used for data acquisition and analyses. GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA) was utilized for the graphing.

Purification and Culture of Retinal Ganglion Cells

Retinas were dissected from P5 Sprague-Dawley rats and RGCs were purified by sequential immunopanning to greater than 99.5% purity as previously reported (Barres et al., 1988; Meyer-Franke et al., 1995) and further described below.

Retinal dissection and dissociation—Retinas were removed from the eye *in situ*. Postnatal rat pups (P5-P7) were decapitated, and skin overlying the eyes, the lens and vitreous humor were taken away. The retina was gently lifted with a small spatula. The membranes were carefully removed under a dissection microscope and retinas were stored at room temperature in Earle's Balanced Salts Solution (EBSS) containing calcium and magnesium, pH 7.4, until all retinas were collected. Retinas were then incubated in papain solution at 34°C for 30 min. Papain solution was prepared by adding 165 units of papain (Worthington) to 10 ml of pre-warmed Dulbecco's phosphate buffered saline (DPBS; Invitrogen) containing phenol red and dissolved in a 37 °C water bath. About 10 min before use, the papain solution was mixed with 2.4 mg of L-cysteine and passed through a 0.22 micron filter into a sterilized universal tube before addition of sterile DNase (100 μ l of 0.4% DNase; Worthington). After digestion, papain solution was aspirated from retinas and retinas were gently rinsed in 3 ml of ovomucoid inhibitor solution (LoOvo) containing ovomucoid (60 mg; Worthington), BSA (60 mg), DNase (0.004%) in DPBS. Rabbit anti-rat antimacrophage serum (80 μ l; Accurate Chemical) was added to 6 ml of LoOvo solution. After the tissue settled, 2 ml of LoOvo + anti-

macrophage was added and tissue was triturated very gently with a P1000 pipetman. After the tissue had settled, the supernatant was transferred to a second tube containing 1 ml of Lo Ovo + anti-macrophage and triturated again. This process was repeated until all cells were in suspension. Supernatant containing dissociated cells were transferred to a final tube, and incubated for 10 min to allow antibodies to bind to cells. Cells were centrifuged cells at 1,000 rpm for 11 min at room temperature. Cells were then resuspended in a solution of ovomucoid inhibitor (HiOvo) containing 120 mg ovomucoid and immediately spun again at 1,000 rpm for 11 min.

Immunopanning—After the final centrifugation in HiOvo solution, the supernatant was discarded and cells were resuspended in panning buffer (DPBS containing 0.05% BSA + 5 µg/ml insulin) and brought up to a final volume of 10 ml. Panning dishes were prepared the night before and stored at 4 degrees (see below). Cell suspensions were added to the first negative panning plate and incubated for 30 min at room temperature on a flat surface (dish was vigorously shaken every 15 min). The remaining suspended cells were then transferred to the second negative panning dish and the process was repeated to immunodeplete the remaining retinal cells. Finally, the cell suspension was filtered through 15 micron Nitex mesh (Tetko Inc.), to remove small cell clumps and then added to the final T11D7 panning plate (30-40 min, room temperature).

Preparation of panning plates—Panning plates were incubated the night before at 4°C with secondary antibodies against panning antibodies. For negative panning with rabbit anti-rat macrophage antibody (Accurate Chemical, AI A51240): two 150 mm petridishes were coated with 10 ml of affinity-purified goat anti-rabbit IgG H+L (30 µl) (Jackson Immunoresearch) in 10 ml TrisHCl (50 mM, pH 9.5). For the positive Thy1/T11D7 dish: 100 mm petridishes were incubated with Goat anti mouse IgM, µ chain specific (Jackson Immunoresearch). The next day, panning dishes were washed 3 times with DPBS before addition of cells. Monoclonal supernatant containing IgM antibodies directed against Thy1.1 (the cell line is available from ATCC, no. TIB 103, name T11D7e2) was added to the positive plate and allowed to bind at room temperature for at least two hours. Immediately before addition of cells, the Thy1/T11D7 monoclonal supernatant was removed from the positive panning plate and the plate was washed 3 times with PBS.

Plating of Purified RGCs—A trypsin solution (0.125%; Sigma) was prepared by adding 200 µl of a 2.5% trypsin (Sigma; T4665) stock solution to calcium- and magnesium-free EBSS containing bicarbonate and phenol red (Invitrogen). Plates were gently washed 6-8 times with DPBS to remove non-adherent cells. Adherent RGCs were then incubated with trypsin solution for 10 min in a 37°C CO₂ incubator. 2 ml of heat inactivated FBS (30% FBS (Invitrogen) diluted in Neurobasal medium) was added to plate and inactivate trypsin. RGCs were then removed by gentle trituration (several rounds) and collected in a final tube containing 1 ml 30% FBS solution. An aliquot was removed for counting before the RGC suspension was centrifuged at 200×g for 11 min. Approximately 35,000 RGCs were cultured per well on glass coverslips (Assistant) coated first with poly-D-lysine (10 µg/ml; Sigma, P6407) and then with laminin (2 µg/ml; CultureX, 3400-010-01). RGCs were cultured in 600 µl of serum-free NB-Sato medium, modified from (Bottenstein and Sato, 1979), containing Neurobasal Medium (Invitrogen), bovine serum albumin (100 µg/ml; Sigma, A4161), sodium selenite (40 ng/ml), putrescine (16 µg/ml), triiodo-thyronine (1.6 µg/ml), apo-transferrin (100 µg/ml; Sigma, T-1147), and progesterone (60 ng/ml). NB-Sato medium was further supplemented with pyruvate (1 mM), glutamine (2 mM), triiodo-thyronine, NAC (N-acetyl-cysteine, 5 µg/ml), insulin (5 µg/ml), B27 (Invitrogen) or NS21 (Table 1), CNTF (10 ng/ml), brain-derived neurotrophic factor (BDNF; 50 ng/ml), and forskolin (10 µM). Chemicals were obtained from Sigma unless otherwise specified.

Preparation of Astrocytes

Cortical glia was prepared as described (McCarthy and de Vellis, 1980). Briefly, newborn rat (postnatal day 1-2) cortices were papain-digested and plated in tissue culture flasks (Falcon) in a medium that does not support neuronal survival (Dulbecco's Modified Eagle Medium, FBS (10%), penicillin (100 U/ml), streptomycin (100 µg/ml), glutamine (2 mM) and Na-pyruvate (1 mM)). Non-adherent cells were shaken off of the monolayer 4 days later and cells were incubated another 2-4 days to allow monolayer to refill. Medium was replaced with fresh medium containing AraC (10 µM; Sigma) and cultures were incubated for 48 hours. Astrocytes were trypsinized and plated onto 24-well inserts (Falcon, 1.0 µm) or 10 cm tissue culture dishes.

Microscopic Imaging and Immunocytochemistry of Retinal Ganglion Cells

RGC cultures were grown in the absence (Fig. 3) and presence (Fig. 4) of an astrocyte feeder layer. The requirement of astrocytes for synapse formation but not survival of RGC cultures is described in (Christopherson et al., 2005). None of our RGC cultures, whether grown in the presence or absence of astrocyte feeder layers, contained serum except for FBS used during the early isolation and plating steps. For immunostaining, cultures were fixed (4% paraformaldehyde; 7 min), washed (PBS), blocked (Blocking Buffer: 50% Antibody Buffer (0.5% bovine serum albumin, 0.5% Triton X-100, 30 mM NaPO₄, 750 mM NaCl, 5% normal goat serum, and 0.4% NaN₃, pH 7.4), 50% goat serum (NGS), 0.1% Triton-X; 30 min), washed (PBS), incubated with primary antibody (rabbit anti-synaptotagmin (cytosolic domain, Synaptic Systems; Goettingen, Germany) and mouse anti-PSD-95 (6G6-1C9 clone, Affinity Bio Reagents) diluted 1:500 in antibody buffer (overnight, 4°C), washed (PBS), incubated with secondary antibodies (Alexa-594 conjugated goat anti-rabbit and Alexa-488 conjugated goat anti-mouse (Molecular Probes) diluted 1:1,000 in antibody buffer (2 h, room temperature), washed (PBS), mounted in Vectashield mounting medium with DAPI (Vector Laboratories Inc.) on glass slides (VWR Scientific), and imaged using a Nikon Diaphot epifluorescence microscopes (Nikon). Phase contrast micrographs were obtained from live RGCs.

Electrophysiological Recordings from Retinal Ganglion Cells

Membrane currents were recorded in the presence of astrocytes by whole-cell patch clamping at room temperature (about 22°C) at a holding potential of -70 mV. Patch pipettes (3 to 10 MΩ) were pulled from borosilicate capillary glass (WPI). For recordings of synaptic glutamate currents, the bath solution contained 120 mM NaCl, 3 mM CaCl₂, 2 mM MgCl₂, 5 mM KCl, and 10 mM Hepes (pH 7.3). The internal solution contained 100 mM K-gluconate, 10 mM KCl, 10 mM EGTA, and 10 mM Hepes (pH 7.3). The holding potential was -70mV. Currents were recorded using pClamp software for Windows (Axon Instruments). Spontaneous excitatory post-synaptic currents (sEPSCs) and mEPSCs were plotted using SigmaPlot (SPSS, Chicago, IL) or Origin (Microcal, Northampton, MA).

Primary Cultures of Mouse Dorsal Root Ganglion Cells

For preparation of DRG cultures (Araki et al., 2004) timed pregnant CD1 mice were obtained from Charles River Laboratories (Cambridge, MA). Gestational day 12.5-13 embryos were removed and DRGs were dissected out and dissociated using 0.5 ml trypsin/EDTA (0.05%/0.02%; Tissue Culture Support Center, Washington University, St. Louis, MO) for no longer than 15 min at 37°C with 2-3 times of gentle shaking by hand. The reaction was stopped by addition of 0.5 ml of DMEM/10% FBS. All subsequent procedures were performed in a laminar flow hood under sterile condition. Cells were gently triturated by pipetting them up and down with a sterile P1000 pipetman less than 10 times. No cell clumps or ganglia should be visible after this trituration. Cell suspensions were centrifuged at 2,000 rpm for 5 min at room temperature. Supernatants were completely removed. Cell pellets were washed twice with 1ml of DMEM/10% FBS. Cells were resuspended in either DMEM/10% FBS or growth medium

and plated directly onto 24-well plates coated with poly-D-lysine (Sigma, P0899; coating was performed in advance with 100 µg/ml poly-D-lysine, dissolved in water, overnight at 37°C; after coating, plates were washed twice with water and completely dried) and laminin (Invitrogen; 3 µg/ml in water for 3 hr at 37°C without any washing steps). Cultures were maintained in Neurobasal medium with B27 (Invitrogen) or NS21, 50 ng/ml NGF, 5 mM glutamine, and antibiotics. Cells were plated in 20 µM 5-fluorouracil as an antimitotic agent for the first 3 days in culture to reduce non-neuronal cell content. Phase contrast images were obtained from live cultures with a Nikon eclipse TE300 microscope with an LWd 20×/0.4 Ph1 DL objective.

For quantification of axonal degeneration, four different fields were chosen for each condition in two independent experiments. Each field contained about 50 axons. Fragmented axons were counted as degenerated. For quantification of somal degeneration, four different fields containing somata were chosen for each condition in two independent experiments. Each field contained about 25 somata.

RESULTS

Primary Hippocampal Cultures

Under optimal conditions pyramidal neurons in primary hippocampal cultures develop multiple, well arborized dendrites. This morphology is illustrated by immunofluorescence microscopy following antibody staining for the Ca²⁺- and calmodulin-dependent protein kinase II (CaMKII) (Figure 1B,C,F,G). CaMKII is one of the most prevalent proteins in neurons. It is localized throughout dendrites and in neurons with fully mature synapses at dendritic spines, the postsynaptic sites of glutamatergic synapses. It is thus an ideal marker to visualize the complete dendritic arborization including dendritic shafts and spines. Using commercial B27 predating 2004 or NS21, neurites exhibited to a comparable degree multiple filopodia-like protrusions that are clearly visible upon staining for CaMKII at 11 DIV (Figure 1B,C; examples are indicated by arrows). These pronounced protrusions that were obvious in cultures grown with NS21, which contains holo-transferrin, were much less numerous when we replaced holo-transferrin with apo-transferrin, as used in B27 (Figure 1A). Cultures with B27 obtained within the last 3 years in the United States did not readily develop filopodia-like structures at 11 DIV in our hands (Figure 1D). A quantitative analysis of the density of filopodia-like structures confirmed the visual impression (Figure 1I). Pyramidal neurons grown in either NS21 or earlier B27 had about 40-50 protrusions per 50 µm dendritic length. If apo-transferrin was used the density dropped by about 50%. More recently purchased B27 yielded fewer than 10 protrusions per 50 µm dendritic length.

Dendritic spines account for the majority of postsynaptic sites of glutamatergic synaptic contacts onto mature pyramidal cells. At 18 DIV, our cultures showed clearly distinguishable dendritic spines with NS21 containing holo-transferrin and with earlier B27 as visualized by CaMKII staining (Figure 1F,G; examples are indicated by arrows). Their density was between 50 and 60 spines per 50 µm dendritic lengths for both conditions (Figure 1J). Dendritic spines are less prominent when using NS21 with apo-transferrin substituted for holo-transferrin (Figure 1E; about 35 spines per 50 µm) and nearly absent with more recent B27 (Figure 1H; less than 10 spines per 50 µm).

To scrutinize whether the presence of dendritic spines reflects the formation of fully developed synapses, AMPA receptor mEPSCs were recorded from pyramidal neurons in primary hippocampal cultures grown in NS21 (Figure 2). These mEPSCs were frequent and showed amplitudes and time courses of neurons with healthy synapses. These findings indicate that NS21 containing holo-transferrin is currently the optimal defined supplement for high quality hippocampal cultures containing fully developed mature synapses.

Retinal Ganglion Cell Cultures

We have developed culture conditions that support the growth and survival of purified RGCs grown in the complete absence of glia or other support cells (Barres et al., 1988; Meyer-Franke et al., 1995). Because of the lack of such support cells, these RGCs are particularly sensitive to the quality of culture medium components. In the past we used commercially available B27 for serum free supplementation of the culture medium. We observed a significant decrease in the survival of purified RGCs that were cultured in the presence of many recent lots of B27 obtained between 2004 and 2007 compared to an older control B27 lot. The most characteristic phenotype associated with the majority of recent commercial B27 lots was a significant fasciculation of RGC neurites and severe clumping of neuronal somata as illustrated for cultures grown in the absence of astrocyte feeder layers (Figure 3A vs. B). RGCs often lifted and peeled off of the substrate after several days *in vitro*. A side by side test of various lots of commercial B27 and custom NS21 revealed that RGCs grown in NS21 appeared healthy and extended axons and dendrites (Figure 3). Synapse formation of RGCs is strongly fostered if an astrocyte feeder layer is placed on top of the RGC cultures. In its presence prominent synapse formation was obvious in cultures grown with NS21 (Figure 4) and comparable to RGC cultures grown with earlier commercial B27 lots (Christopherson et al., 2005; Ullian et al., 2001). As a further indication of good health, astrocytes increased spontaneous EPSCs and mEPSCs in RGCs cultured in standard NB-Sato growth medium supplemented with NS21 (Figure 4D). The amplitude and frequency of these spontaneous EPSCs and mEPSCs were comparable to those observed in RGCs cocultured with astrocytes using earlier commercial B27 lots (Ullian et al., 2001).

Dorsal Root Ganglion Cell Cultures

To examine the efficacy of NS21 on peripheral neuronal cultures, we grew dissociated DRG neurons using B27 or NS21 for 7, 10, and 14 DIV. Neurons grown using media containing recent batches of commercial B27 exhibited axonal beading and fragmentation, a characteristic of degenerating axons, at 10 DIV (Figure 5). At 14 DIV, these neurons displayed complete axonal degeneration. Nearly all axons appear fully fragmented at 14 DIV. Systematic counting of the number of axons that showed clear signs of beading indicates that virtually all axons were degenerated at 14 DIV when using recent B27. Only a few axons displayed signs of beading even after 14 DIV with previous more optimal B27 or NS21 and these axons had extensive arbors indicative of healthy neurons. Substituting apo-transferrin for holo-transferrin strongly increased the number of axons that showed signs of deterioration to about 40% after 14 DIV although this fraction is much smaller at 7 and 10 DIV.

The cell bodies of DRG cells in culture also began to deteriorate by 14 DIV in the presence of recent B27 as evidenced by the increasingly irregular appearance of the boundaries of somata (Figure 6, right panel). At 14 DIV, neurons grown with NS21 containing apo-transferrin rather than holo-transferrin also showed strong signs of somal degeneration (Figure 6, compare left and middle panel). However, neurons grown with NS21 containing holo-transferrin or earlier B27 lots had no signs of degeneration (Figure 6, middle panel). These observations were further confirmed by a quantitative count of the fraction of somata that appeared degenerated. Accordingly, about 80% of DRG cell somata exhibited signs of degeneration at 14 DIV with both, recent B27 and NS21 containing apo- rather than holo-transferrin (Figure 6 bottom). Nearly no cells did so in NS21 containing holo-transferrin.

DISCUSSION

Our work identifies the growth medium component B27 as a major source of variability that has become detrimental to several types of primary neuronal cultures. Our comparisons of the effects of B27 batches obtain before and after 2004 and of NS21 on the quality of hippocampal

cultures are largely based on consecutive rather than parallel experiments as we did not have available earlier B27 after problems became obvious. However, the striking difference between earlier and more recent B27 became clear within the same week in which we changed B27 batches. Furthermore, re-optimization of B27 leading to NS21 rectified the problem especially when holo-transferrin was used. These observations leave no doubt that it is the B27 supplement that is largely if not fully responsible for the low quality of our hippocampal cultures observed with recent B27.

Similarly, RGC and DRG culture quality was strongly impaired upon changing B27 batches from earlier to more recent products. Other components such as FBS, animals, and personnel were the same at the time of these changes. Furthermore, the different B27/NS21 batches were tested in parallel for RGC and DRG cultures as shown in Fig. 3 and Figs. 5 and 6, respectively. Because of the striking differences in the quality of cultures observed with earlier versus more recent B27 batches in the absence of any other obvious changes and because these dramatic results were obtained in three independent laboratories we conclude that it is the currently commercially available B27 that is problematic with respect to obtaining high quality hippocampal, RGC, and DRG cultures.

Several factors can contribute to this variability. First, the quality of the source and lot or batch of each of the 21 individual components can vary. Second, it might be important that the preparation of stocks of the individual components is performed with high care. This aspect might include the proper and careful storage of different components and stock solutions as detailed in Table I. This factor is especially important for lipid stocks, some of which are sensitive to light or oxidation and need to be carefully prepared and stored at -80°C. Third, it might be important how the components are mixed together as detailed in Table I with bovine serum albumin dissolved first before addition of other components. We avoid stirring and only gently but thoroughly swirl the solution after addition of all components. Temporal or local changes in pH or in the concentration of individual components could affect solubility or integrity of other components. It is obviously not possible for us to systematically test the importance of all these factors but given that the protocol outlined in Table I yields NS21 of excellent quality it will be prudent to carefully follow it.

NS21 likely differs from B27 that for most if not all components different sources are used, which are clearly spelled out in Table 1 for NS21. Furthermore, B27 contains apo-transferrin whereas NS21 contains holo-transferrin. The reason for using the latter is due to our observation that hippocampal cultures consistently looked healthier with more numerous dendritic branching, filopodia-like structure, and dendritic spines compared to those cultures that received NS21 in which holo-transferrin was replaced by apo-transferrin (Figure 1). Axons and somata of DRG cells are less healthy if apo-transferrin is used for culturing instead of holo-transferrin (Figures 5 and 6, respectively). We only used one source of apo-transferrin and one source of holo-transferrin and thus cannot be certain whether the superior effect with holo-transferrin is due to its overall rather low total content of iron or other factors such as a more intact batch of holo-transferrin. It is conceivable that transferrin is more stable or easier to purify in an intact form with its ligand iron present.

However, the largest difference with respect to neuronal survival and health is due to the use of a defined bovine serum albumin product. We do not know why bovine serum albumin is critical for the health of neuronal cultures but one possibility is its property to bind various lipids. It thus could serve as a critical carrier for lipids that are important for neuronal survival and development. As given in Table 1, we found that bovine serum albumin from Sigma with the ordering number A4919 is by far better than several other bovine serum albumin sources we tested including another one from Sigma. By now we have used three different charges of

A4919 from Sigma over the last 3 years without noticing any obvious differences. These findings indicate that A4919 is a dependable component for NS21.

While it is certainly convenient to rely on commercially available B27, there are a number of disadvantages. Individual investigators have absolutely no information on or control over any of the above factors including origin and quality of the various components and their handling. The commercial supplier produces a number of different lots per year. It is difficult to carefully and thoroughly test a large number of lots to potentially identify one of suitable quality as would be necessary based on our own results without any certainty that a suitable lot can be identified. Even if this screen succeeds it then is not certain whether a sufficient amount of this same lot can be obtained after the time-consuming tests as the supplier changes lots frequently. Finally, as there is currently only one commercial supplier and the detailed nature of each of the components is confidential, individual investigators have no control over general availability of B27.

Hippocampal cultures are often used as a model to study the effect of oxygen and glucose deprivation. Our hippocampal cultures grown in NS21 are completely suitable for such studies. Cultures undergo massive degeneration within 24-48 hours following a six hour period of oxygen and glucose deprivation (Merrill, Strack, and Hell, data not shown) or a six hour period of glutamate treatment (Stein and Hell, data not shown).

We conclude that the growth of healthy neurons in several different culture systems including our hippocampal, RGC, and DRG cultures is not reliably supported by the currently commercially available B27. However a modified B27 formulation with explicitly defined components including their sources and holo-transferrin that we call NS21 allows culturing of high quality hippocampal, RGC, and DRG neurons.

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ABBREVIATIONS

AMPA, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid; CaMKII, Ca^{2+} /calmodulin-dependent kinase II; DIV, days in vitro; DRG, dorsal root ganglion; NMDA, *N*-methyl-D-aspartate; PBS, phosphate buffered saline; RGC, retinal ganglion cells.

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Figure 1. Morphology of neurons in primary hippocampal cultures

Primary hippocampal cultures were grown in NS21 containing holo-transferrin (**B,F**; labeled “NS21”), NS21 in which apo-transferrin was substituted for holo-transferrin (**A,E**; labeled “NS21 ApoT”), an earlier B27 lot (**C,G**), and a recent B27 lot (**D,H**) at different times. Cultures were fixed after 11 (**A-D**) or 18 DIV (**E-H**), permeabilized, and stained for CaMKII α . CaMKII can be detected throughout dendrites including filopodia-like protrusions at 11DIV (arrows in **B,C**) and spines at 18 DIV (arrows in **F,G**). The density of filopodia-like protrusions and spines of dendritic segments of 11 and 18 DIV cultures, respectively, was quantified by counting those in 50 μ m long segments of proximal dendritic shafts in a blind fashion (**I,J**; given are mean $\pm$ SEM from 15 randomly chosen fields in 2 independent experiments). Only earlier B27 or NS21 containing holo-transferrin fully supported the formation of filopodia-like structure at 11DIV and spines at 18DIV, with NS21 in which holo-transferrin had been replaced with apo-transferrin being less effective and recent B27 only marginally supporting formation of spines at 18DIV and not at all filopodia at 11DIV. Scale bar is 10 μ m.

Figure 2. AMPA receptor mEPSCs in pyramidal neurons cultured with NS21

Recordings were performed from 11 DIV neurons clamped to -70 mV with picrotoxin, MK801, and tetrodotoxin present. Insert at lower right: The average AMPA receptor mEPSC was assembled from multiple events in several recordings.

Figure 3. Comparison of RGC cultures grown with NS21 and commercial lots of B27

Shown are phase contrast micrographs of live retinal ganglion cells (RGCs) grown for 5 days in commercial lots of B27 (Invitrogen) from 2006 (A) and 2004 (B) or in NS21 (C). No astrocyte feeder layers were present in these cultures. NS21 supported the growth and survival of purified RGCs (C) and was comparable to commercial B27 lot from 2004 (B). Note the significant clumping of RGCs grown commercial B27 from 2006 (A), which is typical of cultured supplemented with recent B27 lots. Scale Bar is 30 μ m.

Figure 4. Astrocytes promote formation of functional synapses in RGC cultures grown with NS21
A-C. Immunostaining for the presynaptic marker synaptotagmin (**A**, red) and postsynaptic marker PSD-95 (**B**, green) reveals numerous colocalized synaptic puncta (yellow puncta in **C**) in RGC cultures grown in the presence of a feeder layer of astrocytes. Scale Bar is 30 μ m.
D. Whole cell patch recording of a mixture of spontaneous EPSCs and mEPSCs as observed in the absence of tetrodotoxin from RGCs grown with NS21 with astrocytes present. As expected, astrocytes increased spontaneous EPSCs (large downward deflections) and mEPSCs (small downward deflections) above control (not shown) in RGC cultures supplemented with NS21 to a level that was comparable to that observed earlier with optimal B27 lots (Ullian et al., 2001).

Figure 5. Images of axons in mouse DRG cultures at 7, 10 and 14 DIV

Dissociated DRG cells were cultured with the indicated supplement. Shown are phase contrast images taken from live cultures at 7, 10, and 14 DIV (top) and quantification of the number of axons that displayed beading as a sign of their disintegration (bottom; given are mean $\pm$ SEM from 4 randomly chosen fields in 2 independent experiments). Neurons grown with recent B27 media showed axonal beading at 10 DIV and complete axonal degeneration at 14 DIV. Neurons grown with NS21 containing apo-transferrin ("NS21 ApoT") instead of holo-transferrin also exhibited signs of axonal degeneration in form of little blebs although blebbing was less pronounced. Neuron cultured with NS21 with holo-transferrin or optimal earlier B27 lots showed little evidence of axonal degeneration. Scale Bar is 50 μ m.

Figure 6. Images of somata of mouse DRG cultures at 14 DIV

Dissociated DRG cells were grown using the indicated supplement. Shown are phase contrast images taken from live cultures at 14 DIV (top) and quantification of the number of somata that displayed irregular shapes as sign of their disintegration (bottom; given are mean $\pm$ SEM from 4 randomly chosen fields in 2 independent experiments). Neurons in suboptimal recent B27 media showed massive degeneration of cell bodies at 14 DIV indicated by loss of clearly visible boundaries to neighboring cells and more amorphous overall appearance. Neurons in NS21 containing apo-transferrin also exhibited to a large degree signs of somatic deteriorating. However, neurons grown with NS21 containing holo-transferrin showed virtually no evidence of somal degeneration. Scale Bar is 50 μ m.

Formulation of NS21

Table 1

	Cat. #	Final NB Concentration		Stock(mg/ml)	For 400ml NS21 (20L final medium)
		µg/ml	µM		
Albumin, bovine ³	A4919	2500	37	add as powder	50g
Catalase ²	C40	2.5	0.010	add as powder	50mg
Glutathione (reduced) ³	G6013	1.0	3.2	add as powder	20mg
Insulin ^{3,9}	I1882	4.0	0.6	10 ⁹	8ml
Superoxidase dismutase ^{2,5}	S5395	2.5	0.077	add as powder	50mg
Holo transferrin ^{2,8}	616424	5.0	0.062	add as powder	100mg
T3 (triiodol-L-thyronine) ^{2,5,6}	T6397	0.002	0.0026	2.0	20ul
L-Carnitine ^{4,5}	C7518	2.0	12	add as powder	40mg
Ethanolamine ⁴	E9508	1.0	16	liquid (1g/ml)	20ul
D(+)-galactose ⁴	G0625	15	83	add as powder	300mg
Putrescine ^{4,5}	P5780	16.1	183	add as powder	322 mg
Sodium Selenite ⁴	S9133	0.01435	0.083	1.0	280ul
Ethanollic Stocks^{1,0}					
Corticosterone ^{4,6}	C2505	0.02	0.058	2.0	0.2ml
Linoleic acid ¹	L1012	1.0	3.5	100.0	0.2ml
Linolenic acid ^{1,6}	L2376	1.0	3.5	100.0	0.2ml
Lipoic acid (thioctic acid) ^{1,5,6}	T1395	0.047	0.2	4.7	0.2ml
Progesterone ^{4,6}	P8783	0.0063	0.020	3.2	0.04ml
Retinol acetate ¹	R7882	0.1	0.2	20.0	0.1ml
Retinol, all trans (vit. A) ^{3,6}	95144	0.1	0.3	10.0	0.2ml
D,L-alpha-Tocopherol (vit. E) ^{3,6,7}	95240	1.0	2.3	100.0	0.2ml
D,L-alpha-Tocopherol acetate ^{3,6}	T3001	1.0	2.1	100.0	0.2ml

Footnotes:

Comments:

NB: Neurobasal Medium, BSA: Bovine Serum Albumin

All the components are from Sigma except holo transferrin from Calbiochem, Cat. # 616414

Equilibrate all solid compounds at RT for 1-4 hours before opening.

Start by dissolving 50g BSA in 324ml NB on ice before addition of other compounds. Do not stir.

Finish in 2 hours. Gently swirl after addition of all compounds for careful but thorough mixing.

Store at -20°C, avoid freeze-thawing. Use 10 ml aliquots for 500ml NB medium.

Filter the medium after addition of NS21 (undiluted NS21 is too viscous for effective filtration).

- 1 store at -80°C
- 2 store solid at -20°C
- 3 store solid at 2-6°C
- 4 store solid at room temperature (RT)
- 5 solid is hygroscopic
- 6 Compound is light sensitive
- 7 Compound is O₂ sensitive
- 8 Holo transferrin from Calbiochem
- 9 dissolve in 1% acetic acid
- 10 store ethanolic stocks at -80°C in polyethylene tubes